Basal cell carcinoma

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Basal Cell Carcinoma

EPIGENETICS AND NEW TREATMENT MODALITIES

OUT OF THE BOX

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Basal Cell Carcinoma
EPIGENETICS AND NEW TREATMENT MODALITIES
OUT OF THE BOX

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Chapter 1

General introduction

Skin Cancer

Skin cancer is the most common malignancy in Caucasians worldwide.¹ In literature, skin cancer is often divided into malignant melanoma and nonmelanoma skin cancers (NMSC). Of these, melanoma is the most aggressive and deadly and comprises 11% of all skin cancers.² The term NMSC (or keratinocyte carcinomas) is mainly used for basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). Other primary cutaneous malignancies in the group of NMSC are cutaneous lymphomas, adnexal tumors, Merkel cell tumors and other rare cutaneous neoplasms.¹ Approximately 80% of all NMSCs are BCCs, while 10-20% is SCC. The large number of BCCs to be treated and the correspondingly rising impact on the workload of dermatologists imposes a significant burden on health care systems. Hence, BCC is also a serious health problem. Generally, BCCs are slow-growing indolent tumors with low metastasis rates (0.0028% to 0.55%) and they are rarely fatal.³ However, when incorrectly diagnosed and managed or left untreated, BCCs can be locally destructive, causing substantial morbidity and even mortality. In those cases, they have the potential of progressing to a(n) (locally) advanced BCC (l)aBCC.⁴ In this thesis, studies focused on the pathogenesis and treatment of both non-aggressive BCCs and (l)aBCCs.

Epidemiology

BCC is the most common cancer among Caucasians with worldwide incidence rates rising by about 3-10% annually, reaching epidemic proportions.^{5,6} Incidence rates are estimated because NMSCs are generally poorly registered. In many countries, often only the first BCC in a patient is reported and no distinction is made between BCCs and SCCs.⁷ In the Netherlands, a large observational study found a nearly fourfold increase in age-standardized incidence rates from 40 to 165 per 100.000 males and from 34 to 157 per 100.000 females between 1973 and 2009.⁵ One in five to six Dutch citizens will develop at least one BCC during their lifetime.⁷ In the five years following the first BCC, the cumulative risk of developing subsequent BCC(s) is approximately 40-50%, with the highest risk in the first 6 months after diagnosis of the first BCC.^{1,8} Although BCC is generally considered to be a disease of the elderly, recent literature shows the most rapid increase in its incidence in women aged 40 years or less.⁵ The rising incidence is mainly caused by an increase in recreational (intermittent) ultraviolet radiation (UV(R)) exposure and exposure during childhood.⁹ Other contributing factors are increased patient and physician awareness, better tumor coding and a rise in life expectancy over the years.⁹ BCC mostly affects sun-exposed areas of the skin like the head and neck region. However, the results of a 37-year Dutch observational study show a steep increase in BCCs located on the trunk⁵, possibly caused by sunbathing habits that have gained popularity since Coco Chanel returned suntanned after her holiday in the 1920s.¹⁰

The importance of UVR for NMSC is further reflected by the geographic variation in incidence, with incidence increasing as latitude decreases.¹ With the highest amount of UV exposure, Australia has the highest incidence rates, with 935 of 100.000 persons per year being affected, according to a large observational study between 1997 and 2006.¹¹ It is estimated that two out of three Australians will develop skin cancer. However, because of poor BCC registration in Australia, true incidence rates are unknown.¹²

Risk factors

The risk of developing NMSC, including BCC, depends on a number of intrinsic and extrinsic risk factors. The major and best-known risk factor for BCC development is exposure to UVR. Consequently, persons with a light skin, eye and hair color (Fitzpatrick skin types I and II) and those unable to tan have a higher risk of developing skin cancer. There is a typical latent period of years to even decades between UV exposure and the occurrence of BCC.⁹ However, the exact relationship between UV exposure and BCC is rather complex; literature suggest that both exposure early in life and intermittent (i.e. holidays) and cumulative (i.e. occupational related) UV exposure are important.^{9,13} UVR is divided into UVA (320–400 nm), UVB (290–320nm, sunburn rays), and UVC (200–290 nm), based on wavelengths. While UVC is known to be the most harmful, almost 100% of all solar UVC is absorbed by the atmosphere and the ozone layer. In contrast, even though over 95% of UVA and only 1–10% of UVB radiation reaches the earth's surface¹⁴, UVB is demonstrated to be 10000 times more mutagenic than UVA. This is because UVB directly causes DNA and RNA damage resulting in a characteristic C → T or CC → TT transition mutation (UV-signature). About 50% of all sporadic BCC harbor specific UV-induced mutations in the *TP53* tumor suppressor gene.¹⁵ Still, the underlying mechanisms of UVA-induced DNA damage are less well understood.¹⁶ Primarily, UVA induces mutations indirectly by generating reactive oxygen species capable of causing oxidative DNA damage.^{1,16,17} However, a rather high proportion of BCCs occur on body sites that are generally less exposed to the sun^{5,13}, suggesting that UV radiation alone is not (always) sufficient for the development of BCCs. Photosensitizing medications, such as tetracyclines, thiazide diuretics, NSAIDs and retinoids make the skin more vulnerable to UV damage and in fact increase the risk of especially early-onset BCC. An increased risk is also found after exposure to ionizing radiation in the form of radiotherapy or after exposure to arsenic, which can be present in groundwater. Immune-compromised patients, caused for example by HIV seropositivity or organ transplantation, have a doubled and tenfold risk of BCC development respectively. BCC development is also associated with genetic disorders, such as basal cell nevus syndrome (BCNS), xeroderma pigmentosum (XP) and Bazex–Dupré–Christol syndrome.^{1,18} Of these, the most common syndrome is BCNS, an autosomal dominant disorder causing distinct morphological features including multiple BCCs already at a young age, palmar or plantar pits, jaw cysts tumors and medulloblastomas.¹⁹ The genetic background of BCNS will be further elaborated below.

Pathogenesis

Cells of origin

BCCs are epithelial tumors histologically resembling the basal cells of the epidermis and the outer layer of the hair follicle. Although BCCs are generally referred to as hair follicle tumors, the real origin of the tumor is still controversial. Reliable cell model systems are not available. Transgenic mice expressing the oncogene M2SMO in skin under control of the keratin 5 (K5) promoter developed lesions resembling superficial BCC (sBCC). These were very similar to embryonic hair germs, comprising a focal group of epidermal cells protruding into the underlying dermis of volar skin completely devoid of follicles or other skin appendages.²⁰ A few years ago, it was demonstrated in the same mouse model that these tumors derive from long-lived progenitor cells residing in the interfollicular epidermis (IFE) and not from hair follicle bulge stem cells.²¹ More recently, it was shown in a conditional *PTCH1* knockout (via keratin 14 (K14) promoter) mouse that when BCC-like tumors arise from the IFE, this was only from the touch dome epithelia²², an IFE niche specialized for mechanosensory signaling.²³ On the other hand, Peterson et al. also demonstrated that BCC-like lesions can originate from multiple hair follicle stem cells (upper bulge, lower bulge and isthmus) and all these tumors displayed a similar phenotype, irrespective of cellular origin. In contrast, most stem cells within the IFE did not efficiently form tumors, not even after loss of *TP53*.²² The importance of the hair follicle stem cells in the development of BCCs is also confirmed by Kasper et al. showing that after deletion of *PTCH1* in hair follicle stem cells in *Lgr5Cre-Ptch1^{fl/fl}* mice, BCCs arise from *Lgr5*⁺ stem- and progenitor cells in the hair follicle bulge and secondary hair germ, but not from the IFE.¹⁹ From the previous, it can be speculated that the histological phenotype of BCC reflects its cellular origin as it was previously described that sBCCs arise from the IFE, whereas nBCCs may originate from the hair follicle.²⁴

Genetics and signaling pathways

Although the molecular mechanisms involved in the pathogenesis of sporadic BCCs remain to be fully elucidated, most, if not all, BCCs are characterized by aberrant activation of the Sonic Hedgehog (SHH) pathway due to mutations in one or more of its component genes.²⁵ A schematic overview of the essential signaling pathways in BCCs and a more detailed description of their mode of action is presented in figure 1. The SHH pathway is essential for embryonic development and in adult tissues it regulates tissue stem cells required for organ repair and maintenance.²⁶ Also, SHH plays an essential role in the progression of adult hair follicle morphogenesis past the hair germ stage of development, though SHH is not required for its initiation.²⁷ Loss of growth control after aberrant activation of SHH signaling underlines its importance in adult tissues.²⁶ The identification of a germline mutation in the tumor suppressor gene Patched-1 (*PTCH1*) on chromosome 9q22 in BCNS patients revealed the importance of constitutive SHH signaling in BCC development.^{19,25} In sporadic BCC, inactivating *PTCH1* mutations are found in up to 90% of the patients and an additional 10% have activating mutations in the oncogene Smoothened (*SMO*).²⁵ Mutations in Suppressor of fused (*SUFU*) have been reported in only a few cases.²⁸ Normally, in the absence of SHH

ligand, the transmembrane receptor protein PTCH1 inhibits the transmembrane receptor protein SMO and thereby prevents activation of SHH signaling. Binding of a SHH ligand or loss of functional PTCH1 releases the inhibition of SMO, causing nuclear translocation of the transcription factors GLI1 and GLI2 through a series of interacting proteins, including SUFU, which results in subsequent transcription of tumor-promoting genes.^{25,26} Interestingly, the SHH pathway is also activated by sensory nerves in normal touch domes, while nerve ablation and consequent loss of SHH activity attenuates touch dome-derived tumors. This could implicate that cutaneous nerves are important regulators of stem cell niches.²²

Another important pathway associated with hair follicle development and cycling as well as with (skin) tumor development is canonical (β -catenin dependent) Wingless (WNT) signaling.²⁹ Canonical WNT signaling initiates hair bud formation, while subsequent SHH signaling is required for maturation of the hair follicle by promoting the proliferative expansion of the follicle epithelium.²⁰ Murine BCC models showed that WNT signaling is essential for the tumorigenic response to deregulated SHH signaling in the skin (figure 1).^{20,29} This activation of canonical WNT signaling in BCC is suggested by cytoplasmic and nuclear localization of β -catenin in BCC immunohistochemical stains³⁰, though the accuracy of β -catenin immunohistochemical stains as indicator of active WNT signaling is debatable. Non-steroidal anti-inflammatory drugs (NSAIDs) can suppress the WNT pathway by reducing nuclear β -catenin localization and, consequently, attenuating transcription of target genes in colorectal cell lines.^{31,32} However, the main mode of action of NSAIDs seems to be the suppression of elevated cyclooxygenase (COX) enzyme activity. There are two COX isoforms, COX-1 and COX-2, and it is COX-2 that is elevated in several solid tumors, but also in epithelial cancers like BCC.^{32,33} Additionally, UVR increases COX-2 expression in the epidermis.³⁴ COX-2 catalyzes the synthesis of prostaglandins from arachidonic acid. Prostaglandin E2 (PGE2) is increased by UV-induced COX-2 expression. PGE2 is one of the major cyclooxygenase products implicated in UV-induced skin carcinogenesis by increasing tumor cell proliferation, inhibition of apoptosis, stimulation of an inflammatory response, promotion of immunosuppression and facilitation of tumor invasion.³⁴ Inhibition of COX-2 by NSAIDs leads to apoptosis, mostly by blocking the anti-apoptotic Bcl-2 protein and by activation of the caspase cascade.^{35,33} Interestingly, in a double-blinded, randomized 3-year clinical trial studying the effect of oral Celecoxib (200 mg, twice daily, 24 months) on the development of BCC in 60 patients with BCNS, a trend was found towards reduction in BCC burden in the treatment group ($p=0.069$). When considering only the 60% of patients with less severe disease, Celecoxib actually did significantly reduce BCC number and burden ($p=0.024$).³⁶ Topical 3% diclofenac in 2.5% hyaluronic acid is already approved by the U.S. Food and Drug Administration (FDA) in many European countries, though not in the Netherlands, as treatment of actinic keratosis (AK): pre-cancerous lesions of which it is still assumed that they have the potential to develop into SCC.³⁷ A double-blind, randomized, placebo-controlled multicenter study on the efficacy of twice daily application of 3% diclofenac in 2.5% hyaluronic acid for 60 days in patients with AK showed response rates of 79 % versus 45% and complete clearance in 50% versus 20% in the treatment group versus the control group ($p < 0.001\%$) respectively.³⁸ It is becoming more evident that drugs already available for diseases other than cancer could

serve as anti-cancer agents. Of special interest is the comprehensive mode of action of vitamin D.

Vitamin D signaling and its potential role in the pathogenesis of various human cancers is of increasing interest in cancer research (figure 1). $1\alpha,25(\text{OH})_2\text{D}_3$ (calcitriol) is the active metabolite of vitamin D, with antiproliferative and pro-apoptotic effects.³⁹ Calcitriol can also inhibit angiogenesis and it has shown synergistic effects in cancer treatment in combination with cytotoxic anticancer agents and NSAIDs.³⁹ In addition to these direct effects of calcitriol, the vitamin D receptor (VDR) is thought to be crucial in reducing the proliferative action of tumors by indirectly functioning as a tumor suppressor. Activation of the VDR by calcitriol induces the expression of the transmembrane protein E-Cadherin, which recruits β -catenin to the cell membrane, thereby preventing translocation of β -catenin to the nucleus and so inhibiting canonical WNT signaling. Also, calcitriol directly inhibits SMO in vitro resulting in Gli-mediated repression of SHH signaling.^{39,40}

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Figure 1. Actions of NSAIDs and Calcitriol in Basal Cell Carcinoma: a schematic overview.

Sonic Hedgehog Pathway (green). The extracellular protein Sonic hedgehog (SHH) binds to and inhibits Patched (PTCH1), a transmembrane receptor, which relieves the inhibition of another transmembrane protein, Smoothed (SMO). SMO activates glioma-associated oncogene homolog 1 (GLI1) and GLI2, transcription factors that travel into the nucleus to activate the expression of tumor-promoting genes.²⁵ **Canonical WNT signaling pathway (pink).** Binding of a WNT ligand to its specific receptor complex containing a Frizzled (FZD) family member and LRP5 or LRP6 co-receptors, initiates WNT-β-catenin signaling. Axin relocates to the LRP 5/6 tail at the membrane that is bound to WNT through its interaction with dishevelled (DVL), which forms a complex with GSK3β and prevents β-catenin (β-cat) degradation.³¹ This allows β-catenin to accumulate and enter the nucleus, where it interacts with members of the TCF/LEF family. In the nucleus, β-catenin converts the TCF proteins into transcriptional activators. Suppressor of fused (SUFU) functions as a tumor suppressor by inhibiting both SHH and WNT signaling.²⁰ NSAIDs inhibit WNT signaling by reducing nuclear β-catenin localization.^{31,32} Furthermore, NSAIDs inhibit cyclooxygenase-2 (COX-2), which is overexpressed in basal cell carcinoma (BCC) and catalyzes the conversion of arachidonic acid (AA) to prostaglandins (PGE₂). A subsequent reduction of PGE₂ and a direct down regulation of the anti-apoptotic Bcl-2 family proteins by NSAIDs induces apoptosis.³³ A down regulation of Bcl-2 is also induced by 1α,25(OH)₂D₃ (calcitriol), resulting in caspase cleavage leading to apoptosis. Calcitriol directly inhibits SMO *in vitro*, resulting in repression of SHH signaling. SHH-signaling is also suggested to be directly repressed by the Vitamin D Receptor (VDR) by inhibition of Gli. Finally, activation of the VDR by calcitriol induces the expression of the transmembrane protein E-Cadherin, which recruits β-catenin to the cell membrane and prevents translocation of β-catenin to the nucleus.³⁹⁻⁴¹

Epigenetics

Epigenetics refers to hereditary alterations by mechanisms other than changes in the nucleotide sequence of a gene. It has been suggested that epigenetic modifications occur at least as frequently as genetic mutations in the development of cancer.⁴² Epigenetic modifications are considered to be more tumor specific, while genetic alterations are generally patient specific.⁴³ Of these, DNA-methylation is the best-known, consisting of the addition of a methyl group to cytosine nucleotides followed by a guanine (C-phosphate-G (CpG) dinucleotide), catalyzed by enzymes called DNA methyltransferases (DNMTs).⁴⁴ CpG dinucleotides can be found in clusters (CpG islands), mostly in the promoter region of the gene, which is where DNA transcription is initiated. CpG islands are generally hypomethylated, while the CpG dinucleotides in the majority of the genome are hypermethylated to prevent chromosome instability. CpG island hypermethylation is associated with transcriptional silencing, which in the case of tumor suppressor genes could contribute to the development of cancer.⁴⁴ In cancer cells a shift in the distribution of DNA-methylation can be detected: hypermethylation is mainly found in the promoter regions, while the rest of the genome exhibits general hypomethylation. Besides its role in carcinogenesis, DNA-methylation is also instrumental in the inactivation of the X-chromosome and genomic imprinting.⁴⁵ Epigenetic alterations in BCC have previously been reported, but only in limited sample series and without providing further experimental evidence for the relevance of these changes for BCC carcinogenesis.⁴⁶⁻⁵⁰

Immunology

There is increasing evidence that interactions between tumor cells and the surrounding stroma play an important role in BCC development and progression.⁵¹ The peritumoral stroma of BCCs is well-defined and is composed largely of activated fibroblasts, inflammatory cells and vasculature. Soluble paracrine factors such as WNT-1, HGF and TGF- β are secreted by stromal fibroblasts and generally mediate the complex interplay of the stroma with the developing tumor.⁵² In addition to these paracrine factors, inflammatory cells in the peritumoral stroma can secrete growth factors and reactive oxygen species, which further contribute to tumor growth.^{52,53} Several studies support the idea that BCCs are stroma-dependent and that their growth is limited by the host immune response. They fail to grow in culture and transplantation of human BCC biopsies into mice is only successful in T, B and natural killer (NK) cell deficient mice.⁵⁴ Quantification of the relationship between BCC subtypes and their peritumoral stroma revealed an association between the infiltrative subtype and thicker stroma. Also, elastosis was found more frequently in infiltrative BCCs, which could possibly be explained by the presence of extensive sun damage. So peritumoral stroma seems to differ between the different BCC subtypes.⁵²

Over the past three decades, the importance of inflammation in driving tumor progression has been underscored by the chemopreventive effect of NSAIDs targeting COX in several human tissues. Presence of COX allows maturing dendritic cells (DC) to produce interleukin (IL) 12 and so mediate a Th1 adaptive immune response, while in the absence of COX a Th2 response is initiated.⁵⁵ In brief, the Th1 response is pro-inflammatory whereas the Th2 is

essentially anti-inflammatory and promotes allergic responses.⁵⁶ DCs are important (tumor) antigen presenting cells in the skin and they are thought to be crucial in generating an aggressive immune response against cutaneous cancers.⁵¹ This makes DCs attractive targets for immunotherapies, though most trials investigating the effect of DC vaccines in various human cancers have shown minimal clinical success with respect to tumor regression and overall survival rates.⁵⁷ In BCCs, elevated amounts of DCs were found in the dermis and in the tumor after treatment with imiquimod (an imidazoquinoline), resulting in more efficient tumor clearance.⁵⁷ Comparable enhanced cell proliferation is seen during wound healing after tissue injury.⁵³ Interestingly, biopsy-induced regression of BCCs is a well-known clinical observation which, in one study, was seen in up to 20% of cases, with a mean interval of 46 days.⁵⁸ The Th1 initiated immune response is suggested to be essential in the promotion of spontaneous regression after a biopsy⁵⁹, which is in line with the induced Th1 response after topical application of imiquimod.⁶⁰ Conversely to the initiated Th1 response, Kaporis *et.al.* propose the BCC tumor microenvironment to be predominantly Th2 dominant which suggests a dynamic state within the immune microenvironment associated with BCC.⁶⁰ It is known that SCCs but also BCCs are prone to occur in areas of the skin with chronic ulceration, irritation or inflammation; the so called Marjolin's ulcers.⁶¹ While the exact pathogenesis of this malignant degeneration remains unknown, an attenuated immune response is expected due to chronic inflammation (causing hyperproliferation and DNA-damage), recurrent ulceration and healing, damage of lymph- and blood vessels, indurations and scarring.⁶²

Apparently, inflammatory cells have a dual role in BCCs development by, on the one hand, mechanisms like releasing growth/survival factors, promoting (lymph-)angiogenesis and stimulating DNA damage, and, on the other hand the antitumor effects of anti-inflammatory cytokines. So, the challenge for the future is to selectively suppress pro-inflammatory cytokines and increase those cytokines that are anti-inflammatory.⁵³

Clinical presentation and diagnosis

In general, BCCs are easy accessible for clinical evaluation and a trained dermatologist can often diagnose BCCs based on their macroscopic appearance. However, differentiation between the various subtypes can be challenging. Several classification systems have been devised, generally lacking uniformity of terminology and a clear definition.⁶³ A commonly used simplified classification divides BCC into four subtypes: superficial BCC (sBCC); (micro) nodular ('solid') BCC (nBCC); infiltrative BCC (iBCC), including morphoeic type, mixed type and a combination of the aforementioned types.⁶³ An even more simplified system divides BCCs into indolent-growth (including sBCC and nBCC) or aggressive growth (including iBCC, metatypical and morphoeic) subsets.⁶⁴ Differentiation between the BCC subtypes is important since the different histological subtypes are associated with altered biologic behavior and risk of recurrence after inadequate treatment. Although the diagnosis of a BCC is primarily based on clinical features, a punch biopsy is recommended to confirm the diagnosis and histological subtype.⁶⁴ More than one subtype is seen in

18-49% of the BCCs⁶⁴⁻⁶⁶ and it is the most aggressive one that determines the appropriate treatment.⁶⁷ An adequately taken biopsy correctly predicts the most aggressive subtype of the entire BCC in 84.4% of the cases and tumors with missed aggressive areas on a biopsy have a higher risk of recurrence.⁶⁸ The overall agreement between BCC histological subtype on punch biopsy and on the subsequent surgical excision of primary BCC is only 60.9%.⁶⁸ The three most relevant subtypes and a more rare clinical presentation of BCC, so-called (I)aBCC, will be discussed in more detail below. Figure 2 presents clinical pictures of the most common BCC subtypes with their histological presentation.

Figure 2. Clinical and histological presentation of basal cell carcinoma (BCC).

A,B. superficial BCC; **A** clinical and **B** histological presentation. **C,D.** nodular BCC; **C** clinical and **D** histological presentation. **E,F.** infiltrative BCC; **E** clinical and **F** histological presentation. **G, H.** locally advanced BCC; **G** clinical and **H** histological presentation.

Indolent-growth subtypes basal cell carcinoma

nBCC is the most common subtype and typically presents as a pearly erythematous or skin-colored papule with a raised border and arborizing teleangiectasia. Scaling, crusting, ulceration and bleeding are frequently seen. nBCCs predominantly present in the head and neck region of elderly people. On haematoxylin & eosin (H&E) stained histopathology, large nests of basaloid cells with peripheral palisading and a more chaotic arrangement of the central cells are seen in the papillary or reticular dermis. Retraction spaces sometimes form between the tumor islands and the surrounding stroma. The solid nests, as they are often referred to, are stained intense purple (haematoxylin) as the cells have only little cytoplasm.^{18,69} In pigmented BCCs, functional melanocytes are scattered through the tumor islands and a few melanosomes can be found within the tumor cells. In the stroma of pigmented BCCs, numerous melanophages are present.⁶⁹ Characteristic dermoscopy features are large gray-blue ovoid nests, multiple gray-blue globules, maple-leaf like areas, and spoke-wheel areas.⁷⁰

sBCC is the second most common subtype, occurring more often at a younger age and frequently located on the trunk.^{18,71} A typical sBCC presents as a slowly growing, well-demarcated, erythematous macule or patch, sometimes with a small erosion and a border of translucent small papules, varying in size from a few millimeters to centimeters. In sBCCs, as in nBCCs, variable amounts of melanin pigment may be present. sBCCs can mimic eczema or psoriasis, while lichenoidkeratosis, actinickeratosis and Bowen's disease are further main differential diagnoses.¹⁸

Histologically, in sBCCs small buds (mostly multifocal) of proliferating basal cells spread from the undersurface of the epidermis into the papillary dermis with preservation of attachment to the epidermis. There is palisading of the cells at the periphery and slit-like retraction from the subjacent stroma is frequently seen.⁶⁹

Aggressive-growth subtypes basal cell carcinoma

Infiltrative and morphoeic BCCs present as pink-to-ivory white, shiny, smooth, indurated or depressed atrophic or scar like plaques with poorly defined borders. Although teleangiectasias, erosions, or small crusts may be seen, these signs are frequently absent. Lesions can be missed because their appearance is subtle. Also, they may be misclassified as scar tissue, morphea (localized scleroderma), dermatofibrosarcoma protuberans, Merkel cell carcinoma, amelanotic melanoma, Bowen's disease, eczema, microcystic adnexal carcinoma, and other adnexal neoplasms.^{18,64} Because of their infiltrating behavior, infiltrating and morphoeic BCCs tend to be more aggressive and their propensity to subclinical spread may cause a diagnostic delay.¹⁸ Histologically, elongated strands of basaloid cells one to eight cells thick extend between collagen bundles in the dermis and even into the subcutaneous fat. Additionally, infiltrative BCCs can also form large nodules. Peripheral palisading is mostly absent and retraction artifacts are uncommon.^{18,69} A summary of the prognostic factors classifying BCCs into low-risk versus high-risk categories is presented in table 1.

Table 1. Prognostic factors determining treatment success in basal cell carcinoma⁶⁷

	Low-risk	High-risk
Histological growth pattern	Not-aggressive (superficial or nodular)	Aggressive (infiltrative or micronodular)
Localization	Trunk	H-zone (eyes, ears, lips, nasolabial fold, nose)
Size	<2 cm	>2 cm
Previous treatment	Primary tumor	Recurrent tumor

(Locally) advanced basal cell carcinoma

Aggressive-growth BCCs and indolent-growth BCCs that are neglected for many years or inadequately treated have a greater potential of progressing to (I)aBCC. (I)aBCCs are defined as ‘basal cell carcinoma of American Joint Committee on Cancer (AJCC) stage II or above⁷², in which current treatment modalities (surgery and radiotherapy) are considered potentially contraindicated by clinical or patient-driven factors’. Stage II BCCs are categorized as T2 tumors (larger than 2 cm across, or any size with 2 or more high-risk features) according to the AJCC TNM staging system.⁷² Disease factors leading to a diagnosis of advanced BCCs are, amongst others, tumor size, location, subtype and number. Patient age, performance status and quality-of-life-effects of treatment are examples of patient-driven factors (table 2).⁷³ (I)aBCCs generally show deep tissue invasion, may metastasize and cause substantial morbidity or even mortality.⁷⁴ Histologically they are indistinguishable from common BCC. Additional diagnostics, such as magnetic resonance imaging (MRI) or computed tomography (CT) are often necessary to evaluate the expansion of the tumor and a multidisciplinary approach is recommended to manage these patients. Frequently, these tumors are not eligible for surgery because free resection margins are not likely to be achieved, or because surgery has a high morbidity or can be expected to be mutilating. Contraindications, such as prior radiotherapy can also be present. Prior radiotherapy, location, size or patients restrictions such as the many visits to the hospital may limit the usefulness of radiotherapy. Also, radiotherapy in BCNS patients with (I)aBCC is contra-indicated, because of the risk of inducing new BCC in the irradiated area.⁷⁵ Treatment with cytotoxic agents such as cisplatin has been reported, but there is insufficient evidence to support their efficacy in the treatment of (I)aBCCs and metastatic BCCs (mBCCs).⁷⁶ As mentioned before, BCCs rarely metastasize, however when metastasis does occur, the prognosis is very poor with mean survival ranging from 8 months to 3.6 years.⁷⁷ Lymphogenic metastasis in regional lymph nodes is most common in these cases, followed by haematogenic metastasis to the lungs and the liver.⁷⁸ The development of SMO inhibitors as treatment for (I)aBCCs and mBCCs is expected to be promising, given that the majority of the BCCs are characterized by aberrantly activated SHH signaling. SMO inhibition will be discussed in more detail in the following section.

Table 2. Definition of advanced basal cell carcinoma (BCC), with tumor and patient factors that guide a diagnosis of advanced BCC⁷³**Definition of advanced BCC**

'Basal cell carcinoma of American Joint Committee on Cancer (AJCC) stage II or above, in which current treatment modalities are considered potentially contraindicated by clinical or patient-driven factors.'

Factors guiding a diagnosis of advanced BCC**Disease factors**

Tumor size (e.g., giant BCC)
 Tumor location (e.g., H-zone tumors)
 Number of tumors
 Tumor subtype (e.g., morphoeic tumors)
 Likelihood of successful treatment (e.g., recurrent BCC)

Patient factors

Patient age (e.g., radiotherapy in young patients)
 Patient performance status (e.g., frail patients)
 Quality-of-life-effects of treatment (e.g., poor cosmetic outcome)
 Patient opinions regarding treatment
 Presence of genodermatoses with increased risk for cancer (e.g., Gorlin's syndrome)
 Presence of comorbidities (e.g., organ transplant)

Treatment

Common therapies

For the different types of BCCs, a variety of invasive and non-invasive treatments are available.¹⁸ Conventional surgical excision is the gold standard in the treatment of all BCCs.⁶⁷ According to the Dutch guideline, surgical excision with a clinical safety margin of 3 mm in low risk BCCs and 5 mm in high risk BCCs (table 1) followed by histological examination is an effective treatment.^{67,79} Even though only a small part of the specimen margin is examined, i.e. less than 2%⁸⁰, recurrence rates, five years after conventional surgical excision, are rather low: 4-10% for primary BCCs (pBCCs) and up to 12.1%-17% for recurring BCCs (rBCCs).^{79,81-84} The cumulative probability of recurrence after ten years is still high with 12.2% for pBCCs and 13.5% for rBCCs.⁸⁵ In contrast to conventional excision, Mohs' micrographic surgery provides a three-dimensional histological visualization and 100% evaluation of resection margins.⁸⁶ By assessment of horizontal frozen tissue sections during the surgical procedure, complete assessment of the resection margins can be obtained. Hereby, assessment of resection margins is thought to be more accurate and a further advantage is, that because of the use of frozen sections, reconstruction of the defect can be completed on the same day.⁸⁷ Mohs' micrographic surgery is especially suitable for primary high risk BCCs in the face (five-year recurrence rate of 3-6%) and for rBCCs in the face it is the first-line treatment (five-year recurrence rate of 4,8-10%).^{79,84,87} After ten years, the cumulative probability of recurrence is 4.4% for pBCC and 3.9% of rBCCs.⁸⁵ The func-

tional and cosmetic outcomes are generally better after Mohs' surgery in delicate areas of the face, as more healthy skin is preserved than after conventional surgical excision.⁸⁸ Other invasive treatments, such as cryosurgery or curettage followed by electrocoagulation, can be considered for small low risk BCCs. However, in general these treatments are not recommended because of the higher recurrence rates and worse cosmetic outcome.⁶⁷

Radiotherapy is an effective non-invasive treatment for primary BCCs on all body parts, however it is mainly reserved for irresectable tumors or for patients who cannot undergo surgery.⁸⁹ There is only limited evidence on the long term effects, but in two older studies recurrence rates in primary BCCs after two to four years are found to be 4-7.5%.^{79,90} In rBCCs reported recurrence rates varies between 9.5-16% after five-year of follow-up.⁹¹⁻⁹³ The Dutch guideline recommends radiotherapy particularly for BCC located in the face or around the ear to preserve cosmesis when surgery isn't desired.⁶⁷ Better cosmetic outcome is achieved with lower dosage fractions during a longer period (17- 20 fractions of 3– 3.5 Gy in 4-5 weeks), associated with less post-radiation atrophy, telangiectasias, fibrosis and necrosis.⁶⁷ As already described above, radiotherapy is contra-indicated in patients with BCNS.⁷⁵

Additional non-invasive treatment modalities which include photodynamic therapy (PDT) (photochemical), imiquimod (immune-modulating) cream and 5-fluorouracil (5-FU) (chemotherapeutic) cream, are preferably only used for sBCC only. Tumor-free survival rates in sBCCs vary from 72.8-84.0% (PDT), 83.4-87.3% (imiquimod) and 80.1% (5-fluorouracil), however, they do not reach those of surgical excision.^{94,95} A recently published randomized controlled trial investigated the effect of imiquimod cream in nBCCs and found clearance rates of 81.8% after 3 years.⁹⁶ Non-invasive treatments may have several advantages, such as lower healthcare costs and a decrease in the workload for dermatologists. Also, a better cosmetic outcome with an increase in quality of life for patients can be expected.⁹⁶

Targeted therapies

Targeted therapies are drugs that can block crucial biochemical pathways or mutant proteins that are required for tumor cell growth and survival. With these, further tumor progression can be prevented and even tumor regression can be induced.⁹⁷ The feasibility of blocking SHH was already suggested in the 1950s by an epidemic of sheep with congenital deformities in Idaho.⁹⁸ Sheep that grazed on wild corn lily (*Veratrum californicum*) during gestation gave birth to lambs with cephalic defects and cyclopia (one eye). Cyclopamine (11-deoxojervine), a steroidal alkaloid, was identified as the teratogene.⁹⁹ Because of the similarities between cyclopamine induced developmental malformations and holoprosencephaly (one-lobed brain)-like abnormalities associated with loss of SHH function, it was suggested that cyclopamine is capable of inhibiting the cellular response of the SHH signal.¹⁰⁰ Taipale *et.al.* showed *in vitro* in primary murine fibroblasts lacking functional PTCH (PTCH^{-/-}) that cyclopamine inhibits the SHH pathway by antagonizing SMO.¹⁰¹ Furthermore, several mouse models confirmed the effect of cyclopamine on tumor shrinkage.¹⁰⁰ However, poor oral bioavailability, low affinity for the receptor and inadequate pharmacokinetics¹⁰² all

conspire to make cyclopamine less suitable as treatment for BCCs in daily practice. Though, the discovery of the usefulness of cyclopamine as antagonist of SMO has led to the emergence of several synthetic, cyclopamine-like SMO antagonists, the so-called small molecule inhibitors. SHH and GLI molecularly targeted therapies are also upcoming^{103,104} and all these drugs that interfere with the SHH pathway are frequently referred to as 'Hedgehog Pathway Inhibitors' (HPIs). In 2012 the FDA approved vismodegib 150 mg capsules as first in class for the treatment of (I)aBCCs and mBCCs.¹⁰⁵ Vismodegib is an orally active synthetic cyclopamine-like SMO-inhibitor. In a phase 2 study, complete and partial response to vismodegib was seen in 43% and 30% of (I)aBCCs and mBCCs patients, respectively¹⁰⁶ and recent follow-up results showed increasing response rates of respectively 47.6% and 33.3%.¹⁰⁷ Tumor responses are generally observed within the first 2 months of treatment but can take up to 10 months to manifest.⁷⁷ Only recently, pre-planned interim results of a large international phase 2 study into the safety of vismodegib became available, showing that at least one adverse event was reported by essentially all patients treated (99%). However, the reported adverse events were mainly mild or moderate. The most common adverse events included muscle spasms (64%), alopecia (62%), dysgeusia (54%) and weight loss (33%). Discontinuation of treatment was observed frequently (45%), however the majority of these patients with mild to moderate adverse events had already achieved partial or complete response when they stopped. Serious adverse events were noted in 22%.¹⁰⁸ As the same adverse events are reported in patients treated with sonidegib, another SHH signaling pathway inhibitor, it seems logical to conclude that they are the result of the direct inhibitory effect on this pathway, a phenomenon that is also known as the class effect.¹⁰⁹

Despite the reasonable response rates, initial response to vismodegib followed by secondary tumor progression has been observed more and more in BCCs.^{1,110-113} Acquired resistance to vismodegib has also been described in medulloblastoma, caused by a heterozygous SMO mutation found in the metastatic medulloblastoma that developed during treatment.¹¹⁴ Atwood *et.al.* recently showed that in BCCs, a large number of *SMO* variants are responsible for the maintenance of SHH signaling in the presence of SMO inhibitors.¹¹⁵ More knowledge about the mutational status of pre-treatment and recurrent tumor tissue will help to anticipate on the type of resistance in order to proactively alter treatment. Perhaps in the near future, treatment schedules and combination therapies can then be customized to both patient and tumor characteristics.

Aims of this thesis

The content of this thesis is subdivided into two parts:

Part one, which focuses on the pathogenesis of BCC, and part two, in which new topical and systemic treatments of BCCs are described. The main aim of part one is to evaluate epigenetic alterations, i.e. aberrant DNA-methylation, in both non-aggressive and advanced BCCs. Epigenetic alterations in BCCs have previously been reported, but only in limited sample series.⁴⁶⁻⁵⁰ Promoter hypermethylation results in gene silencing and can precede genetic mutations and genomic instability in tumor development, and may thus not only be crucial for carcinogenesis, but also represent a potential therapeutic target.^{44,116} Assessing the methylation status of tumor suppressor genes that are expected to be involved in BCCs pathogenesis would provide a first insight into the importance of DNA-methylation in BCC. Additional genome-wide analysis of the BCC epigenome and also RNA- expression levels (transcriptome) could further reveal new perspectives in BCC biology, and even in BCC treatment.

Part two aims to address the effectiveness of new topical and systemic treatments in BCCs management, their risks and their use in daily practice. Conventional surgical excision is the gold standard and most effective treatment for all types of BCC.^{66,117} However, lower health-care costs, a decrease in the workload for dermatologists and a better cosmetic outcome with an increase in quality of life for patients can be expected with non-invasive treatments for low-risk BCCs.¹¹⁷ Current research on BCCs focuses on compounds that specifically target key signaling pathways required for tumor growth, however the available non-invasive treatments for low-risk BCCs as such are not specifically targeted. There is increasing evidence that drugs already available for diseases other than cancer, like NSAIDs (anti-inflammation) and calcitriol (psoriasis) can be re-purposed as anticancer-agents.³¹ And since NSAIDs can target the WNT signaling pathway, calcitriol the WNT and SHH pathway and since both drugs can induce apoptosis^{31,39}, we assumed that they could have promising therapeutic effects in BCCs. We additionally hypothesized that simultaneously targeting different signaling pathways or pathway elements may have a synergistic effect, as suggested by several (pre-)clinical studies.¹¹⁸ A combination of NSAIDs and calcitriol would then be a logical step in BCCs treatment.

For (l)aBCCs, an orally active synthetic cyclopamine-like SMO-inhibitor (vismodegib) shows reasonable response rates, however initial response to vismodegib followed by secondary tumor progression has already been observed.¹¹⁰⁻¹¹³ Only recently it was shown that various *SMO* mutations are responsible for the maintenance of SHH signaling in the presence of SMO inhibitors.¹¹⁵ A better insight into the possible rapid occurrence of resistance to vismodegib, apparently caused by different acquired mutations could help to dynamically adapt treatment to both patient and tumor characteristics.

Part one: pathogenesis of BCC

Chapter 2 of this thesis provides a comprehensive analysis of genetic, epigenetic and RNA-expression changes in BCCs, and consists of 3 paragraphs. The genetic background of BCC has been studied extensively, while so far its epigenetic makeup has received comparatively little attention. Epigenetic modifications are heritable changes in DNA structure other than alterations in DNA sequence capable of driving malignant tumor development and progression. To date, DNA hypermethylation is the best-characterized epigenetic mechanism, comprising the addition of a methyl group to a cytosine base adjacent to a guanine base (the CG dinucleotide).⁴⁴ CG-rich areas (CpG islands) located in the promoter region of many tumor suppressor genes become hypermethylated in numerous malignancies¹¹⁹, which results in gene silencing.¹²⁰ The main hypothesis of this chapter is that DNA-methylation plays a significant role in BCC tumorigenesis and leads to differential expression of the affected genes. In **chapter 2.1** we sought to analyze the promoter methylation status of ten putative (tumor suppressor) genes that are associated with SHH, WNT signaling and (hair follicle) tumors in BCC and healthy control samples by methylation-specific PCR. In **chapter 2.2** we aimed to expand the current understanding of BCC biology by combining genome-wide methylated DNA enrichment sequencing (MethylCap-seq) and RNA sequencing on primary BCCs and healthy skin controls. High-throughput next-generation sequencing can provide data about the genome, methylome and transcriptome of malignancies and normal tissue of any organism. **Chapter 2.3** examines a laBCC of the face. (I)laBCCs are tumors capable of deep tissue invasion, have higher metastasis rates and substantial morbidity and even mortality compared to common BCCs.⁷⁴ Since (I)laBCCs are histologically indistinguishable from common BCCs, we looked for differences in methylation between this type of tumor and common BCCs, that would help to elucidate the origin of this relatively uncommon presentation. We discuss the results of genomewide MethylCap-seq and RNA-seq of a laBCC compared with regular BCCs and healthy skin.

The aim of **chapter 3** is to probe hypoxia signaling in benign and malignant hair follicle tumors: trichoepithelioma (TE) and BCC. While the human dermis is well oxygenated and the epidermis is modestly hypoxic, the hair follicle is considered as a moderately to severely hypoxic microenvironment.¹²¹ Based on the hypoxia affecting the hair follicle and the known role of hypoxia related signaling in tumor growth¹²², we hypothesized that hypoxia response pathways are involved in the pathogenesis of TEs and BCCs. In this study, the results of immunohistochemical analysis of the most important target genes in the hypoxia response are presented.

Part two: new topical and systemic treatment modalities for BCC

Chapter 4 comprises two parts. In **chapter 4.1** we discuss the results of a single-blind, phase II, randomized controlled trial comparing topical diclofenac, calcitriol and a combination of both as treatment for sBCCs and nBCCs. Diclofenac and calcitriol are both associated with a reduction of tumor proliferation and induction of apoptosis. Also, diclofenac can inhibit WNT-signaling and the SHH signaling pathway can be inhibited by calcitriol.^{31,33,39,123} We aimed to test the hypothesis that treatment of sBCCs and nBCCs with diclofenac and/or calcitriol would lead to a histologic reduction of the marker Ki-67 (proliferation) and induction of BCL-2 (apoptosis). **Chapter 4.2** discusses reproducibility of semi-automated quantification compared to manual quantification of two well-known immunohistochemical markers in sBCCs and nBCCs using a dedicated software program. In general, manual, pathologist-based quantification remains the current standard for IHC evaluation.¹²⁴ However, a quantitative estimation of percent positivity is a subjective, pathologist-dependent approach and may demonstrate high intra- and inter-observer variability.^{125,126} Additionally, manual quantification is a time-consuming process.¹²⁷ So therefore, the hypothesis underlying this study was that semi-automated quantification is a reproducible and time-efficient manner to analyze established immunohistochemical markers.

The aim of **chapter 5** is to gain insight into the mechanisms underlying resistance against the orally active synthetic cyclopamine-like SMO-inhibitor vismodegib in patients with laBCCs. Vismodegib, a Hedgehog (HH) pathway inhibitor, has been approved in 2012 by the US Food and Drug Administration (FDA) for the treatment of (l)aBCCs and mBCCs.¹²⁸ Recent follow-up results of a phase II trial showed complete and partial response rates of 47.6% ((l)aBCC) and 33.3% (mBCC) of patients, respectively.¹⁰⁷ Initial response to vismodegib followed by secondary tumor progression has already been observed.^{1,110-113} This chapter consists of two parts. In **chapter 5.1** we describe a patient with a laBCC on the dorsal side of her left shoulder. She developed resistance to vismodegib after an initial complete clinical response. We hypothesized that SMO mutations may develop during vismodegib treatment, leading to secondary resistance. Mutational analysis of *PTCH1* and *SMO* was performed on primary tumor, as well as on newly developed tumors and adjacent normal skin in an attempt to confirm our hypothesis. **Chapter 5.2** describes a patient with a laBCC of the nose, right cheek and upper lip, who developed hyperkeratosis and comedo-like lesions after 7 weeks of vismodegib treatment. We hypothesized that the primary BCC had transformed into a SCC. Therefore, immunohistochemical analysis was performed to rule out this possibility.

Chapter 6 provides a general discussion and valorization. In **chapter 7** a summary of the main findings of this thesis is given.

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PART

PATHOGENESIS OF BASAL CELL CARCINOMA

Chapter 2.1

Epigenetic changes in basal cell carcinoma effect SHH and WNT signaling components

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Abstract

Background: The genetic background of Basal Cell Carcinoma (BCC) has been studied extensively, while its epigenetic makeup has received comparatively little attention. Epigenetic alterations such as promoter hypermethylation silence tumor suppressor genes (TSG) in several malignancies.

Objective: We sought to analyze the promoter methylation status of ten putative (tumor suppressor) genes that are associated with Sonic Hedgehog (SHH), WNT signaling and (hair follicle) tumors in a large series of 112 BCC and 124 healthy control samples by methylation-specific PCR.

Results: Gene promoters of *SHH* ($P=0.016$), adenomatous polyposis coli (*APC*) ($P=0.003$), secreted frizzled-related protein 5 (*SFRP5*) ($P=0.004$) and Ras association domain family 1A (*RASSF1A*) ($P=0.023$) showed significantly more methylation in BCC versus normal skin. mRNA levels of these four genes were reduced for *APC* and *SFRP5* in BCC ($n=6$) vs. normal skin ($n=6$). Down regulation of SHH, APC and RASSF1A could be confirmed on protein level as well ($P<0.001$ for all genes) by immunohistochemical staining. Increased canonical WNT activity was visualized by β -catenin staining, showing nuclear β -catenin in only 28/101 (27.7%) of BCC. Absence of nuclear β -catenin in some samples may be due to high levels of membranous E-cadherin (in 94.1% of the samples).

Conclusions: We provide evidence that promoter hypermethylation of key players within the SHH and WNT pathways is frequent in BCC, consistent with their known constitutive activation in BCC. Epigenetic gene silencing putatively contributes to BCC tumorigenesis, indicating new venues for treatment.

Introduction

Basal cell carcinoma (BCC) accounts for 75% of all skin cancers and is the most frequent malignancy in Caucasians. Its incidence is rising by 3-8% each year¹, resulting in an average lifetime risk for Caucasians of developing BCC of 30%.^{2,3} BCC rarely metastasize⁴, but when left untreated they may cause extensive local tissue destruction.^{4,5} Surgical excision is the current standard treatment, with average costs in the Netherlands of €900 per procedure, amounting to a total of €45 million by 2015.^{6,7} The inevitably rising workload can be expected to stress the health care system even further. Hence, BCC is becoming a serious health problem. There is a clear need for a simple and cost-efficient medical treatment. In order to develop one, a thorough understanding of BCC pathobiology will be required and the past few years have witnessed considerable progress in this respect.

Inappropriate activity of the Sonic Hedgehog (SHH) pathway due to mutations of its component genes is firmly implicated in BCC pathogenesis. Inactivating mutations of Patched-1 (PTCH1) are the most common (30-60%) in sporadic BCC⁸⁻¹¹, followed by mutations (10-20%) in Smoothened (SMO) or Suppressor of Fused (SU(FU)).¹¹⁻¹⁶ Even though mutations of the SHH target gene, Glioma-associated oncogene homolog-1 (GLI1), are uncommon, approximately 90% of sporadic BCC overexpress GLI1, which contributes to tumor growth.¹⁷ In addition, Yang and colleagues proved the wingless-type MMTV integration site family (WNT) pathway to be essential in tumorigenic response to deregulated SHH signaling, suggesting crosstalk between the SHH and canonical WNT pathways in BCC.¹⁸ Non-canonical WNT signaling, however, might also be active in the context of repressed canonical WNT signaling in BCC, as recently reported by Pourreya et al.¹⁹ Thus, both WNT signaling pathways might act in a mutually antagonistic fashion in driving BCC growth. In all, the genetic aspects of BCC development seem to be well defined. However, most if not all cancers are characterized by epigenetic alterations in addition to genetic changes.²⁰ Epigenetic modifications are heritable changes in deoxyribonucleic acid (DNA) structure other than alterations in the DNA sequence, and when pathologically altered are capable of driving malignant tumor development and progression. To date, DNA hypermethylation is the best-characterized epigenetic mechanism, comprising the addition of a methyl group to a cytosine base adjacent to a guanine base (the CG dinucleotide).²⁰ CG-rich areas (CpG islands) located in the promoter region of many genes become hypermethylated in numerous malignancies²¹ leading to gene silencing.²² Promoter hypermethylation can precede genetic mutations and genomic instability in tumor development, and may thus not only be crucial for carcinogenesis, but also represent a potential therapeutic target.²³ Indeed, DNA demethylating agents, such as 5-azacytidine (Vidaza®, Celgene) and 5-aza-2'-deoxycytidine (Dacogen™, MGI Pharma) can lead to reactivation of silenced genes.²⁴ Thus, it would be of interest to determine the contribution of promoter hypermethylation to BCC pathogenesis. To date, a very restricted number of studies have addressed this question in a limited number of samples.²⁵⁻³⁰ Therefore, we decided to assess the promoter CpG island methylation status of nine tumor suppressor genes (TSGs). Patched Homolog 1 (PTCH1) is the SHH receptor and is the most commonly mutated tumor suppressor in BCC, whereas adenomatous polyposis coli

(APC), secreted frizzled-related protein 1 (SFRP1), SFRP2, SFRP4, and SFRP5 are all negative regulators of the canonical WNT pathway. Sustained signaling through the canonical WNT pathway contributes to the development of colorectal cancer as well as basal cell carcinoma.^{18,19,31} Since BCC are considered as hair follicle tumors, we additionally analyzed the cylindromatosis (CYLD) and tuberous sclerosis-1 (TSC1) genes, which both are TSGs known to be involved in classic hair follicle tumor syndromes.^{32,33} Additionally, TSC complex proteins are crucial negative regulators of mTOR activity, which is implicated in tumor growth.³⁴ Ras association domain family 1A (RASSF1A) is a well-known TSG, promoter methylation of which has been described as an early and frequent event in several malignancies including basal cell carcinoma.^{28,35} Finally we examined SHH, since we hypothesized that downstream activation of its signaling pathway would permit it to become inactivated through methylation.

We have demonstrated significant hypermethylation affecting several of the selected genes in BCC, supporting the involvement of epigenetic aberrations in the most common skin cancer.

Materials and Methods

Primary tissue specimens

The methylation status of ten selected genes was examined in 112 sporadic BCC samples (107 formalin-fixed paraffin-embedded (FFPE), five fresh frozen (FF)) diagnosed at the Department of Dermatology, MUMC+. All BCC samples were obtained from the Maastricht Pathology Tissue Collection (MPTC). Distinct histological subtypes included were: superficial (sBCC) ($n=37$), nodular (nBCC) ($n=53$), and infiltrative (iBCC) ($n=22$). Thirty of these samples were used for immunohistochemical analysis of the expression of SHH, APC and RASSF1A. β -catenin and E-cadherin were assessed on respectively 101 (30 sBCC, 40 nBCC, 31 iBCC) and 59 BCC (17 sBCC, 27 nBCC, 15 iBCC) independent samples. Patients included 58 men (mean age 68.1 years, SD $\pm$ 11.0) and 54 women (mean age 66.2 years, SD $\pm$ 15.1) A total of 124 healthy control tissue samples (71 FFPE, 53 FF) was collected at autopsies and matched with the 112 BCC patients with regard to age and gender (Table 1). Relatives gave their written consent for usage of the skin tissues. For 31 of these controls, no data were available concerning age and gender. Collection, storage and use of all tissues and patient data were performed in agreement with the "Code for Proper Secondary Use of Human Tissue in the Netherlands". All of the used samples and corresponding data were de-linked and anonymized. Usage of both BCC and control tissue samples was approved by the MPTC scientific committee (MPTC 2009-05).

DNA isolation

From each paraffin tissue block and FF sample, a 4 μ m section was cut and stained with haematoxylin & eosin (H&E) as a part of the routine intake procedure. All H&E sections were reviewed by a dermato-pathologist (AM or VW) to confirm diagnosis and histological subtype. After de-paraffinization of five sections (20 μ m) of each FFPE sample, tumor tissue was macroscopically scraped and collected in 1.5 ml tubes. The sections of the FF samples were directly collected into 1.5 ml tubes. Genomic DNA was extracted from FFPE tissue sections by using a Qiagen microkit for DNA isolation (Qiagen, Venlo, The Netherlands) following the manufacturer's directions.

Bisulfite modification of genomic DNA, methylation-specific PCR and bisulfite genomic sequencing

Promoter CpG island methylation was determined by bisulfite genomic sequencing (BGS) for *SHH*, and by MSP for *PTCH1*, *SHH*, *APC*, *SFRP1*, *SFRP2*, *SFRP4*, *SFRP5*, *CYLD*, *TSC1*, and *RASSF1A*. Sodium bisulfite modification was performed on 500 ng of genomic DNA isolated from the tissue sections by use of an EZ DNA methylation kit (Zymo Research Co, Orange, CA, USA) according to the manufacturer's instructions. The modified DNA was eluted to 50 ng/ μ l in H₂O and stored at -80°C. Nested methylation specific polymerase chain reaction (MSP) was performed on bisulfite-modified genomic DNA with primers specific for methylated DNA and unmethylated DNA as previously described³⁶⁻³⁸. Nested MSP is exquisitely suitable for methylation analysis of FFPE DNA

since it is highly sensitive. Primer sequences and PCR conditions are listed in Table S1. To assess reproducibility of the nested MSP approach, MSP reactions were performed in duplicate starting from DNA amplification with flanking polymerase chain reaction (PCR) primers.

Table 1. Sample characteristics

	Tumor (n=112)	Normal skin (n=124)
Sex		
Male, n (%)	58 (51.3)	50 (53.8)
Female, n (%)	54 (48.2)	43 (46.2)
Unknown, n (%)	0 (0)	31 (25.0)
Age, years mean		
Overall	67.2 ($\pm$ 13.1 SD)	68.1 ($\pm$ 14.0 SD)
Man	68.1 ($\pm$ 11.0 SD)	67.9 ($\pm$ 12.2 SD)
Women	66.2 ($\pm$ 15.1 SD)	68.4 ($\pm$ 16.0 SD)
Tissue Source		
FFPE, n (%)	107 (95.5)	71 (57.3)
FF, n (%)	5 (4.5)	53 (42.7)
BCC subtypes, n (%)		
Superficial	37 (32.7%)	
Nodular	53 (46.9%)	
Infiltrative	22 (19.6%)	
* FFPE= Formalin-fixed, paraffin-embedded, FF= Fresh- frozen		

Non-concordant MSP results were analyzed a third time and concordance in two out of three assays was accepted as end result. The overall reproducibility for the MSP data was 90.3%. For BGS analysis, one μ l of PCR product from bisulfite-modified genomic DNA was cloned into TOP10 bacteria using a TOPO-TA cloning kit (Invitrogen, Breda, The Netherlands). Bacteria were cultured at 37°C overnight. DNA was extracted from at least ten independent bacterial clones and sequenced using an automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing data was analyzed using Sequence Scanner v1.0 software (Applied Biosystems). Mean values were calculated and represented in pie-chart figures per CpG.

RNA isolation, reverse transcription and quantitative reverse transcription PCR (RT-PCR)

Total ribonucleic acid (RNA) from FF samples containing more than 75% BCC cells (n=6) as evaluated from H&E stained sections and FF normal skin samples (n=6) was isolated using the standard procedure for TRIzol® RNA extraction (Invitrogen) and stored at -80°C. Complementary DNA (cDNA) synthesis was performed using the iScript™ cDNA Synthesis kit (Biorad, Veenendaal, The Netherlands) according to the manufacturer's instructions. Quantitative RT-PCR on 30ng cDNA was carried out using primer sets for APC, RASSF1A, SFRP5, and SHH. Primer sequences and PCR conditions are listed in Table S1. Messenger RNA (mRNA) for SHH and RASSF1A could not be detected. APC and SFRP5 expression levels for each BCC sample were normalized to the house-keeping gene Cyclophilin A and average expression levels in normal skin tissues (n=6) by $2^{-\Delta\Delta Ct}$ parameter³⁹. To assure accuracy, all reactions were performed in triplicate.

Immunohistochemistry

For SHH, APC and RASSF1A analysis, FFPE sections (4 µm) were deparaffinized in xylene, rehydrated and incubated in 0.3% hydrogen peroxide (H₂O₂) in methanol for 30 minutes to inactivate endogenous peroxidase activity. Antigen retrieval was performed by microwave treatment at 90 W for 10 minutes in 10 mM citrate buffer (pH 6) (APC and SHH) or Envision Flex target retrieval solution high pH (Dako, Heverlee, Belgium) (RASSF1A). Next, non-specific protein binding was blocked using 3% bovine-serum-albumin (BSA). Subsequently, the sections were incubated for 1h at room temperature with primary antibodies listed in table S2. A horseradish peroxidase (HRP)-conjugated second antibody, either PowerVision+ (ImmunoVision Technology, Brisbane, CA, USA) (APC and SHH) or Envision detection system (Dako) (RASSF1A) was applied for 30 minutes. Bound antibody was visualized by using 3,3-diaminobenzidine (DAB) for 10 minutes. Tissue was counterstained with haematoxylin, dehydrated and sealed with coverslips. Phosphate-buffered saline (PBS) was used throughout for washing steps. For β-catenin and E-cadherin analysis, sections were pre-treated in a pre-treatment module using EnVision FLEX Target Retrieval Solution, High pH (Dako). Staining was performed on a Dako autostainer system using the Dako Envision Flex kit (K8002) for secondary detection. Sections were counterstained with Gill II haematoxylin, dehydrated and sealed with coverslips. For all antibodies, tissue known to be strongly expressing respective protein was included as positive control (Table S2). Negative controls (omission of the primary antibody) were included in all experiments.

Interpretation of staining

A specialized dermato-pathologist of the Department of Pathology, MUMC+ (VW) and an experienced resident of the Department of Dermatology, MUMC+ (TB), examined sections independently. Any discrepancy between the observers was discussed and resolved by consensus. β-catenin staining was assessed with respect to localization (membranous, cytoplasmic, and nuclear). For the other antibodies, the percentage positive tumor cells were determined by assessing ten randomly chosen high-power-fields (magnification 200x) per

slide and the average of both observers' values was used for analysis. Additionally, the intensity of E-cadherin was considered with respect to an internal positive control ranging from 0 (no staining) to 1 (weak), 2 (moderate), and 3 (strong) staining.

Statistics

Statistical analyses were carried out using SPSS version 18.0 software (SPSS, Chicago, IL, USA). Discrete data were analyzed using a Chi-square or Fisher's exact test, where the independent-samples T-test was used for continuous variables. The correlation between two discrete variables was evaluated by the Cohen's kappa.⁴⁰ To evaluate the effect of methylation on the probability of the presence of tumor, multivariate binary logistic regression analyses were performed with presence or absence of tumor as dependent variable. The two way random effect model with absolute agreement intra-class correlation coefficient (ICC) was used as inter-rater reliability analysis to determine consistency among raters.⁴¹ An ICC ≥ 0.75 indicates excellent reproducibility.⁴² All reported *P* values are two-sided, and *P* values ≤ 0.05 were considered statistically significant.

Results

Methylation of SHH and WNT pathway components in BCC

A total of nine candidate TSGs and one oncogene was examined in a series of primary BCC ($n=112$) of three different subtypes and normal skin ($n=124$). Characteristics of the tumor and normal skin samples are listed in Table 1. Primers located in promoter CpG islands were previously determined.³⁶⁻³⁸ Our data showed that promoters of four genes were significantly more frequently methylated in BCC tissue versus normal skin: *SHH* (44/100 (40.0%) vs. 30/122 (24.6%), $P=0.016$), *APC* (64/110 (58.2%) vs. 47/124 (37.9%), $P=0.003$), *SFRP5* (52/109 (47.7%) vs. 28/100 (28.0%), $P=0.004$), and *RASSF1A* (52/112 (46.4%) vs. 39/124 (31.3%), $P=0.023$). The correlation between methylation in *SHH* and *SFRP5* was moderate (kappa 0.68 (95% CI 0.50-0.78)); no further correlations between genes could be detected. After mutual correction of the two genes *SHH* and *SFRP5* by multivariate logistic regression, odds ratios for individual genes were still larger than 1 (O.R. 1.42 ($P=0.304$) and 2.01 ($P=0.032$) respectively), suggesting that methylation of *SFRP5* is significantly associated with the occurrence of BCC. Notably, of the four significant differentially methylated genes, nBCC harbored more frequent methylation for *APC* and *RASSF1A*, whereas *SHH* and *SFRP5* were more frequently methylated in iBCC. Nevertheless, none of these differences were significant because of the low sample numbers. Moreover, although *SFRP4* overall was not significantly hypermethylated in BCC, its methylation frequencies did vary according to the subtype ($P=0.010$ for sBCC vs. nBCC plus iBCC, $P=0.011$ sBCC vs. nBCC) (data not shown). No significant differential methylation could be detected for *PTCH1*, *SFRP1*, *SFRP2*, *SFRP4*, *CYLD*, and *TSC1* (Figure 1A and 1B). When we compared sun-exposed (SE) ($n=47$) with sun-protected (SP) ($n=54$) normal skin tissue, only methylation of *SFRP2* was found to be significantly different, with more frequent methylation in SP skin (SE 2.2% vs. SP 15.1%, $P=0.035$) (Figure 1C). Gender or age-associated methylation patterns were not identified.

As MSP primers of *APC*, *SFRP5*, and *RASSF1A* had been previously established and validated^{38,43,44}, we only validated our MSP results of the fourth differentially methylated gene, *SHH*, by BGS, which is considered the gold standard.⁴⁵ Figure 1D depicts the BGS results of one methylated and one unmethylated *SHH* sample as measured by MSP, and BGS could confirm MSP results.

Figure 1. Methylation analysis in BCC and normal skin of ten candidate genes.

A. Illustration of MSP results of ten candidate genes resolved on a 2% agarose gel. BCC: Basal cell carcinoma; NL, normal; IVD, In Vitro Methylated-DNA; Huvec, Human Umbilical Vein Endothelial Cells; U, unmethylated; M, methylated; H2Oo, water control outside PCR; H2Oi, water control inside PCR. **B.** Illustration of methylation frequencies of ten candidate genes in BCC (n=112) and normal skin samples (n=124). P-values represent the difference between percentage methylation in BCC an normal skin. **C.** Illustration of methylation frequencies of ten candidate genes in BCC, sun-exposed (SE) normal skin and sun-protected (SP) normal skin. P-values represent both the difference between percentage methylation in BCC and SE and SP. **D.** SHH sequence data of bisulfite treated genomic DNA from patients. Upper part shows the SHH promoter region starting 1000 base pairs (bp) upstream of the transcription start site (TSS) to 1000 bp downstream. White boxes indicate putative CpG islands (EMBOSS, <http://emboss.sourceforge.net>). The 256 bp region sequenced stretches from -51 bp from the TSS to +409 bp. Indicated with arrows are the forward and reverse methylation specific PCR (MSP) primers. Vertical bars represent CpG dinucleotides and pie -charts represent the percentage of methylated CpG sites (percentage over at least 10 sequenced clones).

Expression of SHH, APC, SFRP5, and RASSF1A is reduced in BCC

To assess whether the methylation status impacts expression of the differential methylated genes, we performed quantitative real-time PCR on primary BCC (n=6) and normal skin samples (n=6). Whereas we could not detect *SHH* and *RASSF1A* mRNA in BCC and normal skin samples (data not shown), we did observe that *SFRP5* mRNA expression level was significantly reduced in BCC versus normal skin ($P=0.019$). *APC* mRNA expression level was not altered ($P=0.937$) in the samples examined (Figure 2A). Nevertheless, BCC with methylated *APC* showed borderline significant reduction mRNA level compared with BCC in which *APC* was unmethylated ($P=0.050$). Correlation of mRNA expression and methylation was not observed for *SFRP5* ($P=0.355$) (Figure 2B), probably due to the fact that *SFRP5* expression levels in all BCC were very low.

To investigate if we could see an effect of DNA methylation on the protein level, we performed immunohistochemical protein staining for *SHH*, *APC* and *RASSF1A* in a random selection of nodular BCC for which the methylation status had also been determined. After extensive testing, we found that commercially available antibodies for *SFRP5* were not suitable for immunohistochemistry. Using the hair follicle as internal control (set at 100% positivity), the expression of *SHH*, *APC* and *RASSF1A* in tumor cells was significantly lower (all p-values < 0.001) (Figure 2A). The intra-class correlation coefficients (ICC) for the scores of all stainings were high, with 0.87 for *SHH*, 0.8 for *APC* and 0.83 for *RASSF1A*. However, expression levels of methylated samples were not further reduced when compared with unmethylated samples (Figure 2C). Lastly, for *SFRP5* we considered the immunohistochemical data available at the ProteinAtlas website by way of independent comparison with our data. For *SFRP5*, ProteinAtlas has data for one antibody (HPA019840) in six BCC and six squamous cell carcinomas, showing strong cytoplasmic and membranous antibody staining and with strong intensity in > 75% of the tumor cells as well as the overlying skin, which is in contrast with the low mRNA expression levels we found. We think that the ProteinAtlas samples are affected by overstaining. As a result, it is not possible to conclude from these data whether or not *SFRP5* expression in BCC is lower than in unaffected skin, as it is in our series. Furthermore, heterogeneity among immunohistochemical stainings is a frequent problem interfering with any assessment and can be due to several factors including the antibody of choice. To settle this matter, more comprehensive *SFRP5* expression analyses in a larger series of BCC, using both immunohistochemistry and quantitative RT-PCR, would be desirable.

A

B

C

Figure 2. Expression of SHH, APC, SFRP5 and RASSF1A is reduced in BCC versus healthy skin control tissue.

A. Relative expression levels of APC and SFRP5 in BCC tissues as compared to the expression level in normal skin (n=6) (2-ΔΔCt). All reactions were done in triplicates, standard error of the mean (SEM) is shown as error bars. Expression levels were normalized to Cyclophilin A. U, unmethylated sample; M, methylated sample. * p<0.05, **p<0.001. **B.** Relative mRNA expression in unmethylated versus methylated BCC samples for either APC or SFRP5. **C.** Microphotographs of selected samples of SHH, APC and RASSF1A. Bar = 200 μm.

Low levels of nuclear β -catenin coincide with high levels of E-cadherin in BCC

As our results suggest epigenetic involvement of the WNT pathway in BCC, we subsequently analyzed the expression of β -catenin as readout for WNT pathway activity. β -catenin expression was localized at cell membranes of normal epidermis and within the cytoplasm and nuclei of hair follicles, consistent with the known activity of canonical WNT signaling in this structure.¹⁸ In the 101 BCC we examined, β -catenin was present only at cell membranes in 65 (64.4%) tumors, and localized at both the cell membrane and in the cytoplasm in 8 (7.9%) cases. Nuclear staining was predominantly located at the tumor periphery and only seen in 28 (27.7%) of the BCC samples without preference for one of the subtypes (Figure 3A). No correlation could be detected between the methylation status of *APC* or *SFRP5* and the presence or localization of β -catenin. As it is known that the E-cadherin/ β -catenin complex is important in both cell adhesion and canonical WNT signaling⁴⁶, we additionally analyzed the expression of E-cadherin, which is normally expressed throughout all layers of the epidermis, including hair follicles.⁴⁷ Expression of E-cadherin in 59 BCC was significantly lower compared with the normal epidermis ($P=0.001$), but was rated high (94.1%) in the tumor cells. Likewise, intensity of staining was rated as strong (69.5%) in the BCC (Figure 3B). These findings were independent of tumor subtype and in agreement with the generally non-metastatic behavior of BCC.³ The ICC for E-cadherin (0.91) again was high.

Figure 3. Low levels of nuclear β -catenin coincide with high levels of E-cadherin in BCC

A. Microphotographs of selected sample of β -catenin, showing nuclear staining only at the periphery or the tumor. Bar = 200 μ m. **B.** Microphotographs of selected sample of E-cadherin showing lowered expression of the tumor compared with the normal epidermis.

Discussion

To the best of our knowledge, this is the largest study to date addressing epigenetic changes in the most common human malignancy, BCC. We provide data which suggest that the SHH and WNT pathways may be epigenetically involved in BCC pathobiology, since both networks are affected by aberrant promoter methylation of *SHH*, *APC* and *SFRP5*. Aberrant methylation and associated gene silencing of *APC* and *SFRP5* may contribute to the pathogenesis of BCC by impairing negative regulation of WNT pathway activity. In addition, we detected differential methylation of *RASSF1A*, a well-known tumor suppressor modulating a broad range of cellular functions that are essential for normal cell growth. *RASSF1A* expression is lost in high frequency by promoter methylation in a wide variety of human tumors, in fact, it is one of the best-characterized tumor suppressor genes in UV-induced squamous cell carcinoma of the skin^{48,49} and methylation of its promoter had been previously demonstrated in BCC.²⁸

Epigenetic alterations in BCC have previously been reported, but in limited sample series²⁶⁻³⁰ and without providing further experimental evidence for relevance of the epigenetic changes for BCC carcinogenesis. Sathyanarayana *et.al.* investigated promoter methylation of 12 genes in a series of 68 BCC samples and 58 non-malignant lesions (skin tags) and showed that laminin gamma 2 (*LAMC2*), cadherin 1 (*CDH1*) and *RASSF1A* were significantly more often methylated in BCC. These findings are in agreement with our results on *RASSF1A* methylation, even though we found higher methylation frequencies in both BCC and normal skin. A possible explanation for this is that we performed nested MSP, which is a very sensitive technique for methylation analysis on FFPE tissue, while Sathyanarayana *et al.* performed direct MSP, which is less sensitive. In addition, they investigated the effect of sun exposure on promoter methylation in skin tags and cancer, and found similar methylation frequencies for the genes examined. From this, it was concluded that promoter hypermethylation in general was more likely related to sun exposure rather than being tumor-specific. In our study, we could not detect comparable methylation patterns in sun-exposed skin and BCC for *SHH*, *APC*, *SFRP5* and *RASSF1A*. Moreover, normal SE and SP skin samples showed similar methylation patterns, suggesting that the methylation we observed is tumor-specific. It is of interest that methylation patterns in sun-exposed skin did not resemble those in BCC, as sun exposure is the main risk factor in the development of BCC.⁵⁰ Thus, our findings do not support a contribution of UVB exposure to the observed promoter hypermethylation of *SHH*, *APC*, *SFRP5* and *RASSF1A*. The intriguing observation that nBCC and iBCC harbored more frequent methylation for these four genes compared with sBCC might indicate that DNA methylation contributes to the development or evolution of BCC. It is tempting to speculate that increased silencing of putative tumor suppressors correlates with increasingly invasive behavior, a notion that is supported by our finding of differential *SFRP5* methylation in iBCC versus nBCC and sBCC. The increased *SHH* methylation is probably an epiphenomenon, as SHH is no longer required for SMOH activity in the majority of BCC. As an alternative explanation, *SHH* silencing might contribute through as yet unidentified pathways to tumorigenesis.

To confirm functionally relevant methylation, i.e., transcriptional silencing of affected genes, we performed gene expression analyses with RT-PCR on FF and immunohistochemical staining on FFPE samples with verified methylation status. Our mRNA expression analysis of *APC* and *SFRP5* in patient and control samples confirmed a down regulation of these genes in samples harboring methylation. By immunohistochemistry, expression of *SHH*, *APC* and *RASSF1A* (all p-values < 0.001) was lowered in BCC as compared to normal skin. We did not detect a direct correlation between methylation status and expression levels as visualized by immunohistochemistry. We think that this observation can be explained by the two-way detection method in immunohistochemical staining intensifying the original signal. Also, mRNA levels do not always correlate directly with protein levels.⁵¹

These data together support our hypothesis of epigenetic involvement of the SHH and WNT pathways in BCC pathogenesis. Promoter hypermethylation-mediated silencing of negative regulators of WNT signaling is consistent with activity of this pathway, however available literature concerning activity of the WNT pathway in BCC is ambiguous. Therefore we assessed activation of canonical WNT signaling by determining β -catenin intracellular localization. β -catenin, a key WNT effector, is a membrane-bound protein which accumulates in the cytoplasm and subsequently translocates to the nucleus when activated.⁵² In BCC, the literature concerning immunohistochemical analysis of β -catenin is ambiguous.^{19,53-55} El-Bahrawy et al.⁵³ showed nuclear staining in 55% (n=56) of BCC, mostly at the periphery of the tumors, while only 23% of the 86 BCC examined by Saldanha et al.⁵⁴ had β -catenin positive nuclei. No correlation between β -catenin localization and histological subtype was seen in either study. Furthermore, in a paper recently published by Pourreyron et al.¹⁹, nuclear β -catenin was absent in all examined BCC (n=7).¹⁹ In our series, we demonstrated nuclear β -catenin in 28 of the 101 (27.7%) BCC examined, which is consistent with previous reports. It would be of interest in this context to examine Axin 2 expression levels in BCC as an additional marker for canonical WNT activity.¹⁹ We reasoned that the lack of β -catenin nuclear localization might be due to E-cadherin co-expression, as high levels of E-cadherin can prevent nuclear translocation of β -catenin.^{46,56} Indeed our data show high levels of E-cadherin expression in BCC, with 94.1% of the cells being positive, although significantly lowered when compared to adjacent normal epidermis. Thus, the absence of nuclear β -catenin in many cases may be due to high E-cadherin levels, which would also be consistent with the general inability of BCC to metastasize.⁵⁰

It is of considerable interest that the epigenetic changes we found parallel the genetic changes driving BCC growth, in particular the silencing of known WNT pathway inhibitors. These observations support a biological relevance of gene silencing by promoter hypermethylation in BCC. Moreover, they are consistent with a model in which epigenetic changes help to drive BCC tumor growth through deregulation of the WNT pathway, upon initiation of growth by mutations affecting SHH signaling. Previous work has demonstrated that BCC growth requires WNT signaling.^{18,19} WNT pathway effectors are among the target genes of SHH, providing a rationale for this positive feedback mechanism. Our findings uncover a new level of regulation; inactivation of WNT inhibitors may be equally important as increased activity of WNT effectors.

In conclusion, we provide evidence that promoter hypermethylation-mediated silencing of known and putative TSGs is present in BCC. The extent to which these epigenetic changes actively contribute to BCC development will be more fully charted in order to determine whether DNA demethylation could be a viable strategy for BCC treatment.

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Table S1. Primer sequences and PCR conditions*					
Gene	Primer	Sense primer (5'→3')	Antisense primer (3'→5')	Annealing temp (°C)	No. of PCR cycles
PTCH1	Flank	GAAGGYGTAGGGTTTGATTTT	AAACRCTACTAACRCRAAACTACTC	56	35
	U	GGTTTGATTTTGGTAGTGGTGT	CTACTAACCAACAACTACTCAAACTCA	66	30
	M	GATTTTCGGTAGCGGTCGC	ACCGCAAACTACTCGAACTCG	66	30
SHH	Flank	GTGGGGAGYGGTGGAGAGTTTTT	TCTCTTACRCTTTCCCTTCCTC	56	35
	U	GTGGAGAGTTTTTTGTAGTTGTGGT	TCCCTTCCTCACTCCAACTCA	68	25
	M	AGAGTTTTTCGTAGTCGCGGC	CCTTCCTCGCTCCGACTCG	68	25
	B	TCROCCATAAACTAATAACTTCC	GTGGGGAGYGGTGGAGAGTTT	64	40
	qRT-PCR	GCCAGCGGAAGGTATGAAGG	TGAGTGGTGGCCATCTTCTGT	60	40
APC	Flank	TGGYGGGGTTTTGTGTTTTATT	TACRCCACACCCCAACCAATC	56	35
	U	GTGTTTTATTGTGGAGTGTGGGTT	CCAATCAACAAACTCCCAACAA	60	25
	M	TATTGGGAGTGCGGGTC	TCGACGAACTCCCGACGA	60	25
	qRT-PCR	ATGGAAGCCGGGAAGGATC	TCAGCAAGAAGCAATGACCTCTC	60	40

Table S1. Primer sequences and PCR conditions*					
Gene	Primer	Sense primer (5'→3')	Antisense primer (3'→5')	Annealing temp (°C)	No. of PCR cycles
SFRP1	Flank	TTTAGTTTGTAGTTTYYGG GTTAG	CCCCRACCAATAACRACCCCTC	56	35
	U	GTTTTGTAGTTTTTGGAGTTAGTTGTGT	CTCAACCTACAATCAAAAAACAACA CAAACA	66	25
	M	TGTAGTTTTCGGAGTTAGTGTGCGC	OCTACGATCGAAAAACGACGCGAACG	66	25
SFRP2	Flank	TTTGTTTTTYYGGTYGGAGTTTTT	TTATCCORAAACCCRCTCTCTT	56	35
	U	TTTTGGGTTGGAGTTTTTTTGGAGTTGTGT	AACCCACTCTCTTCACTAAATACAAC TCA	66	30
	M	GGGTCGGAGTTTTTCGGAGTTGCGC	CCGCTCTCTTGGCTAAATACGACTCG	66	30
SFRP4	Flank	TTGYGGTTAGAGGGGGTGATG	CTATTTATCCCRACAACCTCCCC	56	35
	U	GGGGGTGATGTTATTGTTTTTGTATTGAT	CACCTCCCCTAACATAAAACTCAAAAACA	66	30
	M	GGGTGATGTTATCGTTTTTTTGTATCGAC	CCTCCCTAACGTAAACTCGAAACG	66	30
SFRP5	Flank	GGGAGAGGGGYGTAAAGATTG	CCTCCACCTCRAAAAC TCAA	56	35
	U	GTAAGATTGGTGTTGGGTGGGATGTTT	AAACTCCAAACCCCAAAACCTCACCATACA	66	25
	M	AAGATTGGCGTTGGCGGGGACGTTT	ACTCAAACCCGAACCTCGCCGTACG	66	25
	qRT-PCR	ACCGCTGGGACAGAAGAATA	GOCCCGTAGAAGAAAGGTA	60	40

Table S1. Primer sequences and PCR conditions*					
Gene	Primer	Sense primer (5'→3')	Antisense primer (3'→5')	Annealing temp (°C)	No. of PCR cycles
CYLD	Flank	GGTTTGTTATAGGGAGGTTTAATTA	CRCACAAAAAATAAAAAAAC	56	35
	U	GGTTTGTTAGGAGTGATTTGG TTTAT	AAAAAACAACAAAAAACAACC A	66	35
	M	CGTTTAGGAGCGTAGTTCGGTTTAC	ACGAAAAACGAAAAAACAACCG	66	35
TSC1	Flank	TYGTAGTTTTTTTTYGGATTTTT	TCCTTCTCRAAAAAAATAAAAC	56	35
	U	TTTTGGATTTTTTTGTGGTTTT	AAAAAATAAACAACCCACACT CA	62	35
	M	CGGATTTTTCGTTGCGTTTC	AAATAAACGACCGCGCTCG	62	35
RASS- F1A	Flank	GTTTAGTTGGATTTTGGGGGAG	CCCRCAACTCAATAAACTCAAACCTC	56	35
	U	GGGGTTTGTTTGTGGTTTGT	AACATAACCCCAATTAAACCCATACCTCA	64	30
	M	GGGTCGTTTGTGTTTCGTTTC	TAACCCGATTAAACCCGTAAGTTTCG	64	30
CYPA	qRT-PCR	GACCTCTGTGGCGACTTCATCT	CGGTAGTGGCAGGTGAAGTTG	60	40
	qRT-PCR	CTCGAATAAGTTTGACTTGTGTTT	CTAGGCATGGGAGGAACA	60	40
PCR, polymerase chain reaction; B, Bisulfite genomic sequencing; U, primers matching originally unmethylated DNA sequence; M, primers matching originally methylated DNA sequence.					

Table S2. Antibody characteristics				
Antibody	Source	Order number	Positive control tissue	Dilution
APC	Sigma-Aldrich	HPA013349	Colon	1:300
SHH	Abcam	AB53281	Kidney	1:50
Rassf1A	Origene	TA502449	Melanoma	1:50
β-catenin	Zymed	18-0226	Internal control (hair follicle)	1:1000
E-cadherin	Dako	IR059	Colon	RTU

Chapter 2.2

Functional epigenomics implicates inflammatory signaling in basal cell carcinoma pathogenesis

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Basal cell carcinoma (BCC) is the most common malignancy in Caucasians.¹ To explore the impact of epigenetic regulation on BCC carcinogenesis, we integrated MethylCap-sequencing² on 5 BCC and 5 healthy human skin samples with RNA-sequencing data from 3 BCC and 3 control samples. The combined data constitute the functional methylome. We correlated methylation with RNA expression and identified 15 differentially methylated or expressed loci in BCC, among which PTCH1¹, PTPRN2³ and IFI30.⁴ In silico pathway analysis using the expression data firmly implicated cytokine signaling in BCC growth. Pathway activity was confirmed with immunohistochemical staining. We identified significant epigenetic modulation of gene expression in BCC and uncovered immune modulatory signaling as a driver in BCC development. We translated these findings into a trial with topical application of cyclooxygenase inhibitors to BCC, yielding excellent treatment responses.

Basal cell carcinoma (BCC) mostly affects people aged 60 years or older. Its incidence is rising by 10% each year worldwide, reaching epidemic proportions.¹ The average lifetime risk for Caucasians to develop a BCC is up to 30%.⁵ About 40-50% of patients presenting with a primary BCC will develop one or more further BCC within 5 years.¹ BCC rarely metastasizes, but can cause extensive local tissue destruction when left untreated.⁶ Surgical excision is the current standard of treatment, with cure rates of roughly 95%.^{1,7} However, surgery is costly and time-consuming. The ever-increasing number of tumors will thus generate high costs and workload, putting a significant burden on health-care systems. Hence, BCC is becoming a serious public health problem and there is a clear need for more cost-effective and simple treatment strategies. In order to develop these, a thorough understanding of BCC biology is required.

It is well established that aberrant Sonic Hedgehog (SHH) signaling due to mutations in one of its components is central to BCC carcinogenesis.⁸ However, a recent study of the BCC exome showed an extremely high mutational load, suggesting that BCC could be a heterogeneous malignancy, whose growth might be sustained by mechanisms other than SHH signaling.⁹

It is known that, in addition to genetic mutations, epigenetic alterations such as DNA methylation play an important role in carcinogenesis, occurring very frequently in a variety of tumor types.¹⁰ DNA methylation consists of adding a methyl moiety to cytosine nucleotides followed by a guanine (CpG dinucleotide). These CpG dinucleotides are clustered (CpG islands) and are usually not methylated when associated with the promoter region of the gene. Cancer-related hypermethylation of the promoter region results in silencing of crucial tumor suppressor genes, in addition to mutational changes.¹¹ Based on results from a pilot study, we reasoned that methylation might contribute to BCC biology.¹² To understand how, we combined as first genome-wide methylated DNA enrichment sequencing (MethylCap-seq)² and single-end RNA sequencing on primary BCC and healthy skin controls. We sequenced methylated CpG (mCpG) enriched DNA of five BCC and five control samples of healthy skin from unaffected persons to obtain an overview of the BCC methylome (see Supplementary Table S1 for patients characteristics and Supplementary methods 1.1). In a first approach, we examined 32979 methylation regions in 22121 genes selected for their potential information content (see Methods). Of these regions, 1 gene was significantly dif-

ferentially methylated between BCC and unaffected skin (False Discovery Rate (FDR) < 0.05) and 12 with FDR < 0.1, see Table 1, see Supplementary Table S2 for full list). Noteworthy is CDKN2A, which codes for two tumor suppressor genes (p14 and p16) and is involved in the negative control of cell proliferation in BCC.¹³ IFI30 is of additional interest, playing a critical immunoregulatory role in melanoma by mediating antitumor immunity via CD4+ T-cells.⁴

Table 1. Significantly differentially methylated loci in bcc tissue versus healthy control tissue.

Ensembl.Gene.Id	Gene Label	Chr	Begin	End	FDR
ENSG00000104879	CKM	19	45826253	45826629	0,0496
ENSG00000147889	CDKN2A	9	21970816	21971287	0,0596
ENSG00000095066	HOOK2	19	12876661	12877365	0,0657
ENSG00000078399	HOXA9	7	27204556	27205337	0,0704
ENSG00000111452	GPR133	12	131513956	131514262	0,0773
ENSG00000187909	AC078925.1	12	131513956	131514262	0,0773
ENSG00000183876	ARSI	5	149681603	149681936	0,0822
ENSG00000100418	PPPDE2	22	41995921	41996177	0,0858
ENSG00000172346	CSDC2	22	41995921	41996177	0,0858
ENSG00000196155	PLEKHG4	16	67322714	67322881	0,0889
ENSG00000186891	TNFRSF18	1	1141672	1142148	0,0921
ENSG00000165588	OTX2	14	57270914	57271235	0,0952
ENSG00000216490	IFI30	19	18282492	18282589	0,0980

Chr: chromosome, FDR: False Discovery Rate

As methylation can influence gene expression levels¹⁴, we decided to correlate the methylation results to global transcriptome data, aiming to determine the 'functional methylome' (see Supplementary Methods 1.2). To obtain genome-wide expression data, we performed rRNA depleted, strand specific, total RNA sequencing (RNA-seq henceforth) on three independent primary BCC samples and three independent, age- and sex-matched, healthy glabrous skin samples. We ranked 51984 Ensembl gene identifiers with detectable expression levels according to differential expression between tumors and controls (see Supplementary Table S3). Fifty-eight differentially expressed genes were identified using FDR < 0.1. The top twenty differentially expressed genes at FDR < 0.05 are listed in Table 2 and include genes affecting inter- and intracellular structure (such as MMP13¹⁵, MMP9¹⁶, ACTN2¹⁷, CDHR1), genes that facilitate apoptotic signaling (GMZK¹⁸, GZMB¹⁹), tumor suppressor genes (DCC²⁰) and immune signaling genes (XCL2²¹, CXCL10²²).

Table 2. Significantly differentially expressed genes in bcc tissue versus healthy control tissue.

Ensembl.Gene.Id	Gene Label	Chr	Begin	End	FDR
ENSG00000137745	MMP13	11	102813724	102826463	0,0199
ENSG00000212195	U3	17	56709003	56709197	0,0255
ENSG00000244734	HBB	11	5246694	5250625	0,0274
ENSG00000202077	RNU1-60P	17	56736510	56736666	0,0290
ENSG00000143185	CXCL2	1	168510003	168513235	0,0304
ENSG00000187323	DCC	18	49866542	51057784	0,0313
ENSG00000118785	SPP1	4	88896819	88904562	0,0323
ENSG00000187689	AMTN	4	71384257	71398459	0,0344
ENSG00000232775	AP000525.10	22	16162066	16172700	0,0356
ENSG00000211526	AL121869.1	X	91715451	91715544	0,0366
ENSG00000100453	GZMB	14	25100160	25103473	0,0382
ENSG00000077522	ACTN2	1	236849754	236927931	0,0395
ENSG00000238274	CASC15	6	22056775	22111157	0,0409
ENSG00000148600	CDHR1	10	85954410	85979377	0,0422
ENSG00000100985	MMP9	20	44637547	44645200	0,0433
ENSG00000113088	GZMK	5	54320081	54330398	0,0445
ENSG00000125869	LAMP5	20	9495008	9511171	0,0456
ENSG00000131203	IDO5	8	39759794	39785963	0,0469
ENSG00000160321	ZNF208	19	22148897	22193745	0,0484
ENSG00000169245	CXCL10	4	76942273	76944650	0,0498

Chr: chromosome, FDR: False Discovery Rate

Based on the RNA-seq data, we repeated the differential methylation analysis in 15983 selected methylation regions located in 643 genes that are likely differentially expressed (see Methods). Fifteen of these regions were significantly differentially methylated ($FDR < 0.1$, listed in Table 3 Supplemental Table S4). Notable hits include PTCH1 (part of SHH pathway), PTPRN2 (methylation marker in squamous lung cell carcinoma³) and IFI30.4. It should be noted that there was little overlap between the top-ranked differentially methylated loci and differentially expressed genes ($FDR < 0.1$) even when preselecting for genes which might be differentially expressed in BCC.

Table 3. Significantly differentially methylated 'functional' loci in bcc tissue versus healthy control tissue.

Ensembl.Gene.Id	Gene Label	Chr	Begin	End	FDR
ENSG00000104879	CKM	19	45826253	45826629	0,0246
ENSG00000185920	PTCH1	9	98218565	98218860	0,0383
ENSG00000186439	TRDN	6	123680870	123681186	0,0481
ENSG00000155093	PTPRN2	7	157916451	157916924	0,0535
ENSG00000155093	PTPRN2	7	158135892	158136224	0,0572
ENSG00000107518	ATRNL1	10	117676548	117676940	0,0602
ENSG00000231200	AC068490.2	2	22404105	22404340	0,0623
ENSG00000216490	IFI30	19	18282492	18282589	0,0655
ENSG00000182870	GALNT9	12	132896405	132896746	0,0714
ENSG00000155093	PTPRN2	7	157486385	157486857	0,0756
ENSG00000105963	ADAP1	7	949072	949345	0,0815
ENSG00000240230	COX19	7	949072	949345	0,0815
ENSG00000155093	PTPRN2	7	157477818	157478450	0,0866
ENSG00000077522	ACTN2	1	236860004	236860257	0,0914
ENSG00000164176	EDIL3	5	83598091	83598405	0,0995
ENSG00000186732	MPPED1	22	43822136	43822777	0,0998

Chr: chromosome, FDR: False Discovery Rate

To further place the RNA expression patterns found in this study in a functional context, we performed pathway analysis of the RNA-seq data with SPIA²³ (see Figure 1, Supplementary Table S5 and methods 2.1) using log2 fold changes of genes that were differentially expressed between BCC and normal tissue (FDR < 0.10). This analysis identified three pathways as potentially activated in BCC (FDR < 0.05): cytokine-cytokine receptor interaction signaling (chemokine (C-X-C motif) ligand 1, chemokine (C-X-C motif) ligand 10, Interleukin 8, Chemokine (C Motif) Ligand 2, inhibin beta A chain Precursor (INHBA)) (KEGG hsa04060), chemokine signaling (Chemokine) (KEGG hsa04062) and Toll-like receptor signaling (Osteopontin, Interferon gamma-induced protein 10, Interleukin 8) (KEGG hsa04620). One pathway is potentially inhibited (Amoebiasis, (chemokine (C-X-C motif) ligand 1, Actinin, Interleukin 8) KEGG hsa05146, FDR<0.05), though given the role of this pathway in infectious diseases, we considered this finding to be of no relevance in BCC. SHH signaling is not in the list of significantly activated pathways based on RNA-seq data. Most of the differentially expressed genes are involved in two or all three of these pathways. Only INHBA, secreted phosphoprotein 1 (SSP1) and Toll-like receptor 8 Precursor (TLR8) were unique for one of each of the respected pathways. We also performed pathway enrichment analysis of the differentially methylated genes obtained in the original gene ranking. This analysis also identified cytokine-cytokine receptor interaction signaling as affected in BCC (Supplementary Table S6). We identified several more pathways when combining the top ranked differentially expressed and methylated genes but cytokine-cytokine receptor interaction signaling was the most significant result (Supplementary Table S7).

Figure 1. Circos plot.

The 'cytokine - cytokine receptor interaction' pathway was the primary candidate to emerge from our data. The central region of this plot shows the pathway ties which are potential affected by differential RNA expression in BCC (red). The circular sections from center to periphery display first the false discovery rate (FDR) for differential expression, secondly the FDR for differential DNA methylation and lastly an ideogram of the chromosomes. FDR values were log transformed and color coded by significance (green for $FDR < 0.05$ and light grey for $FDR \geq 0.50$). Note that the axes for the FDR values are pointed towards each other. Only chromosomes with at least one significantly differentially expressed gene ($FDR < 0.05$) in the cytokine pathway were included.

To validate the pathway analysis predictions, we performed immunohistochemical staining for INHBA, SSP1 and TLR8 on a group of 46 independent BCC samples (Supplementary Table S8). The majority of the BCC samples were positive for INHBA (61.9%). SSP1 (56.8%) and TLR8 (40.9%) positivity was found less frequently. The high expression levels found for INHBA are consistent with the activation of the chemokine-signaling pathway found by SPIA. The relatively low expression levels of TLR8 are in agreement with previous work, reporting no expression of TLR8 in normal human keratinocytes²⁴ and low levels in murine BCC cell lines.²⁵ Of note, topically applied imidazoquinolinamines, of which imiquimod is the most widely used, are effective in the treatment of superficial BCC (sBCC) by initiating a TLR8 mediated innate-immune response.²⁵

Our results are further substantiated by an independently conducted clinical trial in which we tested topical diclofenac, an aselective cyclooxygenase 1 and 2 inhibitor, as treatment for sBCC. After 8 weeks of twice daily application of diclofenac-3% gel, complete tumor regression occurred in 64.3% of patients (chapter 4.1). Further support for the importance of inflammatory signaling in BCC is provided by the results of a 3 year, double blinded, randomized clinical trial in 60 patients with basal cell nevus syndrome, all harboring a germ line mutation in PTCH1. There, oral celecoxib (200 mg, twice daily, 24 months) significantly reduced BCC burden in all subjects.²⁶ Our results suggest that SHH signaling might not be the sole driver of BCC, and immune signaling seems to be important in this respect. Thus, combining SHH-targeted therapy with (topical) anti-inflammatory drugs might increase the efficacy of non-invasive therapy.

In conclusion, we report the first comprehensive correlative analysis of the sporadic BCC methylome and transcriptome. Despite the limited number of samples analyzed, we were able to identify and confirm cytokine-cytokine receptor signaling pathway as a key mechanism in BCC. We used independent samples for MethylCap-seq and RNAseq in order to enhance the robustness of our results. However, this came at the cost of increased biologic variability. Although the MethylCap-seq and RNAseq data point in the same direction at the pathway level, strengthening the biological inference, we observe that congruent results at the gene level were often not retained at stringent FDR cut-off.

Although verification in a larger sample set is required, the combined results suggest that inflammation could be a major driver of BCC development, a notion that is supported by previous clinical work²⁷ as well as by the results of our trial with topical application of diclofenac (unpublished data). Anti-inflammatory drugs are relatively cheap and widely available and inflammation thus could present a very attractive target for future development of drugs to treat BCC.

Methods

MethylCap-seq

Sample selection and preparation

Five fresh frozen (FF) primary basal cell carcinoma samples and five control samples (FF normal sun-exposed (SE) (n=2) and sun-protected (SP) skin (n=3)) samples were matched for gender and age (Supplementary Table S1). DNA was extracted using the Puregene® DNA isolation kit (Gentra Systems, now Qiagen) following the manufacturer's instructions.

Fragmentation and methylation enrichment

Fragmentation was performed on a Covaris S2 to obtain fragments with an average length of 200bp. Maximum 3µg was loaded in 130 µl TE in a microtube with AFA intensifier (Covaris, Woburn, Massachusetts, USA). The DNA was then analysed on the Agilent 2100 (Agilent Technologies) to check the fragment distribution on a high sensitivity DNA chip. Methylated DNA was captured using the MethylCap kit (Diagenode, Liège, Belgium). According to the manufacturer's protocol, 200 ng of starting material was used.

Library preparation, cluster generation and sequencing

We used the DNA Sample Prep Master Mix Set 1 (New England BioLabs, Ipswich, Massachusetts, USA) in combination with the Multiplexing Sample Preparation Oligo Kit (96 samples, Illumina PE-400-1001). We used the total amount of fragmented DNA and followed the NEBNext End Repair Module Protocol. Subsequently adapters were ligated using the NEBNext Quick Ligation Module Protocol. Library size selection was performed on a 2% agarose gel (Low Range Ultra agarose Biorad, Veenendaal, the Netherlands). Fragments of 300 bp +/- 50bp were excised and eluted on a Qiagen Gel Extraction Kit column (Qiagen).

Indexes were incorporated during the amplification step using the 'Multiplexing Sample Preparation Oligo kit' (Illumina). A paired end (PE) flow cell was prepared according to the Cluster Station User Guide. Sequencing was performed according to the GAIIx user guide with a multiplexed paired-end run with 2 x 45 cycles.

Mapping

Paired-end 45bp sequence reads were mapped using bowtie (v0.12.7) software²⁸. Only unique paired reads were retained and both fragments were required to locate within 400bp of each other on the human reference genome (GRCh37/hg19). For each sample duplicate paired reads with the exact same location were discarded.

Data summarization and normalization

The mapped reads were summarized using the Map of the Human Methylome (<http://www.biobix.be/map-of-the-human-methylome/mhm-version-2/>). This location map contains ca. 1.5 million putatively independently methylated regions i.e. “methylation cores”. For each sample, the maximum coverage within a specific methylation core was used as intensity value for that locus. Normalization factors were calculated using the Trimmed Median of M-values (TMM) method described in Robinson & Oshlack²⁹ with standard settings as implemented in the edgeR package (version 2.0.3).³⁰

Differential methylation analysis

For the analyses presented in this research we limited our focus to those loci which were contained within the exons of annotated genes or their promoter region (-2000 bp to +500 bp relative to the TSS). The remaining loci were analyzed using the R statistical package “baySeq”.³¹ We applied a selection to the methylation cores to be analyzed by imposing additional requirements that were independent of the sample grouping (see Supplementary Methods). Briefly, in our first approach we only considered those methylation cores for which the number of unmethylated samples varied between 3 and 7. Analysis settings were identical to those in the RNA results based approach. In a second approach, methylation cores were retained for this analysis only if they were located in genes with a differential expression FDR < 0.5 and had at least 4 read pairs across all samples.

Total RNA Sequencing*Sample selection and preparation*

FF BCC samples containing more than 75% tumor cells as evaluated on haematoxylin and eosin stained sections were selected (n=3). FF normal skin samples (n=3) were matched with regard to gender and age. Total RNA was isolated using the standard procedure for TRIzol® RNA extraction (Invitrogen, now Life Technologies, Carlsbad, California, USA) and stored at -80°C. Patient characteristics are listed in Supplementary Table S1. The extracted RNA was treated with the “DNA free kit” (Ambion, Life Technologies) according to the manufacturer’s instructions to remove any remaining DNA (50 µl reaction volume).

rRNA depletion, Reverse Transcription and Adaptor ligation

The extracted RNA were treated with the Ribo-Zero Gold kit (non-magnetic, Epicentre, Madison, Wisconsin, USA). Reverse transcription and adaptor ligation was performed using the ScriptSeq v2 RNA-Seq Library Preparation Kit (Epicentre). The final library was purified with AMPureXP beads (Beckman Coulter, Brea, California, USA).

Cluster generation and Sequencing

Single read cluster generation was performed on the Cluster Station with the TruSeq v5 kit according to the manufacturer's guidelines (Illumina). All samples were sequenced for 72 cycles on a single Illumina GAIIx run with one sample per lane (no multiplexing) and a Phi-X control lane.

Read mapping

To obtain expression estimates for all known human transcripts we applied Tophat (v 2.0.4) to map reads to the human genome with reference annotation GRCh37.68. Directional information obtained due to the ScriptSeq v2 library preparation was preserved in the mapping.

Data summarization, normalization and differential expression analysis

The mapped reads were summarized by counting the number of reads that mapped (at least partially within known exons and matched the sense of the transcript). The counts were again normalized by the TMM method. Differential expression was assessed using the R statistical package "baySeq".

Pathway analyses

RNA-seq based pathway analysis was performed with SPIA standard settings as implemented in R.²³ MethylCap-seq based pathway analysis was performed with WebGestalt available at "<http://bioinfo.vanderbilt.edu/webgestalt/>". This analysis was performed twice: once with the differentially methylated genes and a second time using the union of genes in the previously mentioned list and the list of differentially expressed genes.

Immunohistochemistry

For a description of the immunohistochemical staining procedure see Suppl. methods 3.1.

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Supplementary

Supplementary	Table S1	Patient characteristics
Supplementary	Table S2	Methylcap-seq results
<i>Additional excel file not provided</i>		
Supplementary	Table S3	RNA-seq results
<i>Additional excel file not provided</i>		
Supplementary	Table S4	Combined methylcap-seq + RNA-seq results
<i>Additional excel file not provided</i>		
Supplementary	Table S5	SPIA pathways
Supplementary	Table S6	Pathway analysis differentially methylated genes
Supplementary	Table S7	Pathway analysis DNA and RNA
Supplementary	Table S8	Antibody characteristics
Supplementary methods		
References

Supplementary Table S1. Patient characteristics.					
DNA			RNA		
Sample number	Gender	Age	Sample number	Gender	Age
BCC 1	F	87	BCC 6	M	81
BCC2	M	63	BCC 7	F	77
BCC 3	F	83	BCC 8	M	69
BCC 4	F	79	Normal skin 6	F	52
BCC 5	M	59	Normal skin 7	M	51
Normal skin 1 (SE)	F	77	Normal skin 8	F	70
Normal skin 2 (SP)	F	77			
Normal skin 3 (SE)	F	47			
Normal skin 4 (SP)	F	47			
Normal skin 5 (SP)	M	69			

DNA: deoxyribonucleic acid, RNA: ribonucleic acid, BCC: basal cell carcinoma, SE: sun exposed, SP: sun protected.

Supplementary Table S5 Pathways enriched in differential expression (SPIA).					
Pathway name	Pathway ID	Genes in pathway	Differentially expressed genes in pathway	FDR	Status
Cytokine-cytokine receptor interaction	4060	265	9	1,10E-06	Inhibited
Chemokine signaling pathway	4062	189	8	3,54E-06	Inhibited
Toll-like receptor signaling pathway	4620	102	6	0,001097925	Activated
Transcriptional misregulation in cancer	5202	179	5	0,215574593	Inhibited
Salmonella infection	5132	85	3	0,508936265	Activated

FDR: False Discovery Rate

Supplementary Table S6. Pathway enrichment analysis of the differentially methylated genes obtained in the original gene ranking.			
Enriched KEGG pathway	Entrez IDs	Genes in pathway	FDR
Taste transduction	2914, 2782	52	0.01
P53 signaling pathway	51246, 1029	68	0.01
Cytokine-cytokine receptor interaction	8784, 3572, 268	265	0.01
Fc gamma R-mediated phagocytosis	56848, 255189	94	0.01

FDR: False Discovery Rate

Supplementary Table S7. Pathway enrichment analysis of genes with differentially methylation or expression.

Enriched KEGG pathway	Entrez IDs	Genes in pathway	FDR
Cytokine-cytokine receptor interaction	8784,3627, 6846, 3572, 268	265	0.0026
Toll-like receptor signaling pathway	7187, 6696, 3627	102	0.0072
African trypanosomiasis	3043, 3620	35	0.0095
Bladder cancer	4318, 1029	42	0.0101
Taste transduction	2914, 2782	52	0.0104
Pathways in cancer	4318, 7187, 1630, 1029	326	0.0104
Chemokine signaling pathway	2782, 3627, 6846	189	0.0117
p53 signaling pathway	51246, 1029	68	0.0126
RIG-I-like receptor signaling pathway	7187, 3627	71	0.0126
Fc gamma R-mediated phagocytosis	56848, 255189	94	0.0194
Leukocyte transendothelial migration	4318, 88	116	0.0261
FDR: False Discovery Rate			

Supplementary Table S8. Antibodies characteristics.

Antibody	Dilution	Positive control	Localization
INHBA	1:250	Pancreas	Nucleus/cytoplasm
SPP1	1:500	Kidney	Cytoplasm
TLR8	1:1000	lung	Cytoplasm
INHBA: inhibin beta A chain Precursor, SPP1: secreted phosphoprotein 1, TLR8: Toll-like receptor 8 Precursor.			

Supplementary Methods

1.1 MethylCap-seq

Sample selection and preparation

Five fresh frozen (FF) primary basal cell carcinoma samples and five control samples (FF normal sun-exposed (SE) (n=2) and sun-protected (SP) skin (n=3)) samples were matched for gender and age (supplementary Table S1). DNA was extracted using the Puregene® DNA isolation kit (Gentra Systems, now Qiagen) following the manufacturer's instructions.

Fragmentation and methylation enrichment

Fragmentation was performed on a Covaris S2, settings: duty cycle 10%, intensity 5, 200 cycles per burst during 190 sec, to obtain fragments with an average length of 200bp. The power mode was frequency sweeping, temperature 6-8°C, water level 12. Maximum 3µg was loaded in 130 µl TE in a microtube with AFA intensifier (Covaris, Woburn, Massachusetts, USA). The DNA was then analyzed on the Agilent 2100 (Agilent Technologies) to check the fragment distribution on a high sensitivity DNA chip. Methylated DNA was captured using the MethylCap kit (Diagenode, Liège, Belgium). According to the manufacturer's protocol, 200 ng of starting material was used. The concentrations of the fragmented and captured DNA were determined on a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany) with the Quant-iT™ Picogreen® dsDNA assay kit (Invitrogen, Bleiswijk, the Netherlands) on 480/520nm.

Library preparation, cluster generation and sequencing

We used the DNA Sample Prep Master Mix Set 1 (New England BioLabs, Ipswich, Massachusetts, USA) in combination with the Multiplexing Sample Preparation Oligo Kit (96 samples, Illumina PE-400-1001). We used the total amount of fragmented DNA and followed the NEBNext End Repair Module Protocol; output was cleaned with the Qiaquick PCR Purification Kit (Qiagen, Manchester, United Kingdom) and eluted in 37 µl Elution Buffer (EB). After applying NEBNext dA-tailing Module Protocol, we purified the product with a Minelute PCR Purification Kit (Qiagen) and eluted in 25µl EB. Subsequently adapters were ligated using the NEBNext Quick Ligation Module Protocol. We used the multiplexing sequencing adapters provided in the Multiplexing Sample Preparation Oligo Kit (Illumina). The ligation product was purified with the Minelute PCR Purification Kit (Qiagen) and eluted in 30 µl EB. Library size selection was performed on a 2% agarose gel (Low Range Ultra agarose Biorad, Veenendaal, the Netherlands). We used a 1Kb Plus ladder (Invitrogen) and ran the gel at 120V for 2 hrs. A fragment of 300 bp +/- 50bp was excised and eluted on a Qiagen Gel Extraction Kit column (Qiagen), then eluted it in 23 µl EB.

Indexes were incorporated during the amplification step using the 'Multiplexing Sample Preparation Oligo kit' (Illumina). The amplified libraries were purified on a Qiaquick PCR Purification column (Qiagen 28101), eluted in 50 µl EB 1:5 diluted and concentrated in a rotary evaporator to 10 µl. 1 µl of each purified library was analyzed on an Agilent 2100 HS DNA chip. Concentration was determined on the Agilent 2100. Samples were subsequently diluted to 15 nM and measured with qPCR according to the 'qPCR Quantification Protocol guide' (Illumina). Phix control was used as a standard. A paired end (PE) flow cell was prepared according to the Cluster Station User Guide. Sequencing was performed according to the GALLx user guide with a multiplexed paired-end run with 2 x 45 cycles.

Mapping

Paired-end 45bp sequence reads were mapped using bowtie (v0.12.7) software¹. Bowtie parameters were set to 0 mismatches in the seed (first 28 nucleotides). Only unique paired reads were retained and both fragments were required to locate within 400bp of each other on the human reference genome (GRCh37/hg19). The mapped PE reads allow us to give every nucleotide in the genome a coverage value. For each sample duplicate paired reads with the exact same location were discarded, as these are most likely amplified from the same DNA fragment.

Data summarization and normalization

The mapped reads were summarized using the Map of the Human Methylome (<http://www.biobix.be/map-of-the-human-methylome/mhm-version-2/>). This location map contains ca. 1.5 million putatively independently methylated regions (200-300bp), i.e. "methylation cores". For each sample, the maximum coverage within a specific methylation core was used as intensity value for that locus. To compensate for artificial differences in read distributions, the original library sizes were multiplied by additional normalization factors calculated using the Trimmed Median of M-values (TMM) method described in Robinson & Oshlack² with standard settings as implemented in the edgeR package (version 2.0.3).³

Differential methylation analysis

For the analyses presented in this research we limited our focus to those loci which were contained within annotated genes or their promoter region (-2000 bp to +500 bp relative to the TSS). The remaining loci were analyzed using the R statistical package "baySeq".⁴ This package assigns likelihoods of differential expression in RNA sequencing experiments and is also suited to determine differential methylation.⁵ Expression data is typically featured by fewer variables and higher coverage per variable than enrichment data, causing the analysis to be prohibitively stringent. To compensate for this property of the analysis tool, we applied a selection to the methylation cores to be analyzed by imposing additional requirements that were independent of the sample grouping. In our first approach the selection criteria were aimed at studying methylation independent of the RNA results without inflating the search space too much. Using a zero count (unmethylat-

ed) versus non-zero count (methylated) classification, we only considered those methylation cores for which the number of unmethylated samples varied between 3 and 7, the sum of read pairs across all 10 samples was 10 or greater and the location was in the promoter region or an exon. Analysis settings were identical to those in the RNA results based approach. In a second approach we utilized the information gained in the RNA-seq experiment. Methylation cores were retained for this analysis only if they were located in genes with a differential expression FDR < 0.5 and had at least 4 read pairs across all samples. Further, standard settings were used with 5-fold sampling for prior estimation and 15 bootstraps for posterior estimation.

1.2 Total RNA Sequencing

Sample selection and preparation

FF BCC samples containing more than 75% tumor cells as evaluated on haematoxylin and eosin stained sections were selected (n=3). FF normal skin samples (n=3) were matched with regard to gender and age. Total RNA was isolated using the standard procedure for TRIzol® RNA extraction (Invitrogen, now Life Technologies, Carlsbad, California, USA) and stored at -80°C. Patient characteristics are listed in Supplementary Table S1. The extracted RNA was treated with the “DNA free kit” (Ambion, Life Technologies) according to the manufacturer’s instructions to remove any remaining DNA (50 µl reaction volume).

rRNA depletion, Reverse Transcription and Adaptor ligation

The extracted RNA were treated with the Ribo-Zero Gold kit (non-magnetic, Epicentre, Madison, Wisconsin, USA) to deplete the ribosomal RNA according to the manufacturer’s specifications. The remaining RNA was purified with the RNA clean and concentrator kit (procedure > 17 nt, Zymo Research, Irvine, California, USA) and eluted in 10 µl UltraPure water. The reverse transcription and adaptor ligation was performed using the ScriptSeq v2 RNA-Seq Library Preparation Kit (Epicentre). The initial cDNA was purified with the Minelute PCR Purification Kit (Qiagen 28004, Qiagen, Hilden, Germany). The final library was purified with AMPureXP beads (Beckman Coulter, Brea, California, USA). Quality control of the libraries was performed on a High sensitivity chip (Agilent Technologies, Santa Clara, California, USA). The concentration was determined with qPCR according to the ‘qPCR Quantification Protocol guide’ (Illumina, San Diego, California, USA). Phi-X control was used as a standard.

Cluster generation and Sequencing

After denaturation with NaOH (end concentration 0.1 M), we further diluted the samples to 11 pM. Single read cluster generation was performed on the Cluster Station with the TruSeq v5 kit according to the manufacturer’s guidelines (Illumina). All samples were sequenced for 72 cycles on a single Illumina GAIIx run with one sample per lane (no multiplexing) and a Phi-X control lane.

Read mapping

To obtain expression estimates for all known human transcripts we applied Tophat (v 2.0.4) to map reads to the human genome. The reference annotation GRCh37.68 from the Ensembl ftp server was provided in gtf format using the “-G” option. The other parameters used were: “--no-novel-juncs” turns off search for unannotated splice junctions, “-m 1” allows 1 mismatch in seed, “-a 10” 10 base pair overhang for mapping over junctions, “-i 40” indicates the minimum length for an intron is 40 bp, “--no-coverage-search”, “--microexon-search”, “--segment-length 24” attempts to map each unmapped read as 3 separate reads of length 24 bp, “--min-segment-intron 40” minimum intron length for segment mapping is also 40 bp and “--library-type fr-secondstrand” to indicate the reads contain directional information due to the ScriptSeq v2 library preparation.

Data summarization, normalization and differential expression analysis

The mapped reads were summarized by counting the number of reads that mapped (at least partially) within the exons and matched the sense of the annotated gene models. The counts were again normalized by the TMM method. Differential expression was assessed using the R statistical package “baySeq”. Two models of interest were defined: equal expression across all samples and differential expression between BCC samples and healthy controls. Genes that did not have at least one read in one of the samples were excluded from the analysis. Standard settings were used with these exceptions: prior estimation was performed by sampling 5-fold over the number of remaining genes and posterior estimation was repeated in 20 bootstraps.

2.1 Pathway analysis

RNA-seq based pathway analysis was performed with SPIA standard settings as implemented in R.⁶ As input we used the Entrez gene identifiers and log fold-changes of all genes, which were found to be differentially expressed with FDR < 0.2. MethylCap-seq based pathway analysis was performed with WebGestalt available at “<http://bioinfo.vanderbilt.edu/webgestalt/>”. As input we used the Entrez identifiers of all genes containing at least one differentially methylated methylation core (FDR<0.2) obtained by the selecting loci with 3 to 7 unmethylated samples. This analysis was performed a second time using the union of genes in the previously mentioned list and the list of differentially expressed genes. In both cases enrichment was calculated relative to the entire human genome based on the KEGG database using the hypergeometric test with Benjamini-Hochberg correction and limiting the results to those pathways with a FDR < 0.05 and at least 2 affected genes.

3.1 Immunohistochemical analyses

Forty-six independent formalin-fixed and paraffin embedded (FFPE) BCC excision specimens were selected from the archives of the Department of Pathology of the Maastricht University Medical Centre. Collection, storage and use of all tissues and patient data were performed in

agreement with the “Code for Proper Secondary Use of Human Tissue in the Netherlands”. All of the used samples and corresponding data were de-linked and anonymized. Usage of tissue samples was approved by the Maastricht Pathology Tissue Collection (MPTC) scientific committee (MPTC 2009-05). Five μm sections were mounted on superfrost plus slides and heated for 15 min at 70°C. After deparaffinization the endogenous peroxidase activity was blocked for 30 min in 3% H₂O₂/methanol. Antigen retrieval was performed by microwave treatment at 90 W for 10 minutes in 10 mM citrate buffer (pH 6). Blocking of non-specific binding and diluting of primary antibodies was performed in 3% bovine-serum-albumin (BSA) in phosphate buffer saline (PBS). Sections were incubated o/n at 5°C with primary antibodies listed in Supplementary Table S8. The secondary antibody from the Dako Real Envision detection system (Dako, Heverlee, Belgium) was used (30min RT) and bound antibody was visualized by using the DAKO DAB chromogen (10 min). Tissue was counterstained with Gill II haematoxylin, dehydrated and coverslipped. In all reactions, appropriate positive and negative controls were included and always showed the expected results. A trained resident of the Dermatology department manually quantified the percentage of positive tumor cells.

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An abstract graphic on the left side of the page. It features a series of thin, pink, curved lines that flow from the top left towards the bottom. Interspersed among these lines are several red circles of varying sizes. Some circles are solid red, while others have a white outline. The overall effect is a dynamic, organic shape that resembles a stylized hair or a cluster of cells.

Chapter 2.3

Locally advanced basal cell
carcinoma has a distinct methylation
and transcriptomic profile

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Experimental Dermatology 2016 Apr;25(4):316-8

Background

Sporadic basal cell carcinoma (BCC) is the most frequent malignant tumor in Caucasians. Generally, BCC grow slowly and rarely metastasize or are fatal.¹ Depending on tumor size, histological subtype ('infiltrative', 'micronodular' and 'morphoeic'), tumor localization (mostly face) and perineural spread, some BCC are considered high-risk.² Those, and neglected or inadequately treated BCC, have a greater potential of progressing to locally advanced BCC (laBCC).² laBCC are at least 5.0 cm in maximum dimension, show deep tissue invasion, may metastasize and cause substantial morbidity or even mortality.³ Since laBCC are histologically indistinguishable from common BCC, they are classified according to phenotypic characteristics as described above.

Essentially all BCC have activated Hedgehog (HH) pathway signaling due to mutations in its component genes¹, however the molecular basis for the aggressive behavior of laBCC remains to be elucidated. The partial response of established laBCC to vismodegib (Erdogre), an orally active synthetic SMOOTHENED (SMO)-inhibitor, suggests that other pathways and mechanisms might be involved in their growth.⁴ As most, if not all, cancers are characterized by epigenetic alterations in addition to genetic changes⁵, this might also be the case for BCC, with epigenetic modifications contributing to its growth. The best-characterized epigenetic mechanism in carcinogenesis to date is promoter hypermethylation, comprising increased methylation of cytosines in promoter CpG islands and resulting in gene silencing⁵, for example of tumor suppressors. Others and we have previously demonstrated significant and relevant promoter hypermethylation in non-aggressive BCC (naBCC).⁶

Question addressed

Genome-wide BCC methylation data ("methylome") and comparisons with laBCC are lacking. We now report the first complete methylome and correlated transcriptome of an laBCC, comparing it with those of naBCC. We find significant differences that might explain the more invasive behavior of laBCC.

Experimental design

A 77-year old woman presented with an ulcerating nodular and infiltrative BCC with squamous differentiation of approximately 12 centimeters in diameter, invading much of the left side of her face, including the orbit, nose and nasal cavity, maxillary bone, and cheek. The tumor had been present for 10 to 15 years (figure 1A, 1B).

Figure 1. A locally advanced basal cell carcinoma of the face.

A. A 77-year old female with extensive facial destruction due to locally advanced BCC of the face existing for 10-15 years. The tumour was very painful and the patient had difficulties speaking and eating. Ultrasound-guided fine-needle aspiration cytology revealed a lymph node metastasis in level II of the left neck. The patient was treated by conventional excision, with a 1 cm margin, nasal amputation and orbital exenteration of the left eye. The resulting defect was closed with use of a free revascularized m. rectus abdominis flap. Adjuvant locoregional radiotherapy and a cervical lymph node dissection of levels I-III were also performed. **B.** Haematoxylin and eosin stain of the tumour showing nests of palisading basaloid cells (20x). **C.** 3D reconstruction based on computed tomography (CT). CT and magnetic resonance imaging (MRI) showed an extensive tumour of 6.5 cm with extensive osteolysis, including the left maxillary sinus, and intraorbital growth. The left eyeball had collapsed, with lateral shifting of the optic nerve. The scans showed no intracerebral growth. **D.** Expression levels as normalized read counts for XCL2, BMS1P18, CDH1R and MMP13. These four genes show an increased expression in this patient (locally advanced BCC) compared to other BCCs and healthy control skin. The 95% confidence interval is indicated for the non-aggressive BCCs and healthy controls. MMP13 expression is particularly heightened, also when compared to non-advanced BCCs.

Computed tomography (CT) and magnetic resonance imaging (MRI) showed destruction of orbit and maxilla (figure 1C). Biopsy samples were taken from both tumor tissue and unaffected skin during excision of the tumor; the patient provided her written informed consent. We sequenced the genes coding for PATCHED 1 (*PTCH1*), SMOOTHENED (*SMO*), and SUPPRESSOR OF FUSED (*SUFU*), all key regulators of Sonic Hedgehog (SHH) signaling and frequently mutated in BCC. Additionally, we used Methylcap-Seq⁷ and RNA-Seq to compare genome-wide DNA methylation and total-RNA expression patterns of this laBCC to those of naBCC and healthy skin of this and other patients (controls) that we had previously obtained. See supplementary methods S1.

Results and Conclusions

In the tumor sample we detected a heterozygous point mutation c.1234C>T in *SMO*, which is predicted to result in a leucine to phenylalanine substitution at position 412 (p.(Leu412Phe)) in the fifth transmembrane domain of the SMO protein (figure S1). No deleterious changes were present in the other genes analysed. p.Leu412Phe has not yet been reported in BCC, but was previously described in medulloblastoma⁸, meningioma⁹ and maxillary ameloblastoma.¹⁰ In ameloblastoma, rare locally destructive benign tumors of odontogenic epithelium histologically resembling BCC, p.Leu412Phe appeared to be an activating mutation.¹⁰ To test whether the tumor could be an ameloblastoma instead of a laBCC, we examined BerEp4, cytokeratin (CK) 14 and CK19 expression. Both BerEp4 and CK14 (markers for BCC) showed robust staining while CK19 (marker for odontogenic epithelium) was negative.^{11,12} Based on these results and the histomorphological characteristics, we considered laBCC the most likely diagnosis. At the time our patient presented, vismodegib was not yet available. However, since the p.Leu412Phe *SMO* mutant is resistant to this SMO-inhibitor¹⁰, the tumor would not have responded.

Limiting the genome-wide DNA methylation results to those regions (n=15, located in 12 genes) that were significantly differentially methylated between naBCC and controls, we found that 7 regions were more similar to naBCC in terms of sequencing coverage, while the remaining 8 resembled the controls (table 1, table S1). For the RNA data, we found that this tumor was similar to naBCC in 15 out of 20 differentially expressed genes (false discovery rate (FDR) <0.05) (table 1, table S2). In the laBCC, *SMO* expression was higher than in the adjacent (unaffected) skin of this patient and, interestingly, also higher compared to naBCC and the naBCC control group. Higher *SMO* expression levels might be an indicator of more aggressive behavior, but this notion needs to be verified in other laBCC. The most dramatic change in expression was observed for matrix metalloproteinase 13 (MMP13), where the laBCC sample showed more than 20-fold increase of expression compared to previously analysed naBCC (figure 1D).

Table 1. Comparison of locally advanced basal cell carcinoma with respect to genes which are significantly differentially methylated and expressed between non-aggressive basal cell carcinoma and normal skin.

Differentially methylated genes	
Methylation pattern similar to naBCC	Methylation pattern similar to normal skin
PTCH1	CKM
PTPRN2	TRDN
ATRNL1	IFI30
AC068490.2	PTPRN2
GALNT9	ADAP1
ACTN2	EDIL3
	MPPED1
Differentially expressed genes	
Expression pattern similar to naBCC	Expression pattern similar to normal skin
MMP13	U3
HBB	U1
XCL2	MMP9
DCC	GZMK
SPP1	IDO1
AMTN	
BMS1P22	
AL121869.1	
GZMB	
ACTN2	
LINC00340	
CDHR1	
LAMP5	
ZNF208	
CXCL10	
naBCC non-aggressive basal cell carcinoma. A single gene may contain multiple methylation sites, but is mentioned only once in the table.	

Immunohistochemical analyses of MMP13 confirmed strong expression in our laBCC and an independent laBCC, compared to mild staining in naBCC (methods S1, figure S2). MMP's are a family of proteolytic enzymes, several of which promote tumor invasion by degrading the

extracellular matrix (ECM) and basement membrane. Higher expression of MMP13 has been reported to correlate with a more invasive histological subtype in facial BCC.¹³ MMP13 is also responsible for osteolysis in various other types of cancer, and may have therefore played a role in the extensive destruction of the maxilla in our patient.¹⁴ Our study provides the first evidence that there are substantial differences in gene methylation and expression levels between laBCC and general BCC, which could contribute to the difference in their behavior. Since there are drugs that can modulate DNA methylation, our findings may have direct therapeutic implications. Finally, the higher level of MMP13 found in laBCC, possibly combined with the higher SMO expression, might explain why laBCC are much more aggressive than naBCC. Thus, MMP13 might be used as a biomarker to distinguish BCC that should be treated and followed up more rigorously to prevent local recurrence or evolution into an laBCC.

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Supplementary methods

MethylCap-seq

Sample selection and preparation

Six fresh frozen (FF) primary basal cell carcinoma samples and five control samples (FF normal sun exposed (SE) (n=2) and sun protected (SP) skin (n=3)) samples were matched with regard to gender and age. DNA was extracted using the Puregene® DNA isolation kit (Gentra Systems, now Qiagen) following the manufacturer's instructions.

Fragmentation and methylation enrichment

Fragmentation was performed on a Covaris S2, settings: duty cycle 10%, intensity 5, 200 cycles per burst during 190 sec, to obtain fragments with an average length of 200bp. The power mode was frequency sweeping, temperature 6-8°C, water level 12. Maximum 3µg was loaded in 130 µl TE in a microtube with AFA intensifier (Covaris, Woburn, Massachusetts, USA). The DNA was then analysed on the Agilent 2100 (Agilent Technologies) to check the fragment distribution on a high sensitivity DNA chip. Methylated DNA was captured using the MethylCap kit (Diagenode, Liège, Belgium). According to the manufacturer's protocol, 200 ng of starting material was used. The concentrations of the fragmented and captured DNA were determined on a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany) with the Quant-iT™ Picogreen® dsDNA assay kit (Invitrogen, Bleiswijk, the Netherlands) on 480/520nm.

Library preparation, cluster generation and sequencing

We made a minor modification to the 'multiplexed paired end ChIP protocol' (Illumina). We used the DNA Sample Prep Master Mix Set 1 (New England BioLabs, Ipswich, Massachusetts, USA) in combination with the Multiplexing Sample Preparation Oligo Kit (96 samples, Illumina PE-400-1001). We used the total amount of fragmented DNA and followed the NEBNext End Repair Module Protocol; output was cleaned with the Qiaquick PCR Purification Kit (Qiagen, Manchester, United Kingdom) and eluted in 37 µl Elution Buffer (EB). After applying NEBNext dA-tailing Module Protocol, we purified the product with a Minelute PCR Purification Kit (Qiagen) and eluted in 25µl EB. Subsequently adapters were ligated using the NEBNext Quick Ligation Module Protocol. We used the multiplexing sequencing adapters provided in the Multiplexing Sample Preparation Oligo Kit (Illumina). The ligation product was purified with the Minelute PCR Purification Kit (Qiagen) and eluted in 30 µl EB.

Library size selection was performed on a 2% agarose gel (Low Range Ultra agarose Biorad, Veenendaal, the Netherlands). We used a 1Kb Plus ladder (Invitrogen) and ran the gel at 120V for 2 hrs. A fragment of 300 bp +/- 50bp was excised and eluted on a Qiagen Gel Extraction Kit column (Qiagen), then eluted it in 23 µl EB. Indexes were incorporated during the amplification step using the 'Multiplexing Sample Preparation Oligo kit' (Illumina). The amplified libraries were purified on a Qiaquick PCR

Purification column (Qiagen 28101), eluted in 50 μ l EB 1:5 diluted and concentrated in a rotary evaporator to 10 μ l. 1 μ l of each purified library was analysed on an Agilent 2100 HS DNA chip. The concentration was determined with smear analysis on the Agilent 2100. Samples were subsequently diluted to 15 nM and measured with qPCR according to the 'qPCR Quantification Protocol guide' (Illumina). Phix control was used as a standard.

A paired end (PE) flow cell was prepared according to the Cluster Station User Guide. Sequencing was performed according to the GAllx user guide with a multiplexed paired-end run with 2 x 45 cycles.

Mapping

Paired-end 45bp sequence reads were mapped using bowtie (v0.12.7) software³⁷. The bowtie parameters were set to 0 mismatches in the seed (first 28 nucleotides). Only unique paired reads were retained and both fragments were required to locate within 400bp of each other on the human reference genome (GRCh37/hg19). The mapped PE reads allow us to give every nucleotide in the genome a coverage value. For each sample duplicate paired reads with the exact same location were discarded, as these are most likely amplified from the same DNA fragment.

Data summarization and normalization

The mapped reads were summarized using the Map of the Human Methylome (<http://www.biobix.be/map-of-the-human-methylome/mhm-version-2/>). This location map contains ca. 1.5 million putatively methylated regions, i.e. "methylation cores".

For each sample, the maximum coverage within a specific methylation core was used as intensity value for that locus. To compensate for artificial differences in read distributions, the original library sizes were multiplied by additional normalization factors calculated using the Trimmed Median of M-values (TMM) method described in (Robinson & Oshlack, 2010) with standard settings as implemented in the edgeR package (version 2.0.3).³⁸

Differential methylation analysis

For the analyses presented in this research we limited our focus to those loci which were contained within annotated genes or their promoter region (-2000 bp to +500 bp relative to the TSS). The remaining loci were analysed using the R statistical package "baySeq".³⁹ This package was designed to assign likelihoods of differential expression in RNA sequencing experiments. It is also suited to determine differential methylation since the underlying data is based on discrete counts (negative binomial distribution). However the package is more apt at detecting quantitative differences whereas promoter methylation often has a qualitative effect on gene expression (i.e. on/off switch, see Jones 2012 for a more nuanced overview of the effects of CpG methylation)¹³. Additionally, expression data is typically featured by fewer variables and higher coverage per variable than enrichment data, causing the analysis to be prohibitively stringent. To compensate for this property of the analysis tool, we applied a selection to the methylation cores to be ana-

lysed, by imposing additional requirements that were independent of the sample grouping. In a first approach we utilized the information gained in the RNA-seq experiment. Methylation cores were retained for this analysis only if they were located in genes with a differential expression FDR < 0.5 and had at least 4 read pairs across all samples. Further, standard settings were used with 5 fold sampling for prior estimation and 15 bootstraps for posterior estimation. The second set of selection criteria were aimed at studying methylation independent of the RNA results without inflating the search space to much. Using a zero count (unmethylated) versus non-zero count (methylated) classification, we only considered those methylation cores for which the number of unmethylated samples varied between 3 and 7, the sum of read pairs across all 10 samples was 10 or greater and the location was in the promoter region or an exon. Analysis settings were identical to those in the RNA results based approach.

Total RNA Sequencing

Sample selection and preparation

FF BCC samples containing more than 75% tumour cells as evaluated on haematoxylin and eosin stained sections were selected ($n=5$). FF normal skin samples ($n=5$) were matched with regard to gender and age. Total RNA was isolated using the standard procedure for TRIzol® RNA extraction (Invitrogen, now Life Technologies, Carlsbad, California, USA) and stored at -80°C . The extracted RNA was treated with the “DNA free kit” (Ambion, Life Technologies) according to the manufacturer’s instructions to remove any remaining DNA (50 μl reaction volume).

rRNA depletion, Reverse Transcription and Adaptor ligation

The extracted RNA were treated with the Ribo-Zero Gold kit (non-magnetic, Epicentre, Madison, Wisconsin, USA) to deplete the ribosomal RNA according to the manufacturer’s specifications. The remaining RNA was purified with the RNA clean and concentrator kit (procedure > 17 nt, Zymo Research, Irvine, California, USA) and eluted in 10 μl UltraPure water. The reverse transcription and adaptor ligation was performed using the ScriptSeq v2 RNA-Seq Library Preparation Kit (Epicentre). The initial cDNA was purified with the Minelute PCR Purification Kit (Qiagen 28004, Qiagen, Hilden, Germany). The final library was purified with AMPureXP beads (Beckman Coulter, Brea, California, USA). Quality control of the libraries was performed on a High sensitivity chip (Agilent Technologies, Santa Clara, California, USA). The concentration was determined with qPCR according to the ‘qPCR Quantification Protocol guide’ (Illumina, San Diego, California, USA). Phix control was used as a standard.

Cluster generation and Sequencing

After denaturation with NaOH (end concentration 0.1 M), we further diluted the samples to 11 pM. Single read cluster generation was performed on the Cluster Station with the TruSeq v5 kit according to the manufacturer’s guidelines (Illumina). All samples were sequenced for 72 cycles on a single Illumina GAIIx run with one sample per lane (no multiplexing) and a Phi-X control lane.

Read mapping

To obtain expression estimates for all known human transcripts we applied Tophat (v 2.0.4) to map reads to the human genome. The reference annotation GRCh37.68 from the Ensembl ftp server was provided in gtf format using the “-G” option. The other parameters used were: “--no-novel-juncs” turns off search for unannotated splice junctions, “-m 1” allows 1 mismatch in seed, “-a 10” 10 base pair overhang for mapping over junctions, “-i 40” indicates the minimum length for an intron is 40 bp, “--no-coverage-search”, “--microexon-search”, “--segment-length 24” attempts to map each unmapped read as 3 separate reads of length 24 bp, “--min-segment-intron 40” minimum intron length for segment mapping is also 40 bp and “--library-type fr-secondstrand” to indicate the reads contain directional information due to the ScriptSeq v2 library preparation.

Data summarization, normalization and differential expression analysis

The mapped reads were summarized by counting the number of reads that mapped within the exons and matched the sense of the annotated gene models listed in the Ensembl database of human genes (v56). Alternative assemblies of genomic regions were ignored for this purpose. The counts were again normalized by the TMM method. Differential expression was assessed using the R statistical package “baySeq”. Two models of interest were defined: equal expression across all samples and differential expression between BCC samples and healthy controls. Genes that did not have at least one read in one of the samples were excluded from the analysis. Standard settings were used with these exceptions: prior estimation was performed by sampling 5-fold over the number of remaining genes and posterior estimation was repeated in 20 bootstraps.

Immunohistochemistry

Collection, storage and use of all tissues and patient data were performed in agreement with the “Code for Proper Secondary Use of Human Tissue in the Netherlands”. We selected 5 BCC BCC, the laBCC described in this paper and an additional laBCC from the Pathologic Anatomical National Automated Archive of the Department of Pathology in the Maastricht University Medical Centre (MUMC). Normal skin samples were obtained during autopsies. Usage of tissue samples was approved by the Maastricht Pathology Tissue Collection (MPTC) scientific committee (MPTC 2009-05). Immunohistochemical stains were performed on 4-µm formalin-fixed paraffin-embedded (FFPE) tissue sections according to standardized laboratory protocols at the Department of Pathology, MUMC+ by using a DakoAutostainer Link 48™ (DAKO Corporation, Carpinteria, CA, USA). Briefly, after heat induced epitope retrieval (K8004 EnVision™ Flex Target Retrieval Solution High pH 9, DAKO), immunohistochemical analysis was performed using DakoAutostainer Link 48™ (DAKO Corporation, Carpinteria, CA, USA). Endogenous peroxidase activity was blocked by peroxidase blocking reagent for 5 minutes. The primary antibodies MMP13 (ab39012, rabbit polyclonal, 1:200 Abcam) was incubated for 20 minutes. The DAKO Envision Flex kit (K8002) was used for secondary detection (+ linker). After secondary detection, all sections were counterstained with haematoxylin. A positive control was included and showed the expected positive result. Slides were assessed by a dermato-pathologist and a trained resident dermatology and scored as mild, moderate or strong positive staining. Discrepancies were resolved by consensus.

Table 1. Patient characteristics immunohistochemical stains

Sample	Gender	Age	Staining
naBCC1	F	64	Mild
naBCC2	F	74	Mild
naBCC3	M	73	Mild
naBCC4	M	58	Moderate
naBCC5	M	75	Moderate
laBCC1	F	77	Strong
laBCC2	F	69	Strong
NL1	M	67	Absent to mild
NL2	M	51	Absent to mild
NL3	F	66	Absent to mild
NL4	M	71	Absent to mild
NL5	F	53	Absent to mild

naBCC non-aggressive basal cell carcinoma, laBCC locally advanced basal cell carcinoma, F female, M male

Figure S1. Sequence analysis of SMOOTHENED.

Top panel indicates normal control trace, while bottom panel shows tumour trace with the mutation indicated by an arrow (c.1234C>T).

Figure S2. Immunohistochemical analysis of MMP13.

MMP13 matrix metalloproteinase 13, naBCC non-aggressive basal cell carcinoma, laBCC locally advanced basal cell carcinoma

Chapter 3

Immunohistochemical analysis of the mechanistic target of rapamycin and hypoxia signaling pathways in basal cell carcinoma and trichoepithelioma

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Abstract

Background Basal cell carcinoma (BCC) is the most common cancer in Caucasians. Trichoepithelioma (TE) is a benign neoplasm that strongly resembles BCC. Both are hair follicle (HF) tumors. HFs are hypoxic microenvironments, therefore we hypothesized that hypoxia-induced signaling pathways could be involved in BCC and TE as they are in other human malignancies. Hypoxia-inducible factor 1 (HIF1) and mechanistic/mammalian target of rapamycin (mTOR) are key players in these pathways.

Objectives To determine whether HIF1/mTOR signaling is involved in BCC and TE.

Methods We used immunohistochemical staining of formalin-fixed paraffin-embedded BCC (n=45) and TE (n=35) samples to assess activity of HIF1, mTORC1 and their most important target genes. The percentage positive tumor cells was assessed manually in a semi-quantitative manner and categorized (0%, < 30%, 30-80% and >80%).

Results Among 45 BCC and 35 TE examined, expression levels were respectively 81% and 57% (BNIP3), 73% and 75% (CAIX), 79% and 86% (GLUT1), 50% and 19% (HIF1 α), 89% and 88% (pAKT), 55% and 61% (pS6), 15% and 25% (pMTOR), 44% and 63% (PHD2) and 44% and 49% (VEGF-A). CAIX, Glut1 and PHD2 expression levels were significantly higher in TE when only samples with at least 80% expression were included.

Conclusions HIF and mTORC1 signaling seems active in both BCC and TE. There are no appreciable differences between the two with respect to pathway activity. At this moment immunohistochemical analyses of HIF, mTORC1 and their target genes does not provide a reliable diagnostic tool for the discrimination of BCC and TE.

Introduction

Basal cell carcinoma (BCC) is the most common cancer in Caucasians. Its incidence is increasing by 3-10% annually, leading to a lifetime BCC risk in Caucasians of approximately 1 in 5-6.¹ Sun-exposed body sites like the head and neck are most commonly affected.¹ Histologically, BCC can be classified into three main subtypes: superficial, nodular and infiltrative.² Youssef et al. were the first to show that BCC are epithelial tumors originating from progenitor cells of the interfollicular epidermis (IFE) and the upper infundibulum.³ Later evidence shows that BCC can also arise from the hair follicle (HF) bulge.⁴ Trichoepithelioma (TE) is a similar, though benign epithelial tumor which expresses markers that are also present in the outer root sheath of the HF^{5,6}, which suggests that TE originate from the HF as well. Two distinct subtypes of TE are recognized: the classic and the desmoplastic type.⁶ Some TE can mimic BCC macroscopically,⁶ making it difficult to differentiate between both tumors and this dilemma can extend to the microscopic level. Histologically, both BCC and TE are characterized by dermal nests of basaloid cells. TE differs from BCC by its absence of peripheral palisading of basaloid keratinocytes, necrosis, retraction artifact, mitotic activity and peritumoral mucin deposition. Also, TE rarely ulcerates. BCC conversely is usually not associated with formation of horn cysts and papillary mesenchymal bodies. However histological distinction is predominantly based on the higher degree of follicular differentiation as found in TE.⁶ Differentiation between these two HF derived tumors is important because of their distinct biologic behavior and therapeutic approach. Decreased oxygen levels are a common feature in many tumors⁷, including cutaneous squamous cell carcinoma and melanoma.⁸ While the human dermis is well-oxygenated and the epidermis is modestly hypoxic, the HF is considered as a moderately to severely hypoxic microenvironment.⁹ A primary mediator of hypoxia-induced gene expression in human HFs is the hypoxia-inducible transcription factor 1 (HIF1).¹⁰ HIF1 is activated in several tumor types,¹¹ and is mainly negatively regulated by prolyl hydroxylase 2 (PHD2); the key oxygen sensor.¹² In hypoxic conditions, HIF1 α regulates the expression of important proteins including vascular endothelial growth factor (VEGF-A), glucose transporter 1 (GLUT1), catalytic enzymes such as carbonic anhydrase IX (CAIX) and pro-apoptotic BCL-2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3). VEGF-A is suggested as a key driver in the angiogenic response and is overexpressed in most solid cancer. As a consequence, inhibition of VEGF-A can suppress tumor growth.¹³ GLUT1 is responsible for glucose uptake and the expression of GLUT1 increases under hypoxic conditions inducing glycolysis.¹⁴ Tumors generally have high rates of glycolysis (the Warburg effect).¹⁵ CAIX is involved in several regulatory process that are beneficial for tumour survival, the most important is pH regulation. Aggressive tumour behavior and poor patient outcome is associated with high CAIX expression levels.¹⁶ Also BNIP3 is associated with poor prognosis in some cancers, promoting apoptosis and even autophagy.¹⁷ Hence, all of these genes play critical roles in tumor growth.¹⁸ Of note, they can also be induced by mechanistic/mammalian target of rapamycin (mTORC1) signaling¹⁹ which is activated in several tumor types.²⁰ Also, mTORC1 enhances the protein levels of HIF via HIF1 α and consequently enhances the expression of HIF target genes.²¹ Dodd et al additionally showed that mTORC1 mediates VEGF-A expression via both HIF1 α dependent and independent mech-

anisms.¹⁹ Conversely, hypoxia promotes tuberous sclerosis complex 2 (TSC2) via 'regulated in development and DNA damage responses 1' (REDD1) that leads to inhibition of mTORC1 and reveals a feedback mechanism where HIF can turn off mTOR complex 1 (mTORC1).²² mTOR, as part of mTORC1, plays an integral role in the coordination of cell growth and division in response to growth factors, nutrients and the energy status of the cell (Figure 1.).^{22 23}

Figure 1: Simplified depiction of pathways affected by hypoxia.

Under hypoxic conditions the expression of prolyl hydroxylase domain proteins (PHDs) is reduced leading to an induction of hypoxia inducible factor 1 α (HIF1 α) expression, which becomes stable and active as a transcription factor, together with hypoxia inducible factor 1 β (HIF1 β). HIF1 activation regulates the expression of several target genes whose products address the needs of oxygen starved cells, such as vascular endothelial growth factor (VEGF-A), BCL-2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3), glucose transporter 1 (GLUT1) and glycolytic enzymes such as carbonic anhydrase IX (CAIX). Hypoxia also influences mechanistic/mammalian target of rapamycin complex 1 (mTORC1) signaling mainly mediated through hypoxic activation of the TSC1-TSC2 complex by REDD1. First, phosphatidylinositol 3 kinase (PI(3)K) and protein kinase B (AKT) have been implicated in the activation of the mTOR protein kinase. One critical target of AKT that regulates mTOR is the tumour suppressor protein, tuberous sclerosis complex 2 (TSC2). TSC2 negatively regulates mTOR signaling, and AKT activation circumvents this inhibition. Constitutive mTOR signaling positively stimulates S6 kinase (S6K), a downstream effector of mTOR pathway, which mainly drives cell growth and proliferation. Also, mTOR enhances the protein levels of HIF and consequently enhances the expression of HIF target genes. Second, under conditions of hypoxia, intracellular ATP levels drop and AMP levels rise. AMP directly binds to a subunit of AMP activated protein kinase (AMPK), which is then phosphorylated by serine/threonine protein kinase 11 (STK11/LKB1). Elevated concentrations of AMPK can cause a complete inhibition of mTOR (mTORC1) activity without affecting PI(3)K-AKT signaling.

Human tumors are characterized by an heterogeneous microenvironment that differs from normal tissue with respect to nutrient supply, pH and oxygenation. As discussed, deficiencies in oxygenation (hypoxia) is strongly associated with tumor development, growth, metastasis and poor response to therapy. HIF and mTORC1 signaling are crucial in here, either directly or indirectly acting on the hypoxia response.²³

Based on the hypoxia affecting the HF and the known role of both HIF and mTORC1 in tumor growth, we have previously hypothesized that hypoxia response pathways are involved in the pathogenesis of HF tumors.¹⁰ If HIF and mTORC1 pathways do contribute to the growth of HF tumors, new opportunities for targeted therapy and diagnostics could emerge.

Therefore, we decided to examine BCC as this is the most prevalent HF tumor and included the very similar TE.^{9,10,24} We analysed the expression of the most important target genes in the hypoxia response using immunohistochemical stains of both BCC and TE. To the best of our knowledge, this is the first large series of BCC and TE to date to be analysed for HIF1 and mTORC1 activity. Our results suggest that HIF1/mTORC1 mediated signaling is active in both tumor types.

Material and Methods

Tissue samples

Collection, storage and use of all tissues and patient data were performed in agreement with the “Code for Proper Secondary Use of Human Tissue in the Netherlands”. We randomly selected 45 BCC and 80 TE haematoxylin-eosin stain (HE) slides from the Pathologic Anatomical National Automated Archive of the Department of Pathology in the Maastricht University Medical Centre (MUMC). A combination of search terms was used to retrieve reports of the specific histological diagnosis. Based on the morphological classification by Rippey, BCC were subcategorized into superficial, nodular and infiltrative during routine histological diagnostic examination.² All TE HE slides were reviewed by three independent investigators (*i.e.* a dermato-pathologist (AM), a general pathologist (BL) and a dermatologist (MT)), because the histopathologic diagnosis of TE can present considerably more difficulties than that of BCC. The 80 slides were reviewed based on the presence or absence of the following characteristics: tumor-stroma cleft formation, ulceration, epithelial primitive structures, small keratinous cysts, inflammatory response, mitosis, necrotic tumor cells, papillary mesenchymal bodies, stromal edema and peritumoral mucin production.⁵ Eventually, 35 TE were found to unambiguously fit the criteria for classic TE and these were used for the analysis, together with 45 BCC. All of the used samples and corresponding data were de-linked and anonymized. Usage of tissue samples was approved by the Maastricht Pathology Tissue Collection (MPTC) scientific committee (MPTC 2009-05).

Immunohistochemistry

Formalin fixed and paraffin embedded (FFPE) biopsies as well as excision specimens were used. Four-micrometer sections were cut and stained with primary antibodies, listed in table 1. HIF1 α , CAIX, Glut-1 and VEGF-A stains were performed on a Dako autostainer system with use of a pre-treatment module using EnVision FLEX Target Retrieval Solution, High pH (Dako, Heverlee, Belgium). The antibodies were applied for 20 minutes at room temperature. For HIF1 α , the slides were additionally incubated with Envision Flex Mouse Linker to amplify the signal. The Dako Envision Flex kit (K8002) was used for secondary detection. For BNIP3, PHD2, pS6, pMTOR2448 and pAKT, slides were deparaffinized in xylene, rehydrated and incubated in 0.3% (PHD2, pMTOR2448) or 3% (BNIP3, pS6) hydrogen peroxide (H₂O₂) in methanol for 30 minutes to inactivate endogenous peroxidase activity. Antigen retrieval was performed by microwave treatment at 90 W for 10 minutes in 10 mM citrate buffer (pH 6) and non-specific protein binding was blocked using 3% bovine-serum-albumin (BSA). The sections were incubated for 1 hour at room temperature (BNIP3, PHD2) or overnight at 4°C (pS6, pMTOR2448, pAKT). For secondary detection an Envision detection system was used and bound antibody was visualized by using 3,3-diaminobenzidine (DAB) for 10 minutes. After secondary detection, all sections were counterstained with Gill II haematoxylin, dehydrated and coverslipped. In all reactions, appropriate positive and negative controls were included and always showed the expected positive resp. negative results.

Table 1. Antibodies used for immunohistochemical analysis

Antibody	Dilution	Producer
BNIP3	1:800	Sigma Aldrich, St. Louis, U.S.A.
CAIX	1:1000	Novus Biologicals, Littleton, U.S.A.
GLUT1	1:200	Thermo Scientific, Landsmeer, the Netherlands
HIF1 α	1:50	BD Transduction Laboratories, Breda, the Netherlands
pAKT	1:25	Cell Signaling technology, Beverly, MA, U.S.A.
pS6	1:50	Cell Signaling technology, Beverly, MA, U.S.A.
pMTOR2448	1:50	Cell Signaling technology, Beverly, MA, U.S.A.
PHD2	1:250	Novus Biologicals, Littleton, US
VEGF-A	1:200	Thermo Scientific, Landsmeer, the Netherlands

BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), carbonic anhydrase IX (CAIX), glucose transporter member 1 (GLUT1), hypoxia-inducible factor 1- α (HIF1 α), phosphorylated -protein kinase B (pAKT), phosphorylated-S6 (pS6), phosphorylated-mechanistic target of Rapamycin (pMTOR), prolyl hydroxylase domain protein 2 (PHD2), vascular endothelial growth factor (VEGF-A).

Interpretation of the stains

A trained medical student of the Department of Dermatology, MUMC (CW) and an experienced resident of the Department of Pathology, MUMC (JE) examined all sections. Both were blinded for patient details. Any discrepancy between the observers was discussed and resolved by consensus. For all tumors, at least four randomly chosen high-power fields (magnification 200x) per slide were assessed to determine the percentage positive tumor cells. The percentage of cell staining was scored as 0 (no staining), 1 (<30% staining), 2 (30-80% staining) and 3 (>80% staining)²⁵. For all assessments, the HF was used as internal standard and considered as 100% positive.

Statistical analysis

Statistical analyses were carried out using SPSS version 20.0 software (SPSS, Chicago, IL, USA). Descriptive data were presented as absolute numbers and percentages for categorical data and as means with standard deviations for continuous data. The Chi-square test for independent proportions was performed to evaluate the differences and similarities in expression of CAIX, BNIP3, GLUT1, HIF1 α , pAKT, PHD2, pMTORC1, pS6 and VEGF-A between BCC and TE specimens. Correlations among the different stainings were assessed by Spearman's correlation coefficient. $P < 0.05$ was considered to be statistically significant.

Results

Sample characteristics

Forty-five BCC (superficial n = 14, nodular n = 24, and infiltrative n = 7) were included. Among the 80 TE cases selected, 35 were unanimously found to be classic type TE. Distribution of patient and tumor characteristics within the TE and BCC group are equal and listed in table 2.

	Trichoepithelioma	Basal cell carcinoma
Number tumours	35	45
Biopsy/excision*	19/16 (54.3/45.7)	0/45 (0.0/100.0)
Gender (m/f)*	16/21 (43.2/56.8)	24/21 (53.3/46.7)
Mean patient age in years	58.8	68.6
Tumour localisation*		
Head	28 (80.0)	18 (40.0)
Nek	3 (8.6)	4 (8.9)
Trunk	3 (8.6)	15 (33.3)
Arm	0.0 (0.0)	2 (4.4)
Leg	1 (2.9)	6 (13.3)
TE Subtype*		
Classic TE	35 (100.0)	
Desmoplastic TE	0 (0.0)	
BCC Subtype*		
Superficial BCC		14 (31.1)
Nodular BCC		24 (53.3)
Infiltrative BCC		7 (15.6)
* Data are presented as n (%)		

Hypoxia target staining patterns in basal cell carcinoma and trichoepithelioma

We investigated the activation status of hypoxia and mTORC1 signaling cascades by immunohistochemical analysis of BCC and TE. Immunohistochemical staining of BNIP3, GLUT1, CAIX, VEGF-A and PHD2 was observed in the suprabasal portion of the overlying epidermis of the BCC and TE tumor islands and in the internal control, the HF. Immunohistochemical staining for HIF1α was negative in epidermis overlying the tumor islands, while the HF showed weak scattered positive nuclear expression. VEGF-A showed expression in the endothelial cells of blood vessels. An overview of all stains is presented in figure 2 and 3.

Overall, about half of all BCC and TE indicate active hypoxia signaling, however the BCC specimens tended to have less CAIX, GLUT1, and PHD2 expression than TEs (Fig. 4a). In TE high expression levels (>80%) of PHD2, GLUT1 and CAIX were observed in 53.3%, 31.0% and 34.4%, respectively (Fig. 4b). Conversely, HIF1 α was more often positive in BCC nuclei than in TE (50.0% vs. 19.4% ($P < 0.05$)). The downstream effectors BNIP3 and VEGF-A showed no significantly different expression levels. When examining different BCC subtypes, nodular BCC harbored more GLUT1 and PHD2 expression compared to superficial and infiltrative BCC with P-values of respectively $P=0.032$ and $P=0.17$. An overview of the expression in BCC and TE is presented in table 3.

Figure 2: Immunohistochemical stains for hypoxia inducible factor 1 α (HIF1 α), phosphorylated mechanistic/mammalian target of rapamycin (pmTOR) and their target genes in basal cell carcinoma (BCC).

BCL-2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3) (a); carbonic anhydrase IX (CAIX) (b); glucose transporter 1 (GLUT1) (c); HIF1 α (d); phosphorylated protein kinase B (pAKT) (e); phosphorylated ribosomal protein S6 (pS6) (f); pmTOR (g); prolyl hydroxylase domain protein 2 (PHD2) (h); vascular endothelial growth factor (VEGF-A) (i). Original magnification: (a–i) $\times 200$.

Figure 3: Immunohistochemical staining for hypoxia inducible factor 1 α (HIF1 α) and phosphorylated mechanistic/mammalian target of rapamycin (pmTOR) and their target genes in trichoepithelioma (TE).

BCL-2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3) (a); carbonic anhydrase IX (CAIX) (b); glucose transporter 1 (GLUT1) (c); HIF1 α (d); phosphorylated protein kinase B (pAKT) (e); phosphorylated ribosomal protein S6 (pS6) (f); p-mTOR (g); prolyl hydroxylase domain protein 2 (PHD2) (h); vascular endothelial growth factor (VEGF-A) (i). Original magnification: (a–i) $\times 200$.

mTORC1 target staining patterns in basal cell carcinoma and trichoepithelioma

An overview of all stains is shown in figure 2,3,4 and table 3. pAKT and pS6 were positive in the epidermis overlying the BCC and TE tumor islands and the HF, while immunohistochemical expression of phosphorylated mTORC1 at Ser-2448 (pmTOR) in the overlying epidermis was negative. The HF showed some weak and scattered pmTOR staining. In contrast, we found pAKT to be highly expressed in 88.9% of BCC and 87.5% in TEs ($P=0.791$). As a downstream effector of pAKT, pmTOR exhibited positive intracytoplasmic and partly membranous expression in only 15.0% of the BCC, while 33.3% of the TE showed positive staining ($P=0.083$). pS6 showed no significant difference between intracytoplasmic staining expression in BCC (55.0%) specimens and TE (63.3%) specimens ($P=0.935$). No differences among the BCC subtypes could be detected.

Spearman's correlation coefficients among HIF and mTORC1 signaling target genes

All stains were statistically analyzed for correlation of expression patterns revealing the following significant correlations. In BCC, significant correlations between CAIX and GLUT1 ($r=0.369$, $P=0.024$), CAIX and pS6 ($r=0.522$, $P=0.001$) and CAIX and PHD2 ($r=0.426$, $P=0.007$) were detected as well as between PHD2 and BNIP3 ($r=0.485$, $P=0.003$) and PHD2 and pS6 ($r=0.399$, $P=0.012$). Furthermore, the expression of VEGF-A correlated significantly with that of pMTOR ($r=0.342$, $P=0.033$). Moreover in TE, PHD2 was significantly correlated with CAIX ($r=0.438$, $P=0.017$) and GLUT1 ($r=0.544$, $P=0.003$). Lastly, pAKT expression correlates significantly with pS6 ($r=0.438$, $P=0.015$) and VEGF-A with pMTOR ($r=0.502$, $P=0.07$) in TE.

Figure 4: Percentage of positive specimens between basal cell carcinoma and trichoepithelioma.

Panel a represents all tissue samples being either positive or negative. In panel b the same results are shown, however here a cut off value of 80% of the tumour cells being positive was used. * ($P < 0.05$), basal cell carcinoma (BCC); trichoepithelioma (TE).

Discussion

The HIF and mTORC1 signaling pathways play crucial roles in many malignancies.²³ To the best of our knowledge, we are the first to perform a systematic analysis of HIF and mTORC1 signaling in both BCC and TE. Although TE showed significantly more expression of CAIX, Glut1 and PHD2, this was only in the proportion of samples with high expression levels of at least 80% and thus not likely to reflect a genuine difference between the two tumor types. The correlations found between components of HIF signaling and the mTORC1 pathway are in line with known crosstalk between both pathways.²³ GLUT1, CAIX and BNIP3 are the most important readouts for HIF1 α signaling²⁶⁻²⁸ and our data show positivity of all three target genes in the majority of both BCC and TE. Positive VEGF-A staining patterns were found in the endothelial cells of blood vessels as well as in BCC/TE tumor cells. However, VEGF-A expression was not specifically tumor related, since some weak staining was also noted in the basal cell layer of epidermis adjacent to the tumors. These low and weak VEGF-A expression levels are in agreement with earlier studies in BCC and other benign and malignant skin tumors^{29,30} and might be explained by the low levels of mTORC1 expression found (see below), since recent evidence indicates that mTORC1 can drive VEGF-A expression.¹⁹ It also fits with the low metastatic behavior of BCC²⁹. Accordingly, strong VEGF-A expression is observed in the more aggressive and potentially metastasizing squamous cell carcinoma and melanoma.^{29,31}

Furthermore, we found expression of PHD2 in 63.3% of TE and 43.9% of BCC specimens, whereas expression of HIF1 α itself was low or even undetectable. Several *in vitro* studies have shown that PHD2 is transiently upregulated in a HIF-dependent manner under normoxic as well as mild hypoxic conditions, which could suggest that HIF might induce an autoinhibitory effect on its own activity.³² Moreover, D'Angelo et al demonstrated that hypoxic upregulation of PHD2 acts as a feedback mechanism to stop hypoxic signaling in reoxygenated cells.³³ Hence, we postulate that the lack of HIF1 α in BCC and TE specimens can be explained by the presence of PHD2.

Activity of the mTORC1 pathway was assessed by use of the upstream regulator pAKT and downstream target pS6. pAKT was positive in 88.9% of BCC and 87.5% of TE, while 55% of BCC and 61.3% of TE specimens were positive for pS6, supporting the presence of active mTOR signaling. The positive stains for pAKT and pS6 found in our study are consistent with the known activity of PI(3)K/AKT signaling in BCC.³⁴ In addition, strong expression of pAKT and pS6 has been observed in a variety of skin neoplasms including Bowen's disease, keratoacanthoma, squamous cell carcinoma and extramammary Paget's disease.^{35,36} However, despite positive staining of pAKT and pS6, hardly any mTORC1 phosphorylation at Ser-2448 was detected in both tumor types. This observation is consistent with two other studies reporting weak positivity of mTOR (Ser-2448) in only 7.7% of BCC³⁷ and 36% positivity among 85 epidermal tumors other than BCC.³⁶ Ser-2448 is the mTORC1 phosphorylation site modified either directly by AKT or by the downstream target of mTORC1, p70S6 kinase, making it the most important marker for activation of mTOR.³⁸ However, the upregulation of up- and downstream target genes of the mTORC1 signaling cascade do

suggest activation of downstream mTORC1 signaling components downstream, which could be attributed to PI(3)K- signaling.³⁹ In addition, it is known that multiple feedback loops exist, for example S6 kinase can dampen growth factor receptor signaling to PI3K.⁴⁰

Overall, the immunohistochemical analysis is consistent with activity of HIF1 and mTORC1 signaling in both BCC and TE, in addition to the known PI(3)K-AKT activity in BCC.³⁴ We also showed that the number of HIF1 α positive cells was significantly higher in BCC than in TE, which might be explained by the fact that BCC tumor nests are generally larger than the TE tumor nests and consequently could be more hypoxic. Nodular BCC tend to have more GLUT1 and PHD2 expression when compared to superficial and infiltrative BCC, which could similarly be due to them being more hypoxic. Advanced insights in molecular pathways active in cancer development have already resulted in the development of novel topical and systemic targeted therapies as a rational approach to the management of many (skin) cancers.⁴¹ Our results suggest that it might be of interest to further explore the contribution of HIF1 and mTORC1 signaling to BCC and TE growth. Deeper insights into such signaling pathways might eventually result in the identification of novel targets for treatment. Finally a better understanding of alterations in gene expression could be used to develop better histological diagnostics, since at this moment immunohistochemical analysis of HIF, mTORC1 and their target genes does not provide a reliable diagnostic tool for the discrimination of BCC and TE.

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PART

TWO

NEW TOPICAL AND SYSTEMIC TREATMENT
MODALITIES FOR BASAL CELL CARCINOMA

Chapter 4.1

The effect of topical diclofenac 3% and calcitriol 3 µg/g on superficial basal cell carcinoma (sBCC) and nodular basal cell carcinoma (nBCC):
A phase II, randomized controlled trial

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Abstract

Background Non-steroidal anti-inflammatory drugs and vitamin D derivatives can target signaling pathways activated in Basal Cell Carcinoma (BCC).

Objective We investigated the efficacy of topically applied diclofenac sodium 3% gel, calcitriol 3µg/g ointment and a combination of both in superficial (sBCC) and nodular (nBCC).

Methods Patients with a primary, histologically proven sBCC (n=64) or nBCC (n=64) were randomized to topical diclofenac, calcitriol, combination of both or no topical treatment (control group). After self-application twice daily under occlusion (8 weeks), tumors were excised. Primary outcome: post-treatment expression levels of proliferation (Ki-67) and anti-apoptosis (Bcl-2) immunohistochemical markers. Secondary outcomes: histological clearance, adverse events, application-site reactions, patient compliance.

Results sBCCs treated with diclofenac showed a significant decrease in Ki-67 ($p < 0.001$) and Bcl-2 ($p = 0.001$), and after combination therapy for Ki-67 ($p = 0.012$). Complete histological tumor regression was seen in 64.3% ($P = 0.0003$) of sBCCs (diclofenac) and 43.8% ($P = 0.007$) of sBCCs (combination therapy) compared to 0.0% of controls. No considerable changes were found in nBCCs. Application-site reactions were mostly mild to moderate.

Limitations The small sample size.

Conclusion Our results suggest that topical diclofenac is a promising new treatment for sBCC. Its mode of action differs from available non-invasive therapies, and thus has an additive value.

Capsule summary

- Non-steroidal anti-inflammatory drugs and vitamin D derivatives can specifically target SHH and/or WNT signaling, activated in BCCs.
- Eight-weeks of topical diclofenac significantly reduced expression of proliferation and anti-apoptosis immunohistochemical markers and resulted in complete tumor regression in nearly two-thirds of treated sBCCs.
- Topical diclofenac is a promising non-invasive treatment for sBCCs.

Introduction

Nonmelanoma skin cancer (NMSC) is the most common cancer among Caucasians. Sporadic basal cell carcinoma (BCC) accounts for 80% of all NMSCs and its incidence is still rising by 3-10% annually.¹ The most common histological subtypes are nodular (nBCC) (40%) and superficial BCC (sBCC) (18-31%), both generally considered to be low-risk tumors.² Conventional surgical excision is the gold standard in treatment of all BCCs, with success rates of 95-98%.^{3,4} However, for low-risk sBCCs, non-invasive treatment modalities such as photodynamic therapy (PDT), imiquimod (immune-modulating) and 5-fluorouracil cream (chemotherapeutic) are frequently prescribed.⁵ Tumor-free survival rates in sBCC vary from 72.8-84.0% (PDT), 83.4-87.3% (imiquimod) and 80.1% (5-fluorouracil).^{5,6} Non-invasive treatments have several advantages, such as lower healthcare costs and a decrease in the workload for dermatologists. Also, a better cosmetic outcome with an increase in quality of life for patients can be expected.⁴

Current research on BCC focuses on treatments that specifically target key signaling pathways required for tumor growth. The Sonic Hedgehog (SHH) signaling pathway is involved in the pathogenesis of essentially all sporadic BCCs⁷ and murine models show that canonical Wntless (WNT) signaling is essential for the tumorigenic response to deregulated SHH signaling in the skin.⁸ Interestingly, a recent study suggests that imiquimod, in addition to its immunomodulating effects, may actually serve as a targeted therapy by directly inhibiting SHH signaling.⁹ As shown in figure 1, both SHH and WNT pathways can either directly or indirectly serve as therapeutic targets for non-steroidal anti-inflammatory drugs (NSAIDs) and the active form of vitamin D (calcitriol (1 α ,25[OH]₂D₃)). NSAIDs were found to inhibit canonical WNT signaling in patients with familial adenomatous polyposis and are suggested to be pro-apoptotic in BCC cell lines in a cyclooxygenase-2 (COX-2) dependent and independent manner (fig 1).¹⁰⁻¹² COX-2 is highly expressed in several solid tumors, including BCC.¹³ In a phase II clinical trial, systemic NSAIDs reduced both the number and burden of BCCs in patients with basal cell nevus syndrome.¹⁴ Additionally, topically applied diclofenac induced a clinical response in the majority of the patients with actinic keratosis, which can be a precursor of squamous cell carcinoma.¹⁵ The anti-tumor effects of calcitriol, as seen in model systems of several human malignancies such as cancer of the prostate, ovary and lungs, are mainly attributed to stimulation of the vitamin D receptor (VDR).¹⁶ In keratinocytes, the VDR has a regulatory role in SHH and WNT signaling by acting as a tumor suppressor, resulting in reduction of proliferation and differentiation and induction of apoptosis (programmed cell death) (fig 1).¹⁶

We sought to investigate the efficacy of topical application of either a NSAID or a vitamin D analogue on low-risk BCCs, by evaluating the effects on proliferation and apoptosis. Both are well-accepted drugs already available for treatment of diseases other than cancer, like muscle pain (NSAIDs) and psoriasis (calcitriol).¹⁰ We also investigated the effect of combination of both therapies, because we hypothesized that simultaneously targeting different signaling pathways or pathway elements may have a synergistic effect as suggested by several preclinical and clinical studies.¹⁷

Figure 1. Actions of NSAIDs and Calcitriol in Basal Cell Carcinoma: a schematic overview.

Sonic Hedgehog Pathway (green). The extracellular protein Sonic hedgehog (SHH) binds to and inhibits Patched (PTCH1), a transmembrane receptor, which relieves the inhibition of another transmembrane protein, Smoothened (SMO). SMO activates glioma-associated oncogene homolog 1 (GLI1) and GLI2, transcription factors that travel into the nucleus to activate the expression of tumor-promoting genes.⁷ Canonical WNT signaling pathway (pink). Binding of a WNT ligand to its specific receptor complex containing a Frizzled (FZD) family member and LRP5 or LRP6 co-receptors, initiates WNT-β-catenin signaling. Axin relocates to the LRP 5/6 tail at the membrane that is bound to WNT through its interaction with dishevelled (DVL), which forms a complex with GSK3β and prevents β-catenin (β-cat) degradation.¹⁰ This allows β-catenin to accumulate and enter the nucleus, where it interacts with members of the TCF/LEF family. In the nucleus, β-catenin converts the TCF proteins into transcriptional activators. Suppressor of fused (SUFU) functions as a tumor suppressor by inhibiting both SHH and WNT signaling.⁸ NSAIDs inhibit WNT signaling by reducing nuclear β-catenin localization.^{10,31} Furthermore, NSAIDs inhibit cyclooxygenase-2 (COX-2), which is overexpressed in basal cell carcinoma (BCC) and catalyzes the conversion of arachidonic acid (AA) to prostaglandins (PGE₂). A subsequent reduction of PGE₂ and a direct down regulation of the anti-apoptotic Bcl-2 family proteins by NSAIDs induces apoptosis.¹¹ A down regulation of Bcl-2 is also induced by 1α,25(OH)₂D₃ (calcitriol), resulting in caspase cleavage leading to apoptosis. Calcitriol directly inhibits SMO in vitro, resulting in repression of SHH signaling. SHH-signaling is also suggested to be directly repressed by the Vitamin D Receptor (VDR) by inhibition of GLI. Finally, activation of the VDR by calcitriol induces the expression of the transmembrane protein E-Cadherin, which recruits β-catenin to the cell membrane and prevents translocation of β-catenin to the nucleus.^{16,32,33}

Methods

Protocol

We performed a phase II, single-blind, randomized-controlled intervention trial. Patients from the dermatology outpatient clinic of the Maastricht University Medical Centre (MUMC), Maastricht, the Netherlands, were included between November 1st 2011 and February 15th 2013. Histologically proven primary sBCCs or (micro) nodular BCCs ≥ 4 mm, not located in the face or on the hairy scalp, were eligible for inclusion and were asked to participate in the trial (appendix p 2). In case of mixed superficial and nodular BCC, tumors were categorized according to the most aggressive component (nBCC). Patients were excluded if they were using oral NSAIDs more than four days a week (chronic users)¹⁸ or vitamin D (containing) supplements in the preceding 30 days. The local medical ethics and scientific committee approved the protocol and two following amendments. The study was performed in accordance with the Declaration of Helsinki. All participants provided written informed consent.

Enrolled patients with a superficial BCC or nodular BCC were randomly assigned to receive either topical diclofenac sodium-3% gel in hyaluronic acid 2.5% (Solaraze®, Almirall, Barcelona, Spain), calcitriol ointment 3 µg/g (Silkis®, Galderma, Rotterdam, the Netherlands) (henceforward called diclofenac and calcitriol respectively), a combination of both (combination therapy), or no medication (control group).

The primary outcome measure was the post-treatment expression of Ki-67 and Bcl-2. Ki-67 is a proliferation marker, which can be detected in the nuclei of proliferating cells. Apoptosis is regulated by the proto-oncogene Bcl-2, which is overexpressed in the majority of the BCC.¹⁹ Immunohistochemical analysis is a reliable approach to evaluate treatment effects^{20,21} and was used to evaluate the percentage of cells expressing Ki-67 or Bcl-2. Secondary outcomes were histological tumor regression, adverse events, application-site reactions and patient compliance.

Assignment and masking

Randomization was stratified for histological tumor type and performed via a computer-generated random allocation scheme. Random permuted blocks of eight were used to ensure concealment of allocation. Patients as well as investigators prescribing study treatment and collecting clinical data were not blinded to treatment assignment. The pathologists who assessed expression levels of Ki-67 and Bcl-2 and histological tumor regression were blinded to assigned treatment.

Participant flow and follow-up

Patients received the study medication from the investigator directly after randomization. The vehicle was self-applied on the tumor with a radius of 0.5cm and covered with an occlusive sheet (Tegaderm®, 3M, Leiden, The Netherlands) twice a day for eight weeks. This period was generally the time patients waited for surgical excision. In case of combination therapy, diclofenac gel application was followed by calcitriol ointment with a two-minute interval.

Treatment was continued until the day before surgery. In case of a severe local skin reaction, surgery was postponed. Surgical excision was performed with a 3-5mm safety margin. Expression of Ki-67 and Bcl-2 was evaluated in both baseline biopsies and excision specimens (appendix p2). No study related follow-up visits were planned after surgical excision.

Treatment reactions were evaluated by a phone interview two weeks after the start of the treatment. Furthermore, secondary outcome parameters were collected from a patient diary, which was filled-out once a week during the course of the treatment. The patient diary included questions on pain expressed on a visual analogue scale, local skin reactions and compliance. Standardized photographs of all lesions were taken with a ruler and pantone color card (Danes-Picta, Praha, Czech Republic) on day one and 56. Every consultation was documented. Compliance was defined as the number of actual applications as a percentage of the total prescribed number of applications.

Statistical analysis

In total, we aimed to include 64 nBCC and 64 sBCC patients to enable comparison of the four study arms separately for sBCC and nBCC (appendix p2). Continuous variables were presented as a mean with $\pm$ standard deviations (if normally distributed) or as a median with an interquartile range (if not normally distributed). Differences in proportions between groups were tested using the Fisher's exact test. Analysis of covariance (ANCOVA) was used to compare post-treatment expression of Ki-67 and Bcl-2 between treatment groups and control group. Variables indicating treatment group and baseline expression levels of Ki-67 and Bcl-2 were entered as independent variables. The regression coefficients associated with the treatment groups represent the difference in post-treatment expression level between the corresponding treatment group and the control group. In case of skewed baseline distributions of the of Ki-67 and Bcl-2 expression levels data were log transformed to normalize distributions and normality of the distribution of residuals was checked using a normal probability plot. Statistical analyses were carried out using SPSS 20.0 software and www.openepi.com. All reported P values are two-sided, and P values ≤ 0.05 were considered statistically significant. This study is registered as a controlled trial at clinicaltrials.gov, number NCT01358045, since May 17th 2011.

Results

Patients

One hundred and twenty-eight patients were included (64 nBCC and 64 sBCC) and randomly assigned to one of the four study arms (fig 2). The distribution of the baseline demographic and tumor characteristics was comparable between treatment groups (table 1). One patient withdrew directly after treatment allocation and thus information on all outcomes was missing. The primary outcome was not available for eight patients. One patient had a BCC that required treatment by Mohs' micrographic surgery and another patient had a syringoma. For six other patients, the biopsy or excision specimens did not include sufficient paraffin embedded tumor tissue to enable immunohistochemistry for Ki-67 and Bcl-2. The subjects with missing primary outcome were evenly distributed among the treatment groups. A total of 119 patients were included in the statistical analysis of the primary outcome (n=59 sBCC and n=60 nBCC, fig 2). No crossovers occurred.

Figure 2A: CONSORT diagram.

Abbreviations: BCC=Basal Cell Carcinoma. Diclofenac=diclofenac sodium-3% gel, Calcitriol=Calcitriol 3µg/g ointment, Combination therapy=diclofenac and calcitriol.

Nodular BCC **Figure 2B: CONSORT diagram.**

Abbreviations: BCC=Basal Cell Carcinoma. Diclofenac=diclofenac sodium-3% gel, Calcitriol=Calcitriol 3µg/g ointment, Combination therapy=diclofenac and calcitriol.

Table 1. Demographic and tumor characteristics of the study patients at baseline, according to treatment assignment and subgroup*

	Diclofenac (n=16)	Calcitriol (n=16)	Combination therapy (n=16)	Control (n=16)
Superficial BCC				
Sex				
Male	12 (75%)	13 (81%)	9 (56%)	11 (69%)
Female	4 (25%)	3 (19%)	7 (44%)	5 (31%)
Age in years	63.0 (54-68)	65.5 (55-72)	67.5 (60-74)	61.5 (49-68)
Localization BCC				
Neck	2 (13%)	0	0	0
Upper Extremity	5 (31%)	8 (50%)	5 (31%)	6 (38%)
Trunk	6 (38%)	8 (50%)	10 (63%)	5 (31%)
Lower Extremity	3 (19%)	0	1 (6%)	5 (31%)
Other	0	0	0	0
Histology biopsy				
Superficial BCC	16 (100%)	16 (100%)	16 (100%)	16 (100%)

Table 1. Demographic and tumor characteristics of the study patients at baseline, according to treatment assignment and subgroup*

Tumor surface in mm ²	61.7 (30.0-84.4)	54.2 (44.8-87.6)	46.7 (37.1-101.3)	59.7 (43.6-74.2)
Percentage Ki-67 expression	20.0 (6-37)	5.5 (4-32)	21.0 (12-49)	10.0 (6-35)
Percentage Bcl-2 expression	8.5 (4-52)	10.0 (4-63)	19.0 (5-65)	5.0 (1-25)
Nodular BCC				
Sex				
Male	12 (75%)	12 (75%)	11 (69%)	9 (56%)
Female	4 (25%)	4 (25%)	5 (31%)	7 (44%)
Age in years	78.5 (62-82)	68.5 (61-75)	71 (68-79)	66 (56-73)
Localization BCC				
Neck	0	1 (6%)	2 (13%)	1 (6%)
Upper Extremity	1 (6%)	3 (19%)	6 (38%)	3 (19%)
Trunk	9 (56%)	10 (63%)	4 (25%)	9 (56%)
Lower Extremity	6 (38%)	2 (13%)	3 (19%)	3 (19%)
Other	0	0	1 (6%)	0
Histology biopsy				
Nodular BCC	16 (100%)	13 (81%)	15 (94%)	14 (88%)
Micro nodular BCC	0	1 (6%)	0	1 (6%)
Mixed type	0	2 (13%)	0	1 (6%)
Tumor surface in mm ²	49.5 (31.4-70.7)	59.7 (34.3-87.6)	44.8 (33.0-53.8)	53.4 (39.1-98.4)
Percentage Ki-67 expression	10.0 (5-13)	14.5 (6-21)	10.5 (9-28)	8.5 (6-14)
Percentage Bcl-2 expression	22.0 (4-69)	47.5 (6-80)	28.5 (5-76)	42.5 (8-80)

Continuous variables are expressed as median (interquartile range) and categorical variables as n (%). Abbreviation: BCC=Basal Cell Carcinoma. Diclofenac=diclofenac sodium-3% gel, Calcitriol=Calcitriol 3µg/g ointment, Combination therapy=diclofenac and calcitriol. * Percentages have been rounded and therefore do not always total 100.

Immunohistochemical analysis of proliferation and apoptosis

Figure 3 illustrates the median expression levels of Ki-67 and Bcl-2 in tumor cells before and after treatment for sBCC and nBCC in the four randomized groups. With respect to sBCCs, this figure shows that in the control group median values of endpoint Ki-67 expression levels increased slightly when compared with baseline levels. There was a substantial decrease in Ki-67 expression after treatment with diclofenac and combination therapy and a small increase in the calcitriol group. Median values in Bcl-2 expression show a small increase in the control group, whereas there was a slight decrease in tumors treated with diclofenac, calcitriol and combination treatment. In the appendix photomicrographs of the changes on the immunohistochemical stains were given (appendix pp 3 and 4).

superficial bcc

nodular bcc

Figure 3: Pre- versus post-treatment scatterplot of changes in Ki-67 and Bcl-2 expression.

Data are median %. Abbreviation: BCC=Basal Cell Carcinoma. Diclofenac=diclofenac sodium-3% gel, Calcitriol=calcitriol 3µg/g ointment, Combination therapy=diclofenac and calcitriol. Pre- versus post-treatment changes in Ki-67 and Bcl-2 expression after 8 weeks treatment according to treatment groups.

The distributions of Ki-67 and Bcl-2 expression levels were skewed and a $\log(x+1)$ transformation was used to normalize the distributions. ANCOVA analyses were used to adjust for imbalances in baseline values of Ki-67 and Bcl-2. The mean differences between the treatment groups and the control group were back transformed from the logarithm scale to raw scale and are presented in Table 2. Ki-67 expression was significantly lower in sBCCs treated with diclofenac and combination therapy, when compared to the post treatment levels in the control group ($p < 0.001$ and $p = 0.012$, respectively, table 2). Also, Bcl-2 expression was significantly lower in sBCCs treated with diclofenac ($p = 0.001$, table 2). Post treatment expression levels in sBCCs treated with calcitriol did not differ significantly from those in the control group. With respect to nBCCs, there were no statistically significant differences in expression levels in excision specimens, neither for Ki-67 nor for Bcl-2, between treatment groups and the control group (Table 2).

Table 2: Mean difference in post treatment percentage of cells with expression of Ki-67 or Bcl-2 between treatment and control group, after adjustment for baseline differences using ANCOVA

	Ki-67			Bcl-2		
	Mean difference	95% CI	P	Mean difference	95% CI	P
Superficial BCC (n=59)						
Diclofenac	-6.37	-19.01 to -1.72	<0.001	-6.63	-21.67 to -1.57	0.001
Calcitriol	-0.70	-2.77 to 0.30	0.179	-0.45	-3.33 to 1.07	0.492
Combination therapy	-3.61	-13.81 to -0.44	0.012	-0.97	-8.14 to 1.35	0.372
Nodular BCC (n=60)						
Diclofenac	-0.59	-2.55 to 0.40	0.246	-0.59	-3.50 to 0.78	0.367
Calcitriol	+0.39	-0.54 to 1.97	0.386	+0.82	-0.71 to 4.71	0.290
Combination therapy	-0.60	-3.12 to 0.62	0.321	-1.12	-5.72 to 0.50	0.193

Data represent mean difference in post treatment percentage of cells with expression between treatment and control group, after adjustment for baseline differences. P-values are calculated with ANCOVA.
 Abbreviation: BCC=Basal cell carcinoma. Diclofenac=diclofenac sodium-3% gel, Calcitriol=calcitriol 3µg/g ointment, Combination therapy=diclofenac and calcitriol.

Clinical response and compliance

In the sBCC subgroup, histologically complete tumor regression was seen in 64.3% (9 of 14) and in 43.8% (7 of 16) of the tumors treated with diclofenac and combination therapy respectively (fig 4). The difference with the control group (0 of 16) was statistically significant ($P=0.0003$ and $P=0.007$, respectively). None of the participants in the calcitriol group showed complete regression. In the nBCC subgroup 31.2% (5 of 16), 6.2% (1 of 16), 33.3% (5 of 15) showed histological complete regression in patients treated with diclofenac, calcitriol or combination therapy, respectively. No residual tumor was observed in 18.8% (3 of 16) of the nBCC control group (fig 4). Differences between active treatment groups and the control group were not statistically significant. Evaluation of excision specimens of the sBCC subgroup with no complete tumor regression revealed a nodular BCC component in four tumors assigned to the combination therapy group. In the calcitriol group two tumors with a nodular component and one tumor with an invasive BCC were found. Data on compliance were available for 95 patients. Median compliance rates were generally high (92.7%-98.2%) and comparable between groups (appendix p 5).

superficial bcc

Figure 4: Complete histologic tumor regression rates after 8 weeks of topical treatment.

Abbreviation: BCC=Basal Cell Carcinoma. Diclofenac=diclofenac sodium-3% gel, Calcitriol=calcitriol 3µg/g ointment, Combination therapy=diclofenac and calcitriol. P-values were calculated with Fisher exact test. Complete histologic tumor regression for superficial BCC and for nodular BCC according to treatment groups, compared to control group.

Adverse events

Adverse events were comparable for sBCC and nBCC, with mostly mild to moderate severity (appendix pp 7-10). Erythema, pruritus and erosions at the target tumor site were the most frequently reported application-site reactions (table 3). In eight cases the severity of the application-site reactions led to discontinuation of the therapy. A topical antimicrobial cream was prescribed in those cases. In 19.4% (6 of 31) patients treated with diclofenac and 9.4% (3 of 32) patients treated with combination therapy, surgical excision had to be postponed two weeks, due to the severity of investigator-observed application-site reactions. Three patients had serious adverse events requiring treatment and were hospitalized (table 3), but none of these serious adverse events were considered to be related to the study medication (appendix p 9). No adverse events were reported in the control group.

Table 3. Adverse events according to treatment groups*

	Diclofenac (N = 31)	Calcitriol (N = 32)	Combination therapy (N = 32)	Control (N = 32)
Event				
Any adverse event	28 (90.3%)	27 (84.4%)	28 (87.5%)	0 (0%)
Serious adverse event ¶	2 (6.5%)	0 (0%)	1 (3.2%)	0 (0%)
Adverse event leading to permanent discontinuation	6 (19.4%)	2 (6.2%)	8 (25.0%)	-
Pain at application site	8 (25.8%)	4 (12.5%)	13 (40.6%)	-
Burning sensation at application site	12 (38.7%)	9 (28.1%)	12 (37.5%)	-
Application-site reactions §				-
Erythema	21 (67.7%)	20 (62.5%)	22 (68.8%)	-
Swelling	12 (38.7%)	7 (21.9%)	10 (31.2%)	-
Wounds	12 (38.7%)	9 (28.1%)	13 (40.6%)	-
Crusts	11 (35.5%)	5 (15.6%)	10 (31.2%)	-
Vesicles	11 (35.5%)	7 (21.9%)	7 (21.9%)	-
Scaling	6 (19.4%)	3 (9.4%)	4 (12.5%)	-
Pruritus	16 (51.6%)	11 (34.4%)	14 (43.8%)	-
Paresthesia	5 (16.1%)	2 (6.2%)	5 (15.6%)	-

Data are n (%). Diclofenac=diclofenac sodium-3% gel, Calcitriol=Calcitriol 3µg/g ointment, Combination therapy=diclofenac and calcitriol.

* The events included are those that occurred at the time the first dose of the study drug was administered or any time thereafter, up to 14 days after excision. ¶ Serious adverse events where as follows: hospitalization due to fever and hypotension probably associated with a non-Hodgkin lymphoma, hospitalization due to fever associated with recurrent urinary tract infections after kidney transplantation, hospitalization due to fever associated with erysipelas, 3 days after excision. || Pain and burning sensation scores were assessed using a 10cm Visual Analogue Scale (VAS) where 0 represented 'no pain' and 10 represented, 'most severe pain imaginable'. We computed variables into mild (VAS 1-3), moderate (VAS 4-6) and severe (VAS 7-10) (appendix p 6). § Application-site reactions were reported by the participants in a week diary and scored by mild, moderate or severe (appendix p 6).

Discussion

This phase II trial provides evidence that topical diclofenac has the potential to clear sBCC, with complete histologic tumor regression in 64.3% (9 of 14) and 43.8% (7 of 16) of the patients treated with diclofenac and combination therapy, respectively. These results are in line with significant decreases of expression levels of the proliferative marker Ki-67 and the anti-apoptotic marker Bcl-2 in these treatment groups. Although, application-site reactions were reported frequently, most reactions were of mild to moderate severity, which is in accordance with the literature.²² Similar reactions are seen in other non-invasive therapies for sBCC, such as imiquimod and 5-fluorouracil cream. Those reactions are postulated to be necessary to achieve tumor regression and are indicators of a therapeutic effect.^{3,5} Repetitive occlusion may have attributed to the severity of the skin reactions.

Our results do not corroborate effectiveness of calcitriol. In calcitriol treated sBCCs, slight increases in expression levels of the proliferative marker Ki-67 and slight decreases in the anti-apoptotic marker Bcl-2 between baseline levels and end point levels were detected, and differences in endpoint levels when compared with the control group were not statistically significant. We found no evidence for the hypothesized synergistic effect of the combination of diclofenac and calcitriol. The observed clinical effect of the combination therapy in sBCC is probably due to the effect of diclofenac. A lower diclofenac concentration following dilution by the calcitriol or the relatively high ratio of nodular BCC components in four tumors diagnosed as sBCC in the combination therapy group are possible explanations for clinical treatment failure. In more than 15% of BCC of mixed histology the most aggressive histological subtype is missed by punch biopsy.²³ An uneven distribution of misdiagnosed subtypes among treatment groups despite randomization is probably due to the relatively low sample size within treatment groups.

In the nBCC subgroup, we did not detect significant effects of treatment on Ki-67 and Bcl-2 expression levels or in histological tumor clearance. However, when comparing post treatment expression levels of Ki-67 between the calcitriol and the control group (fig. 3), calcitriol treated nBCCs had slightly, although not significantly, higher Ki-67 and Bcl-2 expression. Previous *in vitro* studies found that high doses of calcitriol can inhibit keratinocyte proliferation, while lower doses may stimulate proliferation.^{24,25} As topical application of low dose calcitriol to mouse skin can even stimulate epidermal proliferation²⁶, this might have been the case in the nBCCs treated with calcitriol.

An explanation for the lack of effect of calcitriol in general may be that the calcitriol ointment did not contain hyaluronic acid, whereas the diclofenac sodium-3% gel contains 2.5% hyaluronic acid. Excipients such as hyaluronic acid may enhance the penetration and bioavailability of a substance.²⁷ It is possible that a higher concentration of calcitriol and/or a different vehicle might be needed to obtain clinical tumor regression.

A complete tumor regression was seen in 18.8% of the controls in the nBCC subgroup. As we included tumors $\geq 4\text{mm}$ and punch biopsies were 3mm, total tumor clearance could be a result of a previously reported biopsy-induced local immune response²⁸, or a lack of sensitivity of regular histological techniques to detect small amounts of residual tumor. The presence of “therapeutic biopsy”, found in the control group is an event that could have occurred in all other treatment groups and underlines the importance of a control group in clinical trials. The difference in response between nBCC and sBCC is in agreement with reports from other studies investigating non-invasive therapies and is presumably caused by insufficient penetration of the drug into the deeper dermis.²⁹

In this study, the use of readily available diclofenac and calcitriol compounds was explored, without previous studies of dose-response effects for this indication. The clinical effect of diclofenac was higher than expected, but not as high as effects of currently available non-invasive BCC treatments. However, the expected major benefit of the investigated therapies is their ability to attack different molecular pathways activated in BCC. Evidence has already suggested that simultaneously targeting SHH and other signaling pathways may have a synergistic effect.¹⁷ Combination of therapies is therefore a logical next step in improving topical treatments. From this perspective, combining diclofenac with imiquimod cream could be promising. Imiquimod is known to inhibit the SHH-pathway and is currently the most effective non-invasive therapy for BCC. However, with a 1-year efficacy of 83.4%³⁰, it is still not as effective as surgery, the gold standard. By adding a drug targeting a different pathway, such as diclofenac cream, the imiquimod resistant cells could be attacked, resulting in higher long-term cure rates.

Limitations of the study are the small sample size and imbalances in baseline levels of Ki-67 and Bcl-2. Despite the small sample size, significant effects of diclofenac treatment in targeting key signaling pathways could be demonstrated and ANCOVA was used to adjust for the differences in baseline levels.

This trial provides evidence that topical application of diclofenac 3% gel in 2.5% hyaluronic acid in sBCC significantly reduces proliferation, induces apoptosis and moreover results in significant histological clearance compared to the control group. We therefore conclude that although surgical excision remains the gold standard for all BCC, topical diclofenac may be a promising new treatment for low risk sBCC. Efficacy of topical calcitriol was not observed. Other trials using different concentrations, excipients or combinations of both investigated drugs may be useful to optimize treatment strategies. Also, given the effectiveness of diclofenac gel in treatment of both BCCs and actinic keratosis with only limited side effects, it is tempting to speculate that there might be a role for topical diclofenac as a prophylactic agent in NMSC. This might be an interesting hypothesis to test in future studies.

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Appendix

Eligibility criteria

Patients with a histologically proven primary superficial BCC or (micro)nodular BCC with a diameter of at least 4 mm, not located in the face or hairy scalp were eligible for inclusion and were asked to participate in the trial. Only one BCC per patient was included in order to facilitate the patients' compliance. In patients with two or more BCC eligible for inclusion, the tumour most accessible for topical application of the cream by the patient him/herself was chosen for inclusion. When all BCCs were equally accessible, the largest tumour was included. The main exclusion criteria were: proven or suspected malignancy of other organs; genetic skin cancer disorders; tumors in the face or on the hairy scalp; incapable of comprehending treatment instructions; use of oral NSAIDs (> 4 days a week) or vitamin D (containing) supplements during the trial period or within 30 days before inclusion.

Power analysis

For calculation of the sample size it was assumed that in non-treated BCCs the mean percentage of cells expressing Ki-67 would be 25% with a standard deviation of 8%. Assuming an alpha of 5% with a power of 90%, 13 patients per study arm were required to detect a minimally relevant clinical difference of at least 10% in the percentage of cells expressing Ki-67 at the end of the treatment when comparing active treatment with control. Taking into account a potential lost to follow up of 20%, we planned to include 16 patients per randomized group.

Immunohistochemical staining procedure and assessment

Formalin-fixed and paraffin-embedded (FFPE) 4µm tissue sections of both biopsy and excision specimens were collected onto one slide to ensure congruent staining results. Immunohistochemistry (IHC) was performed with the primary antibodies Ki-67 and Bcl-2 (both mouse monoclonal antibodies [mAbs]). After heat induced epitope retrieval (K8004 EnVision™ Flex Target Retrieval Solution High pH 9, DAKO), immunohistochemical analysis was performed using DakoAutostainer Link 48™ (DAKO Corporation, Carpinteria, CA, USA). Endogenous peroxidase activity was blocked by peroxidase blocking reagent for 5 minutes. The primary antibodies Ki-67 (Ki-67, clone MIB-1, ready to use Antibody, DAKO) and Bcl-2 (Bcl-2, clone 124, ready to use Antibody, DAKO) were incubated for 20 minutes. The DAKO Envision Flex kit (K8002) was used for secondary detection. After secondary detection, all sections were counterstained with Haematoxylin. In all IHC procedures positive controls were included. Two independent and blinded dermatopathologists, manually quantified the percentage of tumor cells positive for Ki-67 and Bcl-2 at x200 magnification. Zero percent expression was scored if there were no tumor cells in the excision specimens (complete histologic tumor regression). Discrepancies in the quantification were resolved in a follow-up joint review of the slides and discussion by both pathologists.

Comprehensive description of serious adverse events

The first participant (sBCC, diclofenac sodium-3% gel) was hospitalized with fever and hypotension associated with a recurrent non-Hodgkin lymphoma. This participant already had a medical history of recurrent Hodgkin lymphomas treated with radio- and chemotherapy more than 5 years ago. The second participant (nBCC, diclofenac sodium-3% gel) was hospitalized with fever due to a urinary tract infection. The patient had a history of kidney transplantation and known recurrent urinary tract infections. Both patients continued their study medication during their hospitalization. The third participant (nBCC, combined therapy) was hospitalized with fever due to erysipelas at the excision site two days after excision of the target lesion. This participant already discontinued the study medication after trial week 6 due to erythema of the target lesion. The erythema had resolved at the time of the excision.

Figure S1. Clinical and histological responses.

A. Superficial basal cell carcinoma (sBCC) at baseline. **B.** sBCC after 8 weeks treatment with diclofenac sodium-3% gel and calcitriol 3 µg/g (combined therapy). **C.** Specific nuclear (brown) Ki-67 expression at baseline in an sBCC. **D.** Reduction of Ki-67 expression after 8 weeks treatment with combined therapy in an sBCC as assessed by immunohistochemistry (IHC). **E.** Specific cytoplasmic (brown) Bcl-2 expression at baseline in an sBCC. **F.** Reduction of Bcl-2 expression after 8 weeks treatment with combined therapy in an sBCC as assessed by IHC. Fig A-F are from the same patient.

Figure S2. Clinical and histological responses in biopsy versus excision specimens.

G. Nodular basal cell carcinoma (nBCC) at baseline. **H.** nBCC after 8 weeks without topical treatment (control group). **I.** Specific nuclear (brown) Ki-67 expression at baseline in an nBCC. **J.** No reduction of Ki-67 expression after 8 weeks without topical treatment in an nBCC as assessed by immunohistochemistry (IHC). **K.** Specific cytoplasmic (brown) Bcl-2 expression at baseline in an nBCC. **L.** No reduction of Bcl-2 expression after 8 weeks treatment without topical treatment in an nBCC as assessed by IHC. Figures G-L are from the same patient.

Figure S3. Clinical and histological responses after 8 weeks.

A. Superficial basal cell carcinoma (sBCC) after 8 weeks treatment with topical diclofenac sodium-3% gel. **B.** sBCC after 8 weeks without topical treatment (control group). **C.** Specific nuclear (brown) Ki-67 expression after 8 weeks treatment with diclofenac in an sBCC as assessed by immunohistochemistry (IHC). **D.** Specific nuclear Ki-67 expression after 8 weeks without topical treatment in an sBCC as assessed by IHC. **E.** Specific cytoplasmic (brown) Bcl-2 expression after 8 weeks treatment with diclofenac in an sBCC. **F.** Specific cytoplasmic Bcl-2 expression after 8 weeks without topical treatment in an sBCC as assessed by IHC. Figures A, C and E and B, D and F are from the same patients.

Table S1: Median compliance rates according to treatment assignment

	Median Compliance (%)
Superficial BCC	
Diclofenac sodium-3%	93.18% (0 to 100)
Calcitriol	98.18% (15.45 to 100)
Diclofenac sodium-3% and Calcitriol	96.36% (10.91 to 100)
Nodular BCC	
Diclofenac sodium-3%	91.82% (23.64 to 100)
Calcitriol	96.36% (0 to 100)
Diclofenac sodium-3% and Calcitriol	93.64% (0 to 100)
Data are median % (range). BCC=Basal Cell Carcinoma. Diclofenac=diclofenac sodium-3% gel, Calcitriol=Calcitriol 3µg/g ointment, Combined therapy=diclofenac and calcitriol Compliance was calculated as the number of applications actually applied by the patient as percentage of the total prescribed number of applications.	

Supplementary Table S2. Adverse events												
Event	Diclofenac (N = 31)			Calcitriol (N = 32)			Combined therapy (N = 32)					
	Mild	Moderate	Severe	Mild	Moderate	Severe	Mild	Moderate	Severe			
Any adverse event	28 (90.3%)			27 (84.4%)			28 (87.5%)			n		
Serious adverse event ¶	2 (6.5%)			0 (0%)			1 (3.2%)					
Adverse event leading to discontinuation	6 (19.4%)			2 (6.2%)			8 (25.0%)					
Application-site reactions §												
Erythema												
Week 1	29 n	8 (27.6%)	4 (13.8%)	2 (6.9%)	30 n	2 (6.9%)	6 (20.0%)	1 (3.3%)	28 n	4 (14.3%)	3 (10.7%)	1 (3.6%)
Week 2	28	13 (46.4%)	2 (7.1%)	1 (3.3%)	30	1 (3.3%)	6 (20.0%)	1 (3.3%)	28	5 (17.9%)	5 (17.9%)	2 (7.1%)
Week 3	26	10 (38.5%)	3 (11.5%)	0 (0.0%)	29	0 (0.0%)	5 (17.2%)	1 (3.4%)	27	6 (22.2%)	7 (38.5%)	2 (27.6%)
Week 4	27	6 (22.2%)	6 (22.2%)	2 (7.4%)	29	2 (7.4%)	5 (17.2%)	0 (0.0%)	28	4 (14.3%)	4 (14.3%)	2 (7.1%)
Week 5	25	5 (20.0%)	4 (16.0%)	2 (8.0%)	29	2 (8.0%)	4 (13.8%)	0 (0.0%)	29	6 (20.7%)	4 (13.8%)	3 (10.3%)
Week 6	23	3 (13.0%)	7 (30.4%)	0 (0.0%)	28	0 (0.0%)	3 (10.7%)	1 (3.6%)	28	4 (14.3%)	4 (14.3%)	5 (17.9%)
Week 7	21	3 (14.3%)	6 (28.6%)	0 (0.0%)	28	0 (0.0%)	2 (7.1%)	0 (0.0%)	27	6 (22.2%)	5 (18.5%)	3 (11.1%)

Supplementary Table S2. Adverse events

Event	Diclofenac (N = 31)				Calcitriol (N = 32)				Combined therapy (N = 32)			
Week 8	19	3 (15.8%)	6 (31.6%)	1 (5.3%)	26	1 (5.3%)	2 (7.7%)	0 (0.0%)	23	5 (21.7%)	7 (30.4%)	0 (0.0%)
Swelling												
Week 1	30	0 (0.0%)	3 (10.0%)	1 (3.3%)	30	0 (0.0%)	0 (0.0%)	0 (0.0%)	28	2 (7.1%)	1 (3.6%)	0 (0.0%)
Week 2	29	0 (0.0%)	3 (10.3%)	0 (0.0%)	29	4 (13.8%)	1 (3.4%)	0 (0.0%)	27	2 (7.4%)	3 (11.1%)	0 (0.0%)
Week 3	26	1 (3.8%)	1 (3.8%)	0 (0.0%)	28	2 (7.1%)	0 (0.0%)	0 (0.0%)	27	3 (11.1%)	1 (3.7%)	0 (0.0%)
Week 4	27	5 (18.5%)	0 (0.0%)	1 (3.7%)	28	2 (7.1%)	0 (0.0%)	0 (0.0%)	27	4 (14.8%)	3 (11.1%)	0 (0.0%)
Week 5	26	3 (11.5%)	1 (3.8%)	1 (3.8%)	28	2 (2.4%)	0 (0.0%)	0 (0.0%)	28	6 (21.4%)	1 (3.6%)	0 (0.0%)
Week 6	24	2 (8.3%)	2 (8.3%)	0 (0.0%)	28	1 (3.6%)	0 (0.0%)	0 (0.0%)	28	4 (14.3%)	3 (10.7%)	0 (0.0%)
Week 7	20	1 (5.0%)	1 (5.0%)	0 (0.0%)	28	0 (0.0%)	0 (0.0%)	0 (0.0%)	26	5 (19.2%)	1 (3.8%)	1 (3.8%)
Week 8	19	0 (0.0%)	2 (10.5%)	0 (0.0%)	17	1 (3.7%)	0 (0.0%)	0 (0.0%)	23	4 (17.4%)	0 (0.0%)	0 (0.0%)
Wounds												
Week 1	29	0 (0.0%)	2 (6.9%)	2 (6.9%)	29	4 (13.8%)	1 (3.4%)	0 (0.0%)	28	0 (0.0%)	0 (0.0%)	0 (0.0%)
Week 2	28	0 (0.0%)	2 (7.1%)	1 (3.6%)	30	4 (13.3%)	3 (10.0%)	1 (3.3%)	27	0 (0.0%)	2 (7.4%)	1 (3.7%)
Week 3	26	4 (15.4%)	1 (3.8%)	0 (0.0%)	29	3 (10.3%)	1 (3.4%)	1 (3.4%)	27	2 (7.4%)	1 (3.7%)	0 (0.0%)
Week 4	27	3 (11.1%)	1 (3.7%)	1 (3.7%)	28	3 (10.7%)	1 (3.6%)	0 (0.0%)	27	4 (14.8%)	1 (3.7%)	0 (0.0%)
Week 5	26	3 (11.5%)	1 (3.8%)	1 (3.8%)	28	4 (14.3%)	1 (3.6%)	0 (0.0%)	28	5 (17.9%)	1 (3.6%)	0 (0.0%)
Week 6	24	5 (20.8%)	1 (3.6%)	0 (0.0%)	28	1 (3.6%)	2 (7.1%)	0 (0.0%)	28	4 (14.3%)	3 (10.7%)	3 (10.7%)
Week 7	21	3 (14.3%)	1 (4.8%)	0 (0.0%)	28	2 (7.1%)	1 (3.6%)	0 (0.0%)	26	4 (15.4%)	4 (15.4%)	2 (7.7%)
Week 8	19	3 (15.8%)	0 (0.0%)	0 (0.0%)	27	0 (0.0%)	1 (3.7%)	0 (0.0%)	23	3 (13.0%)	3 (13.0%)	1 (4.3%)
Crusts												

Supplementary Table S2. Adverse events													
Event	Diclofenac (N = 31)				Calcitriol (N = 32)				Combined therapy (N = 32)				
Vesicles	Week 1	29	2 (6.9%)	1 (3.4%)	0 (0.0%)	30	1 (3.3%)	0 (0.0%)	0 (0.0%)	28	0 (0.0%)	0 (0.0%)	0 (0.0%)
	Week 2	28	1 (3.6%)	1 (3.6%)	0 (0.0%)	29	0 (0.0%)	2 (6.9%)	0 (0.0%)	27	1 (3.7%)	2 (7.4%)	0 (0.0%)
	Week 3	26	3 (11.5%)	0 (0.0%)	0 (0.0%)	29	1 (3.4%)	1 (3.4%)	0 (0.0%)	27	2 (7.4%)	0 (0.0%)	0 (0.0%)
	Week 4	26	0 (0.0%)	1 (3.8%)	1 (3.8%)	28	1 (3.6%)	0 (0.0%)	0 (0.0%)	27	1 (3.7%)	0 (0.0%)	0 (0.0%)
	Week 5	26	0 (0.0%)	2 (7.7%)	1 (3.8%)	28	0 (0.0%)	0 (0.0%)	0 (0.0%)	28	1 (3.6%)	2 (7.1%)	0 (0.0%)
	Week 6	24	1 (4.2%)	2 (8.3%)	0 (0.0%)	28	1 (3.6%)	0 (0.0%)	0 (0.0%)	27	1 (3.7%)	0 (0.0%)	2 (7.4%)
	Week 7	20	1 (5.0%)	0 (0.0%)	0 (0.0%)	28	1 (3.6%)	0 (0.0%)	0 (0.0%)	26	0 (0.0%)	3 (11.5%)	0 (0.0%)
	Week 8	19	1 (5.3%)	0 (0.0%)	0 (0.0%)	27	1 (3.7%)	0 (0.0%)	0 (0.0%)	23	0 (0.0%)	3 (13.0%)	0 (0.0%)
Vesicles													
Week 1	29	1 (3.4%)	1 (3.4%)	0 (0.0%)	0 (0.0%)	30	2 (6.7%)	0 (0.0%)	0 (0.0%)	28	1 (3.6%)	0 (0.0%)	0 (0.0%)
Week 2	28	0 (0.0%)	1 (3.6%)	1 (3.6%)	0 (0.0%)	29	2 (6.9%)	0 (0.0%)	0 (0.0%)	27	1 (3.7%)	0 (0.0%)	1 (3.7%)
Week 3	26	3 (11.5%)	1 (3.8%)	0 (0.0%)	0 (0.0%)	28	0 (0.0%)	2 (7.1%)	0 (0.0%)	27	0 (0.0%)	0 (0.0%)	1 (3.7%)
Week 4	27	2 (7.4%)	2 (7.4%)	2 (7.4%)	0 (0.0%)	28	0 (0.0%)	0 (0.0%)	1 (3.6%)	27	0 (0.0%)	1 (3.7%)	0 (0.0%)
Week 5	26	3 (11.5%)	2 (7.7%)	1 (3.8%)	0 (0.0%)	28	1 (3.6%)	1 (3.6%)	0 (0.0%)	28	2 (7.1%)	1 (3.6%)	1 (3.6%)
Week 6	24	1 (4.2%)	2 (8.3%)	0 (0.0%)	0 (0.0%)	28	3 (10.7%)	0 (0.0%)	0 (0.0%)	28	1 (3.6%)	2 (7.1%)	2 (7.1%)
Week 7	21	2 (9.5%)	1 (4.8%)	0 (0.0%)	0 (0.0%)	28	1 (3.6%)	0 (0.0%)	0 (0.0%)	26	0 (0.0%)	6 (23.1%)	0 (0.0%)
Week 8	19	2 (10.5%)	1 (5.3%)	0 (0.0%)	0 (0.0%)	27	1 (3.7%)	0 (0.0%)	0 (0.0%)	23	1 (4.3%)	2 (8.7%)	0 (0.0%)
Scaling													
Week 1	29	1 (3.4%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	30	1 (3.3%)	0 (0.0%)	0 (0.0%)	28	0 (0.0%)	0 (0.0%)	0 (0.0%)
Week 2	28	3 (10.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	29	1 (3.4%)	0 (0.0%)	0 (0.0%)	27	1 (3.7%)	1 (3.7%)	0 (0.0%)

Supplementary Table S2. Adverse events

Event	Diclofenac (N = 31)			Calcitriol (N = 32)			Combined therapy (N = 32)		
Week 3	Week 3	26	1 (3.8%)	0 (0.0%)	0 (0.0%)	28	2 (7.1%)	0 (0.0%)	0 (0.0%)
	Week 4	27	0 (0.0%)	1 (3.7%)	0 (0.0%)	28	1 (3.6%)	0 (0.0%)	0 (0.0%)
	Week 5	26	0 (0.0%)	2 (7.7%)	0 (0.0%)	28	0 (0.0%)	1 (3.6%)	0 (0.0%)
	Week 6	23	2 (8.7%)	0 (0.0%)	0 (0.0%)	28	2 (7.1%)	0 (0.0%)	0 (0.0%)
	Week 7	20	0 (0.0%)	0 (0.0%)	0 (0.0%)	28	2 (7.1%)	0 (0.0%)	0 (0.0%)
	Week 8	19	2 (10.5%)	0 (0.0%)	0 (0.0%)	27	1 (3.7%)	0 (0.0%)	0 (0.0%)
	Pruritus								
	Week 1	29	4 (13.8%)	1 (3.4%)	0 (0.0%)	30	4 (13.3%)	4 (13.3%)	0 (0.0%)
Week 2	Week 2	28	4 (14.3%)	0 (0.0%)	1 (3.6%)	30	5 (16.7%)	0 (0.0%)	1 (3.3%)
	Week 3	26	3 (11.5%)	3 (11.5%)	0 (0.0%)	29	6 (20.7%)	1 (3.4%)	1 (3.4%)
	Week 4	27	9 (33.3%)	3 (11.1%)	1 (3.7)	28	4 (14.3%)	2 (7.1%)	0 (0.0%)
	Week 5	26	5 (19.2%)	3 (11.5%)	1 (3.8%)	29	3 (10.3%)	2 (6.9%)	0 (0.0%)
	Week 6	23	5 (21.7%)	2 (8.7%)	0 (0.0%)	29	3 (10.3%)	1 (3.4%)	0 (0.0%)
	Week 7	20	3 (15.0%)	1 (5.0%)	0 (0.0%)	28	2 (7.1%)	3 (10.7%)	0 (0.0%)
	Week 8	19	4 (21.1%)	2 (10.5%)	1 (5.3%)	27	1 (3.7%)	2 (7.4%)	0 (0.0%)
	Paraesthesia								
Week 1	Week 1	29	0 (0.0%)	1 (3.4%)	0 (0.0%)	30	1 (3.3%)	0 (0.0%)	0 (0.0%)
	Week 2	28	0 (0.0%)	1 (3.6%)	0 (0.0%)	29	0 (0.0%)	1 (3.4%)	0 (0.0%)
	Week 3	26	2 (7.7%)	0 (0.0%)	0 (0.0%)	29	0 (0.0%)	1 (3.4%)	0 (0.0%)

Event	Diclofenac (N = 31)				Calcitriol (N = 32)				Combined therapy (N = 32)			
Week 4	27	1 (3.7%)	0 (0.0%)	0 (0.0%)	28	1 (3.6%)	0 (0.0%)	0 (0.0%)	27	1 (3.7%)	1 (3.7%)	0 (0.0%)
Week 5	25	3 (12.0%)	0 (0.0%)	0 (0.0%)	28	0 (0.0%)	0 (0.0%)	0 (0.0%)	28	1 (3.6%)	0 (0.0%)	0 (0.0%)
Week 6	23	0 (0.0%)	0 (0.0%)	0 (0.0%)	28	0 (0.0%)	0 (0.0%)	0 (0.0%)	27	1 (3.7%)	1 (3.7%)	0 (0.0%)
Week 7	20	0 (0.0%)	0 (0.0%)	1 (5.0%)	28	0 (0.0%)	0 (0.0%)	0 (0.0%)	26	1 (3.8%)	1 (3.8%)	0 (0.0%)
Week 8	19	1 (5.3%)	0 (0.0%)	0 (0.0%)	27	0 (0.0%)	0 (0.0%)	0 (0.0%)	23	0 (0.0%)	0 (0.0%)	0 (0.0%)
Pain II												
Week 1	30	1 (3.3%)	1 (3.3%)	1 (3.3%)	31	1 (3.2%)	0 (0.0%)	0 (0.0%)	31	0 (0.0%)	0 (0.0%)	2 (6.5%)
Week 2	29	0 (0.0%)	2 (6.9%)	1 (3.4%)	30	1 (3.3%)	2 (6.7%)	0 (0.0%)	31	1 (3.2%)	1 (3.2%)	0 (0.0%)
Week 3	30	1 (3.8%)	0 (0.0%)	0 (0.0%)	26	0 (0.0%)	1 (3.4%)	0 (0.0%)	30	0 (0.0%)	2 (6.7%)	0 (0.0%)
Week 4	27	2 (7.4%)	0 (0.0%)	0 (0.0%)	29	0 (0.0%)	0 (0.0%)	0 (0.0%)	29	1 (3.4%)	1 (3.4%)	0 (0.0%)
Week 5	26	0 (0.0%)	1 (3.8%)	0 (0.0%)	29	0 (0.0%)	0 (0.0%)	0 (0.0%)	29	5 (17.2%)	0 (0.0%)	0 (0.0%)
Week 6	25	0 (0.0%)	0 (0.0%)	0 (0.0%)	29	0 (0.0%)	0 (0.0%)	0 (0.0%)	29	3 (10.3%)	0 (0.0%)	0 (0.0%)
Week 7	22	0 (0.0%)	0 (0.0%)	1 (4.5%)	28	0 (0.0%)	0 (0.0%)	0 (0.0%)	29	2 (6.9%)	1 (3.4%)	1 (3.4%)
Week 8	21	1 (94.8%)	0 (0.0%)	2 (9.5%)	27	0 (0.0%)	0 (0.0%)	0 (0.0%)	26	2 (7.7%)	1 (3.8%)	2 (97.7%)
Burning sensation II												
Week 1	30	1 (3.3%)	1 (3.3%)	0 (0.0%)	30	2 (6.7%)	0 (0.0%)	0 (0.0%)	31	2 (6.5%)	1 (3.2%)	1 (3.2%)
Week 2	29	0 (0.0%)	2 (6.9%)	2 (6.9%)	30	1 (3.3%)	1 (3.3%)	1 (3.3%)	31	1 (3.2%)	1 (3.2%)	1 (3.2%)
Week 3	26	1 (3.8%)	2 (7.7%)	0 (0.0%)	29	3 (10.3%)	1 (3.4%)	0 (0.0%)	30	1 (3.3%)	2 (6.7%)	2 (6.7%)
Week 4	27	4 (14.8%)	0 (0.0%)	0 (0.0%)	29	1 (3.4%)	1 (3.4%)	0 (0.0%)	28	3 (10.7%)	1 (3.6%)	0 (0.0%)

Supplementary Table S2. Adverse events										
Event	Diclofenac (N = 31)				Calcitriol (N = 32)				Combined therapy (N = 32)	
Week 5	26	5 (19.2%)	0 (0.0%)	1 (3.8%)	29	1 (3.4%)	1 (3.4%)	0 (0.0%)	29	3 (10.3%)
Week 6	24	5 (20.8%)	0 (0.0%)	0 (0.0%)	29	2 (6.9%)	1 (3.4%)	0 (0.0%)	29	2 (6.9%)
Week 7	22	1 (4.5%)	1 (4.5%)	1 (4.5%)	28	2 (7.1%)	0 (0.0%)	0 (0.0%)	29	2 (6.9%)
Week 8	20	3 (15.0%)	1 (5.0%)	2 (10.0%)	27	0 (0.0%)	0 (0.0%)	0 (0.0%)	25	2 (8.0%)
Data are n (%). Diclofenac=diclofenac sodium-3% gel, Calcitriol=Calcitriol 3µg/g ointment, Combined therapy=diclofenac and calcitriol. * The events included are those that occurred at the time the first dose of the study drug was administered or any time thereafter, up to 14 days after excision. † Serious adverse events where as follows: hospitalization due to fever and hypotension probably associated with a non-Hodgkin lymphoma, hospitalization due to fever associated with recurrent urinary tract infections after kidney transplantation, hospitalization due fever associated with erysipelas, 3 days after excision. ‡ Pain and burning sensation scores were assessed using a 10cm Visual Analogue Scale (VAS%) where 0 represented 'no pain' and 10 represented, 'most severe pain imaginable'. We computed variables into mild (VAS 1-3%), moderate (VAS 4-6%) and severe (VAS 7-10%). ‡ Application-site reactions were reported by the participants in a diary and scored by mild, moderate or severe.

An abstract graphic on the left side of the page. It features a series of red circles of varying sizes, some of which are outlined in white. These circles are connected by a network of thin, light pink lines that form a larger, elongated shape. The lines are more densely packed in some areas and more sparse in others, creating a sense of flow and movement. The overall effect is a modern, artistic representation of a biological or molecular structure.

Chapter 4.2

A comparison of semi-automated
versus pathologist-based manual
quantification of immunohistochemical
stains in basal cell carcinoma

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Abstract

Immunohistochemical analysis of important markers for proliferation (Ki-67) and apoptosis (Bcl-2) is crucial to evaluate therapeutic efficacy of experimental treatments in basal cell carcinoma (BCC). Manual pathologist-based quantification (MQ) is currently still the most frequently used technique, however it is subjected to high intra- and inter-observer variability, it is time-consuming and labor-intensive. Evaluation of 122 superficial and nodular BCC shows that semi-automated quantification (SAQ) generates reasonable high inter-observer agreements with 95% limits of agreement between -9.2% and 10.8% for Ki-67 and between -12.2% and 20.0% for Bcl2. However, large discrepancies were seen between SAQ and MQ especially at higher expression levels (more than 20% positive tumor cells) of Ki-67 and Bcl-2. This finding points to the need for a further study wherein consensus evaluation by more than one pathologist will be compared with SAQ measurements.

Introduction

Immunohistochemical (IHC) analysis is being used more often to gain insight in the underlying mode of action or efficacy of new targeted therapies for basal cell carcinoma (BCC).¹⁻³ Manual pathologist-based quantification (MQ) is currently the most frequently used technique for IHC evaluation⁴ and is considered as the gold standard. However, estimation of the percentage positively stained tumor cells is a subjective approach and may demonstrate high intra- and inter-observer variability.⁵⁻⁷ Moreover, MQ is time-consuming and labor-intensive.⁸ Dichotomized scoring scales are frequently used to standardize quantification of immunohistochemical stains.^{9,10} However, a major disadvantage of these categorized scoring methods is that they are less sensitive to detect limited changes in expression.

BCC is the most common malignancy in Caucasians, leading to a social and economic burden on health care facilities.^{11,12} There is an urgent need for cost-efficient and patient friendly (targeted) non-invasive treatments. The proliferation marker Ki-67 and anti-apoptosis marker Bcl-2 have previously been used in research settings to evaluate therapeutic efficacy of experimental treatments in BCC.^{1,2} The nuclear protein Ki-67 is expressed in all active cell cycle phases, except G₀.¹³ Ki-67 is generally accepted as the most accurate cell proliferation marker.¹⁴ The proto-oncogene Bcl-2 encodes the largest known anti-apoptotic cytoplasmic protein¹⁵ and inhibits programmed cell death (apoptosis). For reliable evaluation of IHC analysis in the development of BCC treatments, there is a need for a reproducible and time-efficient method. Digital image analysis techniques offer an objective approach with a high sensitivity for change and the possibility for increased reproducibility.^{16,17} However, comparative studies that prove the assumption of high reproducibility of digital image analysis in BCC are lacking.

A digital image analysis program was used to quantify the expression of Ki-67 and Bcl-2 in BCC. The aim of this study was to evaluate reproducibility of semi-automated quantification (SAQ) of skin tissue sections in patients with superficial and nodular BCC using a self-developed software program. Another aim was to evaluate the agreement of SAQ scores with MQ scores.

Materials & methods

Immunohistochemistry

Tissue sections were derived from patients ($n=128$) enrolled in a single blind, exploratory phase II, randomized-controlled trial at the Maastricht University Medical Center (MUMC+) registered at clinicaltrials.gov, number NCT01358045 (clinical trial chapter 4.1). All patients gave their written informed consent before enrolment. This study was conducted according to the Declaration of Helsinki principles. Haematoxylin and eosin histological sections from all formalin-fixed paraffin-embedded (FFPE) punch biopsy tumor blocks were re-evaluated by an investigator and a dermato-pathologist and per patient the tumor block including the most tumor cells was selected for further analysis. Immunohistochemical stains were performed on 4- μ m tissue sections according to standardized laboratory protocols at the Department of Pathology, MUMC+ by using a DakoAutostainer Link 48™ (DAKO Corporation, Carpinteria, CA, USA). Briefly, after heat induced epitope retrieval (K8004 EnVision™ Flex Target Retrieval Solution High pH 9, DAKO), immunohistochemical analysis was performed using DakoAutostainer Link 48™ (DAKO Corporation, Carpinteria, CA, USA). Endogenous peroxidase activity was blocked by peroxidase blocking reagent for 5 minutes. The primary antibodies Ki-67 (IR614, clone MIB-1, mouse monoclonal, ready to use Antibody, DAKO) and Bcl-2 (IR626, clone 124, mouse monoclonal, ready to use Antibody, DAKO) were incubated for 20 minutes. The DAKO Envision Flex kit (K8002) was used for secondary detection. After secondary detection, all sections were counterstained with haematoxylin. In both IHC procedures positive controls were included and all showed the expected positive result.

Quantification of immunohistochemical staining

One dermato-pathologist and two investigators analyzed the same set of 122 biopsies stained with Ki-67 and Bcl-2 antibodies. MQ was performed independently by one dermato-pathologist blinded for order, patient and clinical data. BCC nests were identified manually in all fields. The percentage positively stained tumor cells (brown staining) within the total number of tumor cells was manually estimated in all tumor nests on the entire slide at x200 magnification.

SAQ was performed independently by two trained, but previously inexperienced, investigators blinded for order, patient and clinical data. All slides were analyzed by using a so-called operator-dependent computerized thresholding technique (Leica Qwin version 3.5.1, Leica Microsystems, Cambridge, UK). This technique is an extensively evaluated method, especially for mammographic density.^{18,19} All tumor nests were manually identified under low-power magnification. From each section, two color photomicrographs of 2088x1550 pixels red-green-blue (RGB) format were obtained with a Leica DFC320® digital camera coupled to a microscope (Leica DM3000) using a x10 objective for both markers. In case there was only one tumor nest on the biopsy, one picture was taken. The images were stored as tagged image file (TIF). For each new session the microscope, camera and computer were calibrated according to a standardized procedure. Both stains were performed on parallel tissue sections. All images were analyzed using Leica Qwin version 3.5.1. For each image, the following

procedure was performed. The investigator defined the tumor area to be quantified by encircling the area manually with a mouse. Color thresholds were manually calibrated to establish a positive color threshold for each immunohistochemical staining. The threshold values of each staining were held constant during the assessment of all images. The percentage nuclear (Ki-67) or cytoplasm (Bcl-2) immunopositive area was calculated automatically by dividing the sum of the pixels of the immunopositive area by the sum of the pixels of the total tumor area multiplied by one hundred. Outcomes were automatically stored in an Excel-file.

Statistical analysis

Statistical analyses were performed by using SPSS version 20.0 (IBM SPSS Statistics, Chicago, IL, USA). Agreement between observers on SAQ scores as well as agreement between SAQ and MQ measurements was visualized by Bland-Altman plots. The difference in measurements between observers are plotted against the mean of measurements.²⁰ If the mean difference deviates from zero, this deviance can be interpreted as systematic error. The standard deviation (SD) of the mean difference is used to calculate 95% limits of agreement ($1.96 \times \text{SD}$) which represent the range wherein 95% of discrepancies between measurements are situated. The limits of agreement give an indication of the size of random measurement error.²¹ Additionally, the inter-observer reproducibility for SAQ was calculated by using the intra-class correlation coefficient (ICC). An ICC < 0.4 indicates poor reproducibility, ≥ 0.4 and < 0.75 fair to good reproducibility and ≥ 0.75 excellent reproducibility.²²

Results

Semi-automated quantification shows reproducible expression levels of Ki-67 and Bcl-2 in basal cell carcinoma

Of 128 available punch biopsy specimens, 122 (58 superficial BCC and 64 nodular BCC) included sufficient paraffin embedded tumor tissue to enable staining with Ki-67 and Bcl-2 antibodies, respectively. All 122 slides were assessed by SAQ and MQ. Figure 1 provides an overview of an example of a superficial/nodular BCC assessed using an operator-dependent computerized thresholding technique (Leica Qwin version 3.5.1).

For Ki-67, mean percentage of positive tumor cells was 16.2 ± 10.0 for investigator 1 and 15.4 ± 10.3 for investigator 2 (Table 1). A Bland-Altman plot of the SAQ results for Ki-67 indicates a mean difference between investigators of 0.8 ± 5.1 with 95% limits of agreement ranging from -9.2 to 10.8 (Table 2 and Figure 2a). The intra-class correlation coefficient (ICC) was 0.87 [95% CI 0.82-0.91].

Mean expression levels of Bcl-2 were 12.9 ± 13.8 for investigator 1 and 9.0 ± 10.5 for investigator 2 (average 10.9 ± 11.6) (Table 1). The mean difference between the two assessors was 3.9 ± 8.2 . The 95% limits ranged from -12.2 to 20.0. The ICC was of 0.74 [95% CI 0.58-0.84] (Table 2 and Figure 2b).

Figure 1. Overview of operator-dependent computerized thresholding technique (Leica Qwin version 3.5.1, Leica Microsystems, Cambridge, UK) for Ki-67 immunohistochemical stains.

An overview of Ki-67 semi-automated quantification in a basal cell carcinoma using an operator-dependent computerized thresholding technique. (a) Immunohistochemical stain for Ki-67 in a superficial and nodular basal cell carcinoma. (b) Manually drawn tumor area. (c) Detection of positively stained tumor cells. (d) Detection of negatively stained tumor cells. (e) Positively and negatively stained cells per within the tumor area.

Table 1. Percentages of positively stained cells for semi-automated (SAQ) and manual quantification (MQ) measurements.

Ki-67	Mean % $\pm$ SD	Average of mean % SAQ scores $\pm$ SD
SAQ <i>investigator 1</i>	16.2 $\pm$ 10.0	15.8 $\pm$ 9.8
SAQ <i>investigator 2</i>	15.4 $\pm$ 10.3	
MQ	19.3 $\pm$ 20.1	
Bcl-2		
SAQ <i>investigator 1</i>	12.9 $\pm$ 13.8	10.9 $\pm$ 11.6
SAQ <i>investigator 2</i>		9.0 $\pm$ 10.5
MQ		34.6 $\pm$ 32.9
SD: standard deviation.		

Table 2. Agreement between semi-automated quantification (SAQ) derived by two investigators and agreement between SAQ and manual quantification (MQ) by one pathologist.

SAQ (investigator1 vs investigator 2)	Mean difference $\pm$ SD	95% limits of agreement
Ki-67	0.8 $\pm$ 5.1	-9.2 to 10.8
Bcl-2	3.9 $\pm$ 8.2	-12.2 to 20.0
MQ minus SAQ		
Ki-67	3.5 $\pm$ 14.4	-24.7 to 31.7
Ki-67 $\leq$ 20% positive cells	-2.2 $\pm$ 7.4	-16.7 to 12.3
Ki-67 > 20% positive cells	19.0 $\pm$ 17.3	-14.9 to 52.9
Bcl-2	23.7 $\pm$ 26.8	-28.8 to 76.2
Bcl-2 $\leq$ 20% positive cells	3.3 $\pm$ 9.2	-14.7 to 21.3
Bcl-2 > 20% positive cells	51.0 $\pm$ 16.0	19.64 to 82.36
SD: standard deviation of mean difference. The results presented are percentages.

Figure 2. Bland-Altman plot for semi-automated quantification of Ki-67 and Bcl-2 immunohistochemical stains in basal cell carcinoma showing high reproducibility.

Semi-automated quantification by two independent investigators for Ki-67, (a) and Bcl-2, (b). The average percentage positive tumor cells is represented on the x-axis. The y-axis (difference in Ki-67 and Bcl-2 respectively) represents the difference in percentage of positively stained tumor cells assessed by assessor 1 minus assessor 2. The middle continuous line represents the mean difference of all measurements. The two dashed lines represent the 95% limits of agreements (± 1.96 Standard Deviation).

Manual quantification versus semi-automated quantification of Ki-67 and Bcl-2 in basal cell carcinoma shows limited agreement, especially at high percentage positive tumor cells

When comparing SAQ with MQ, the mean percentage of Ki-67 positive cells according to MQ was 19.3 ± 20.1 SD and higher than the average of the two investigators on SAQ of 15.8 ± 9.8 (Table 1). The mean difference was 3.5 ± 14.4 and 95% of the differences fell between -24.7 and 31.7 with (Table 2 and Figure 3a). However, the Bland-Altman plot indicated that the magnitude of differences did not remain the same over the whole range of mean values. Limited agreement was seen especially at higher expression levels of Ki-67. Therefore, tumors were categorized into subgroups according to percentage of positive tumor cells. A cut-off point of 20% was chosen. Within tumors with $\leq 20\%$ positive cells, MQ was scored lower than SAQ (mean difference -2.2 ± 7.4) with 95% limits of agreement between -16.7 and 12.3 (Table 2 and Figure 3b). Within tumors with $> 20\%$ positive cells higher scores for MQ compared to SAQ were seen with a mean difference of 19.0 ± 17.3) and wider 95% limits of agreement ranging from -14.9 to 52.9) (Table 2 and Figure 3c).

Figure 3. Bland-Altman plot for manual quantification versus semi-automated quantification of Ki-67 immunohistochemical stains in basal cell carcinoma showing variable agreement, especially at high percentages of positive cells.

Manual quantification (MQ) by one pathologist versus semi-automated quantification (SAQ) of the average of two independent investigators for Ki-67. (a) Overall results. (b) Results separately for percentages of positive tumor cells $\leq 20\%$. (c) Results separately for percentages of positive tumor cells $> 20\%$. (a, b, c) The average percentage positive tumor cells is represented on the x-axis. The y-axis represents the difference in percentage of positively stained tumor cells assessed by MQ minus SAQ. The middle continuous line represents the mean difference of all measurements. The two dashed lines represent the 95% limits of agreements (± 1.96 Standard Deviation).

For Bcl-2, the mean percentage positive cells according to MQ was 34.6 ± 32.9 versus an average of 10.9 ± 11.6 according to SAQ (Table 1). The mean difference was 23.7 ± 26.8 and 95% of the differences fell between -28.8 and 76.2. Again, differences between SAQ and MQ were seen especially at high expression levels of Bcl-2 (Table 2, Figure 4a). In BCC with $\leq 20\%$ positive tumor cells, the mean difference was 3.3 ± 9.2 (95% limits of agreement between -14.7 and 21.3) (Table 2, Figure 4b). The mean difference was much higher within the subgroup with $> 20\%$ positively stained cells (MD 51 ± 16.0) and the 95% limits of agreement were wide (19.64 to 82.36) Table 2, Figure 4c).

Figure 4. Bland-Altman plot for manual quantification versus semi-automated quantification of Bcl-2 immunohistochemical stains in basal cell carcinoma showing variable agreement, especially at high percentages of positive cells.

Manual quantification (MQ) by one pathologist versus semi-automated quantification (SAQ) of the average of two independent investigators for Bcl-2. (a) Overall results. (b) Results separately for percentages of positive tumor cells $\leq 20\%$. (c) Results separately for percentages of positive tumor cells $> 20\%$. (a, b, c) The average percentage positive tumor cells is represented on the x-axis. The y-axis represents the difference in percentage of positively stained tumor cells assessed by MQ minus SAQ. The middle continuous line represents the mean difference of all measurements. The two dashed lines represent the 95% limits of agreements (± 1.96 Standard Deviation).

Discussion

In this study, we used a dedicated software program that we have developed to evaluate the reproducibility of SAQ of superficial and nodular BCC cells expressing Ki-67 and Bcl-2, and we compared SAQ scores with MQ scores. Our results show that the 95% limits of agreement between SAQ scores of two observers lie between -9.2% and 10.8% for Ki-67 and between -12.2% and 20.0% for Bcl2. The random measurement error may be acceptable for evaluation of treatments with large effects. However, in case of studies on therapies with more subtle effects it may be advisable to use the average score of two observers to increase the precision.

When we compared SAQ to MQ, large discrepancies between SAQ and MQ scores were observed especially in tumors with higher levels of expression of Ki-67 or Bcl2. In tumors with a mean of $\geq 20\%$ positively stained cells, the MQ score tended to be systematically higher than the SAQ score. Whether this should be considered as overestimation of MQ or underestimation of SAQ remains debatable. True reference values are required in order to determine the accuracy of a measurement, but for expression of Ki-67 and Bcl-2 in BCC no true reference values are available. Although the evaluation by the pathologist is still considered as the gold standard, the results obtained in our study suggest that MQ could overestimate the amount of positive cells. This is line with literature describing that positive cells are more prone to capture the attention of the assessor as compared to unstained cells.²³ It has previously been reported that when comparing MQ and SAQ in breast tissue, higher scores for MQ were mainly seen in highly positive tissue.¹⁹ In our study, discrepancies were even larger for Bcl-2, which is a cytoplasmic stain and cytoplasmic stains might be even harder to assess manually because the distinction with “background staining” can be difficult.

Another explanation for the lack of agreement between SAQ and MQ could be related to a variation in the selection of tumor area to be counted. As BCC are characterized by (multiple) small nests of basaloid cells spreading into the epidermis, MQ is often based on the estimation of positive cells in a limited area of the tumor in one or a few fields of view. Then, this estimation is averaged and extrapolated to the total tumor nest. Since it is known that BCC is a highly heterogeneous tumor, this approach may not be representative for the total of all tumor nests.²⁴ Visual recognition remains a subjective method and, even for the experienced pathologist, the assessment is performed in a more approximate rather than an accurate manner.^{16,25} SAQ has the advantage of measuring a large area of positive cells, negative cells and stromal tissue. In case of BCC, all tumor nests will be included in the measurement. This large number of evaluated cells reduces the risk of over- or underestimation. Furthermore the computer objectively measures the percentage positively stained tumor cells.

Another major advantage of SAQ is that even investigators with little experience in using the software, can achieve excellent reproducibility,¹⁹ as is also confirmed in this study. Additionally, the software program can obtain extra measurements like a quantitative staining intensity measurement or other details, which can help to distinguish for example background staining from surrounding positive tissues. All measurements are automatical-

ly stored in an Excel-file, which make the data easy accessible for statistical analyses. In contrast to fully automated quantifications, SAQ, with the technique we used, is dependent on the selection of color thresholds by the observer. However, the threshold is set once before the start of the measurements and is saved for all following slides, so in case of different observers, this would lead to a systematic difference between observers rather than random measurement errors. Some authors warn for the fact that the software program also requires input from the observer for tumor selection. However, human input as in SAQ even seems to be necessary since a human observer may be better at recognizing non-tumor or stromal areas in the sample than a fully automated image analysis system.²⁶

There are some limitations of the study. Currently, assessment by the pathologist is still regarded as the gold standard in the assessment of immunohistochemical stains, but assessment of MQ by only one pathologist is a considerable disadvantage of this study. Because of the lack of agreement between SAQ and MQ measurements, we were not able to derive definite conclusions on accuracy of SAQ. Further study is needed which enable comparison of SAQ scores with MQ scores by multiple pathologists. Such a study design not only offers the opportunity to evaluate the reproducibility of pathologist based measurements and to compare it with reproducibility of SAQ scores but also provides a more valid approach to the concept of assessment by the pathologist as gold standard. Evidence from a well-designed study that would confirm that SAQ is a reproducible and accurate method for assessing immunohistochemical stains is very relevant for research. The use of a semi-automated technique would be especially useful when large amounts of slides needs to be assessed, which is often the case in clinical trials studying new treatment modalities. Pre- and post-treatment expression levels of important immunohistochemical markers are more and more used as important outcome measures. Assessing the change in expression values is less subjected to random errors of individual observers which makes SAQ very suitable for these studies. Even the rather inexperienced young researches would be able to generate valid results and at and this could relieve the workload of the pathologist in the daily practice of patients diagnostics.

In this study, reasonable high inter observer agreement was demonstrated for both Ki-67 and Bcl-2 antibody assessment with SAQ. The large discrepancies between SAQ and MQ measurements can partly be explained by systematic error. Systematic error is not a large problem, because adjustment for this type of error is easy. More worrying are the wide 95% limits of agreement especially in highly positive tumors. This finding points to the need for studies wherein consensus evaluation by more than one pathologist will be compared with SAQ measurements.

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Chapter 5.1

Acquired Resistance to the Hedgehog Pathway Inhibitor Vismodegib due to smoothened Mutations in Treatment of locally advanced basal cell carcinoma

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Abstract

Background: Basal cell carcinoma (BCC) is the most common cancer in Caucasians. Aberrant activation of the Sonic Hedgehog (SHH) signaling pathway is a key event in the development of BCCs. Smoothened (SMO) activates SHH signaling and can be inhibited by small molecule inhibitors, such as vismodegib. However subsequent acquired resistance is an upcoming problem.

Objective: To analyze the acquired resistance in a 68-year old woman with a locally advanced BCC, who developed tumor regrowth 20 weeks after an initial response to vismodegib.

Methods: Mutation analysis of *PTCH1* and *SMO* was performed on primary tumor tissue as well as on secondary developed tumors and on clinically responding skin.

Results: Histologic examination confirmed the presence of BCC in the newly developed tumors. A *PTCH1* mutation was found both in the primary tumor and in the two newly developed tumor nodules. The recurrent tumor nodules harbored two *SMO* mutations (p.Trp281Leu and p.Val321Met) which were neither found in the primary BCC nor in the responding skin.

Limitation: We performed no functional analysis.

Conclusion: Our findings suggest that in this patient, two novel acquired SMO mutations resulted in resistance of the BCC to vismodegib. Alternating treatment schedules or combined therapies can be promising in this respect.

Introduction

Sporadic basal cell carcinoma (BCC) is the most frequent malignancy in Caucasians. It mostly behaves indolently, however when neglected it can become very extensive and destructive. Such locally advanced BCC (laBCC) are usually located in the head and neck area and surgical excision is the primary choice of treatment.^{1,2} However, surgery can be very mutilating or disabling and radiotherapy as alternative treatment is not always desirable.² BCC metastases are rare (0.003 to 0.55%), usually affecting lymph nodes, liver or lungs, and are characterized by a poor prognosis with median survival rates of 8-14 months.³ Reports of successful treatment with cisplatin based chemotherapy for laBCC and metastatic BCC (mBCC) are scarce.⁴

The SHH pathway is essential for embryonic development and in adult tissues it regulates tissue stem cells required for organ repair and maintenance.⁵ Upregulation of SHH signaling is crucial in the development of almost all BCCs and also plays a role in the pathogenesis of other human malignancies, such as brain and pancreatic cancer (Fig A and B in the Supplementary Appendix).⁶ Hedgehog ligands bind to the tumorsuppressor transmembrane receptor protein Patched homologue 1 (PTCH1), suppressing inhibition of the oncogenic transmembrane protein Smoothened (SMO) and so activates downstream effectors.⁵ Approximately 90% of sporadic BCCs have identifiable mutations in *PTCH1*, and an additional 10% have activating mutations in *SMO*.⁵

In 2012 the U.S. Food and Drug Administration (FDA) approved vismodegib 150 mg capsules for the treatment of laBCC and mBCC.⁷ Vismodegib is an orally active synthetic cyclopamine-like SMO-inhibitor. In a phase 2 study, complete and partial response to vismodegib was seen in 43% (laBCC) and 30% (mBCC) of patients, respectively⁸ and recent follow-up results showed increasing response rates of respectively 47.6% and 33.3%.⁹ Initial response to vismodegib followed by secondary tumor progression has already been observed.¹⁰⁻¹⁴ Acquired resistance to vismodegib has also been described in medulloblastoma, caused by a heterozygous *SMO* mutation found in the metastatic medulloblastoma that developed during treatment.¹⁵ Here, we describe a patient with laBCC who developed resistance to vismodegib after an initial response. To unravel the mechanism of this resistance, we performed mutation analysis on pre-treatment tumor tissue as well as on responding tissue and recurrent tumors after treatment with vismodegib.

Case description

A 68-year-old woman presented with an extensive, deeply ulcerating tumor on the dorsal side of her left shoulder, with a diameter of 15 centimeters, which initially started as a small asymptomatic red papule twenty years ago (Fig. 1A). She had never sought medical care before. Histological examination showed an infiltrative BCC (Fig. 2A) and magnetic resonance imaging (MRI) confirmed extensive tumor invasion of the shoulder girdle, including the infraspinatus, teres major and minor and also in the proximal latissimus dorsi muscles and a spina scapulae fragment with part of the deltoid muscle (Fig 1B, 1C). There was no evidence for lymphogenic or visceral metastasis. Because curative surgery would imply a significant risk of impaired shoulder function, our patient decided to participate in a trial with vismodegib. She was enrolled in a clinical trial and treated with vismodegib 150 mg once daily. Treatment resulted in dramatic clinical tumor regression within 8 weeks of treatment. After 16 weeks, a complete clinical response was achieved; only sclerotic tissue remained (Fig 1D). Treatment was continued, but at a control visit in week 20 multiple nodules were found in the former tumor area (FIG 1E). On MRI multifocal tumor recurrence was seen in the skin and underlying muscles (Fig 1F, 1G). Biopsies of two of these nodules revealed infiltrative BCC while in clinically responding skin no BCC cells were detected (Fig. 2B and 2C). Because of this progressive disease, vismodegib was discontinued approximately 26 weeks after treatment initiation. Extensive surgical excision was performed, including resection of involved musculature, resulting in tumor free margins. The defect was successfully closed with a rotation flap.

For mutation analyses, 8-mm biopsy samples were taken from both clinically tumor-free sclerotic tissue and two newly developed tumor nodules during surgery. Each (tumor) tissue sample was coated with Tissue-Tek® O.C.T. (Sakura) and immediately frozen and stored at -80°C. From all tissue samples a 4 mm section was stained with haematoxylin & eosin (H&E) as part of routine diagnostic procedure. All H&E sections were reviewed by a dermato-pathologist to confirm diagnosis and histological subtype. We collected a saliva sample (Oragene-DNA, DNA GenoTek, Kanata, Ontario, Canada). Written informed consent was obtained.

Figure 1. Clinical and magnetic resonance imaging (MRI) measured response of the tumor.

A. Clinical picture basal cell carcinoma (BCC) before treatment with vismodegib. **B.** Corresponding T1 MRI and T1 with fat suppression (Spectral Presaturation with Inversion Recovery (SPIR)) MRI after gadolinium are shown in panel **C**. **D.** Complete granulation of defect, 16 weeks after start of treatment. **E.** Newly developed tumors after 27 weeks of treatment with vismodegib and in panel **F** and **G** the corresponding T1 MRI and T1 SPIR MRI after Gadolinium.

Figure 2: Histopathological examination of pre- and post-treatment tissue.

A. Tumor biopsy before treatment with vismodegib, showing infiltrative palisading basaloid cells reaching into the reticular dermis fitting the diagnosis of BCC (haematoxylin-eosin (HE) magnification x2,5). **B.** Biopsy from newly developed tumor nodule after 27 weeks of treatment with vismodegib, showing infiltrative basal cell carcinoma. (HE, magnification 2,5x). **C.** Biopsy of responding tissue after 27 weeks of vismodegib, showing extensive fibrosis with an increased number of fibroblasts, neovascularization, a sparse lymphocytic infiltrate, but no residual BCC (HE, magnification x2,5).

Materials en methods

Nucleic acid extraction

Genomic DNA was extracted from fresh frozen (FF) biopsy tissue by using NucleoSpin® Tissue XS (Machery-Nagel) and from saliva by using Oragene-DNA kit (DNA GenoTek), following the manufacturer's directions.

Mutation analysis

All coding exons and immediate flanking intron sequences of PTCH1 were analysed by PCR (AmpliTaq 360, Life Technologies Europe BV, Bleiswijk Netherlands) and direct sequencing (BigDye® Terminator v1.1 Cycle Sequencing Kit and 3730 DNA Analyzer, Life Technologies) in DNA isolated from a primary tumor biopsy sample (protocol and primer sequences available on request). Targeted sequence analysis of the detected PTCH1 mutation was tested in all other tissue samples.

Loss of heterozygosity (LOH) was confirmed in the primary tumor with allele specific sequencing by using cloning of the PCR product (PGEM-T Easy cloning systems, Promega Benelux, Leiden, Netherlands) and quantitation of the specific cloned allele by PCR and subsequent restriction fragment analysis with BstUI (New England BioLabs, Whitby, ON, Canada), digesting the C-allele of the rs2066836 SNP. Sequence data of the cloned fragments showed that the C-allele of the SNP is linked to the PTCH1 deletion/insertion mutation.

All coding exons and immediate flanking intron sequences of SMO were analysed by PCR (Taq DNA polymerase, New England Biolabs) and direct sequencing in DNA isolated from secondary tumor biopsy samples (protocol and primer sequences available on request). Targeted sequence analysis of the detected SMO mutations was tested in all other tissue samples.

Results

Histologic examination of biopsies of the primary tumor as well the two newly developed nodules revealed infiltrative BCC (Fig. 2.). The biopsies of the sclerotic tissue showed extensive fibrosis with an increased number of fibroblasts, neovascularization, a sparse lymphocytic infiltrate, but no residual BCC (Fig 2C). Genomic analysis of the *PTCH1* coding exons in DNA isolated from the primary BCC identified a heterozygous c.1728_1728+1delinsAA mutation (GenBank RefSeq NM_000264.3) (Fig. 3), putatively affecting the splice donor site of intron12 (5 different splice site prediction programs included in Alamut version 2.3) (Fig 2 in the Supplementary Appendix). As expected, this mutation was not present in DNA isolated from buccal mucosa (saliva). Interestingly, a heterozygous polymorphism present in exon 12 (dbSNP 137: rs2066836) showed an equal peak distribution in buccal mucosa while the pattern was skewed (T<C) in tumor tissue. Similar skewed peak distributions in tumor DNA were noted at different location within the *PTCH1* gene (dbSNP 137: rs2297088, rs2297086, rs2066829, rs2274692, rs2236406). This skewed allelic distribution may indicate loss of heterozygosity (LOH). DNA sequencing is not a quantitative method; therefore the *PTCH1* exon 12 PCR amplicon derived from the tumor was cloned and quantified by linking the mutation to the predominant SNP C-haplotype. In 15% of clones (5 out of 33 clones) the wild type allele linked to the SNP T-haplotype was found, clearly showing underrepresentation of this allele and thus being indicative of LOH. The *PTCH1* mutation was found in the two newly developed tumor nodules (Fig. 3.), as was the skewed nucleotide distribution. In the sclerotic tissue (2 biopsies) remaining after treatment with vismodegib, the c.1728_1728+1delinsAA mutation was not present and the flanking rs2066836 SNP showed the same allelic distribution as buccal mucosa. Vismodegib resistance in medulloblastoma is caused by acquired mutations in *SMO*¹⁴⁻¹⁷ and we reasoned that similar mutations might occur in BCCs to confer resistance. The coding exons of the *SMO* gene (GenBank RefSeq NM_005631.4) were analyzed in the two biopsy samples from the newly developed tumors, unveiling a heterozygous c.842G>T mutation (p.Trp281Leu) in exon 4 in one recurrent BCC nodule. The other BCC nodule revealed a heterozygous c.961G>A mutation (p.Val321Met) in exon 5 (Fig 4.). Both missense mutations are novel and are located respectively in the second and third transmembrane domain of the smoothened protein. The importance of these amino acid residues is reflected by the fact that they are completely conserved between species. Neither mutation was found in the laBCC biopsy specimen obtained before initiation of vismodegib therapy, in the biopsy specimens of sclerotic skin after discontinuation of therapy or in the buccal swab (not shown).

Figure 3. Genomic analysis of PTCH1 coding exons.

The top panel confirmed the absence of a germline mutation in buccal swab. The primary tumor and resistant tumor, panel 2 and 4 respectively both harbored the same mutation and skewed nucleotide distribution. In the scar tissue after vismodegib treatment no PTCH1 mutation could be identified (panel 3).

Figure 4. Sequence traces from SMO exon 4 and 5 of resistant tumor tissue.

Top panels represent resistant tumor 1 and lower panels resistant tumor 2. Arrows indicate the mutations in either exon 4 (left panels) for resistant tumor 2 or exon 5 (right panels) for resistant tumor 1. If the mutation is present in one tumor, it is absent from the other and vice versa. Normal tissue, primary tumor and responding tumor tissue display similar wild type sequence (not shown).

Discussion

We describe a patient with a laBCC, who developed resistance to vismodegib after an initial complete clinical response, so-called acquired resistance. Molecular profiling of the primary BCC revealed a novel somatic mutation in *PTCH1*. In addition, there was evidence for LOH. The same *PTCH1* mutation was present in the recurrent tumor nodules, strongly suggesting that despite a clinical complete response, cells from the original tumor had survived. The mutation could not be detected in the responding tissue, which is consistent with the absence of BCC on histopathology. In the recurrent BCC we detected two different heterozygous missense *SMO* mutations (codon 281 and codon 321) that have never been reported before in vismodegib resistant BCC. This precludes a clonal origin of the resistant BCC nests supporting tumor heterogeneity, even though they arose from the presumably clonal primary tumor.

One of the most common mechanisms of resistance to targeted drugs involves secondary genetic alterations in the drug target, disrupting drug binding. There is evidence that these kinds of mutations are already present at a low level before treatment and undergo positive selection during treatment.¹⁸ Acquired mutations of *SMO* have already been observed as a cause of secondary tumor growth during treatment with vismodegib in medulloblastoma and BCC.^{10,14,15} In fact, *SMO* codon 321 was already described by Dijkgraaf et al. as a possible site critical for vismodegib binding.¹⁴ Codon 281 has never been related to vismodegib resistance and we suggest that our findings extend the *SMO* domains important for vismodegib binding beyond transmembrane domains (TM) 3, TM5, TM6, TM7 and the third extracellular loop domain including adjacent amino acids (Fig 3 in the Supplementary Appendix). Since the mutations we found were only present in the newly developed tumor nodules, a causal relationship with the development of secondary resistance is very likely. We cannot exclude that there are even more *SMO* mutations underlying tumor regrowth in this case, because not all new tumor nodules were biopsied.

Preliminary data from the largest study to the efficacy and safety of vismodegib showed complete (17.5%) and partial response (39.8%) in 251 assessable advanced BCCs (aBCCs).¹⁹ Thus resistance to the drug is actually quite common and insight in the mechanism of resistance is important to develop alternative treatment strategies for this group of patients. Possible approaches are combination therapy with different small molecule inhibitors, sequential or rotational therapy with non-treatment periods or alternating different small molecule inhibitors. Advantages of combination therapy in systemic treatment of laBCC or mBCC can be synergistic therapeutic effects, lower dosage application and, possibly, prevention of (acquired) resistance.²⁰ The concept of rotational therapy is currently being investigated in basal cell carcinoma (NCT01815840, clinicaltrials.gov) and in metastatic renal cell carcinoma (NCT01408004, clinicaltrials.gov). A drug holiday or altered dosing can also prevent the emergence of lethal drug resistance as was found in melanoma cell lines treated with vemurafenib.²¹ For combination of sequential therapy, several compounds with a different mode of action than vismodegib are available. Dijkgraaf et al. already found that *SMO*-antagonist compound 5, from the bis-amide class, was

effective in the inhibition of vismodegib-resistant mutant SMO in murine allograft models.¹⁴ Itraconazole, an FDA-approved antifungal agent, inhibits SHH signaling by targeting SMO at a different site from cyclopamine and vismodegib, and offers an alternative systemic treatment of BCC.²² GLI-antagonists target the SHH pathway downstream of SMO and may be effective in vismodegib resistant BCC as well. Arsenic Trioxide (ATO) is such a GLI antagonist and already FDA-approved. ATO effectively targets GLI downstream of SMO by blocking accumulation of GLI in the primary cilia and is currently used in treatment of acute promyelocytic leukemia.²² The combination of itraconazole and ATO in *in vitro* and *in vivo* medulloblastoma murine models was superior to either agent alone.²²

The phosphatidylinositol 3-kinase / v-akt murine thymoma viral oncogene homologue (PI3K /AKT) pathway is activated in many human cancers, including vismodegib resistant BCC.¹⁰^{23,24} Simultaneous administration of SMO antagonists and the PI3K inhibitor NVP-BKM120 delayed the onset of resistance in *in vivo* and *ex vivo* medulloblastoma murine models.²⁵ Hence, pharmacological inhibition of both SHH and PI3K signaling in tumors resistant for SMO antagonists might be useful and combined therapy should be considered in vismodegib resistant BCC.

It is important to be aware of the possible rapid occurrence of resistance to vismodegib, apparently caused by different acquired mutations. Maybe in the near future, mutational analysis on pre-treatment and recurrent tumor tissue will help to anticipate on the type of resistance, in order to proactively alter treatment. Treatment schedules and combination therapies can then be customized on both patient and tumor characteristics.

In this paper we described a patient with laBCC, in whom two different acquired SMO-mutations underlay the development of resistance to vismodegib, supporting heterogeneity of vismodegib resistant BCC. To our knowledge, to date this phenomenon has not been reported before.

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Supplementary Figure 1: Schematic presentation of the Hh signaling pathway.

A. Hh signaling during normal development. The 12-transmembrane protein Patched 1 (PTCH1) inhibits Smoothened (SMO), thereby preventing translocation of Gli to the nucleus and suppressing Hh signaling. **B.** Hh signaling in basal cell carcinoma. Inactivating mutations in PTCH1, preventing inhibition of SMO, or activating mutations, in SMO lead to upregulation of Hh signaling, resulting in transcription of target genes and tumor proliferation.

Supplementary Figure 2: Alamut output for *PTCH1*.

Analyses of the heterozygous c.1728_1728+1delinsAA *PTCH1* mutation using Alamut version 2.3, which included 5 different splice site prediction program. Mutated sequences ends in a STOP codon, confirming the pathogenicity of the mutation.

Supplementary Figure 3: Schematic representation of SMO.

Schematic representation of the SMO protein with the 7 transmembrane (tm) helices. Tm 3, 5, 6 and 7 are thought to be most important in vismodegib binding. Shown are codon 281 and 321, which are the mutation sites in our patient.

Supplementary material: Methods

Our patient provided written informed consent for the use of both clinical pictures and tissue specimens for research purposes. Biopsies were taken from tumor tissue before treatment with vismodegib. After the occurrence of resistance, biopsies were taken from clinically responding and non-responding skin. Each (tumor) tissue sample was coated with Tissue-Tek® O.C.T. (Sakura) and immediately frozen and stored at -80°C. From all tissue samples a 4 µm section was stained with haematoxylin-eosin (HE) as part of routine diagnostic procedure. All HE sections were reviewed by a dermatopathologist to confirm diagnosis and histological subtype.

Genomic DNA was extracted by using NucleoSpin® Tissue XS (Machery-Nagel), following the manufacturer's directions. We extracted DNA from 10 sections of 10 µm of each tissue specimen. Mutation analysis of the *PTCH1* gene and *SMO* gene was performed to unveil the primary mutation leading to development of BCC. LOH of the *PTCH1* gene was confirmed with allele specific sequencing by using cloning and restriction fragment analysis showing that the C-allele containing clone also contains the deletion/insertion mutation and only 15% of the clones (5 clones out of 33 clones) contain the T-allele. Primer sequences are available upon request.

Chapter 5.2

Hyperkeratosis, epidermal cyst formation and tumor resistance in a patient treated with vismodegib for locally advanced basal cell carcinoma

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Introduction

Vismodegib, a Sonic Hedgehog (SHH) pathway inhibitor, was approved by the US Food and Drug Administration (FDA) in 2012 for the treatment of locally advanced (la) and metastatic (m) basal cell carcinoma (BCC). Interim results of the largest clinical study of vismodegib have shown complete (17.5%) or partial (39.8%) response, and stable (39.0%) or progressive (2.8%) disease in 251 cases of laBCC and mBCC.¹ Formation of squamous cell carcinoma (SCC) within the tumor area, as well as occurrence of SCC at other body sites, has been reported in patients treated with vismodegib.²⁻⁶ To our knowledge this is the first report of formation of epidermal cysts and hyperkeratosis within the tumor area during treatment with vismodegib.

CASE REPORT

A 52-year-old man was referred to our hospital with a recurrent BCC that was treated with cryotherapy 15 years previously, followed by multiple incomplete excisions. An area of scar tissue and minimal hyperkeratosis was visible on the nose, extending to his right cheek and upper lip (Fig. 1A). Several biopsies were taken, and showed infiltrative BCC invading the deep dermis; there was subtle hyperkeratosis, but no evidence of SCC or BCC with squamous differentiation (Fig. 2A).

Figure 1.

A. Clinical picture before treatment with vismodegib. Biopsy sites (*) were positive for infiltrative basal cell carcinoma (BCC). Crusts from earlier biopsy sites (↓), also positive for BCC. **B.** Clinical picture with extensive hyperkeratosis and comedo-like lesions after 7 weeks' treatment with vismodegib. Biopsy site (*).

To avoid mutilating surgery, the patient was enrolled in a clinical trial with oral vismodegib 150 mg daily.⁷ This trial was approved by the ethics and scientific committee of the Maastricht University Medical Centre. After 7 weeks of treatment, hyperkeratosis increased and comedo-like lesions developed on the nose (Fig. 1B). Sequential skin biopsies showed epidermal cysts, but no residual BCC was found. Because of the impressive hyperkeratosis, we feared for progression into SCC despite the absence of malignancy in repeated biopsies, taking the possibility of a sampling error into account. Therefore, we performed Mohs' micrographic surgery 5 months after initiation of vismodegib therapy, which was continued until the day before surgery. Five stages of Mohs' surgery with 19 frozen sections, including nose amputation, were necessary to achieve clear margins. Histological examination

showed residual infiltrative BCC, accompanied by epidermal cyst formation and some hyperkeratosis, but no evidence of SCC (Fig. 2B). Immunohistochemical analysis confirmed residual BCC cells by positive staining for Ber-Ep4, whereas epidermal cysts stained negative.

Figure 2. Histological examination.

A. Skin biopsy before treatment with vismodegib, showing infiltrative basal cell carcinoma (BCC). **B.** Skin excision specimen with epidermal cyst (*) formation and infiltrative BCC (↓). Haematoxylin-eosin stain (20x).

Discussion

Pseudocyst-like structures as a result of tumor regression during the use of vismodegib have been reported previously; however, they were characterized by a different histology with so-called pseudocystic areas consisting of extensive fibrosis and some residual BCC cells but without keratin formation.⁸ Formation of SCC within the initial BCC has been reported in several patients treated with vismodegib as well as development of SCC and keratoacanthoma on other body sites.²⁻⁶ It is therefore important to be aware of the possibility of occurrence of SCC during vismodegib treatment and to perform sequential biopsies from suspicious areas. Tumor heterogeneity may underlie the emergence of SCC within the tumor area. Squamous differentiation is common in BCC and a subset of malignant squamous cells could become predominant when the BCC is treated with vismodegib. However, this does not explain the occurrence of squamous neoplasms on other sites of the body or, as in our case, the development of benign squamous neoplasia. Furthermore, the epithelial cell marker Ber-EP4 negative immunohistochemical staining of the epidermal cysts in our patient speaks against the survival of cells from the original tumor, as BCC and basosquamous carcinoma stain Ber-EP4 positive. A possible explanation for the emergence of the benign squamous neoplasms could be an effect of vismodegib on keratinocyte differentiation. In essentially all BCC, loss of function mutations in the tumor suppressor gene *Patched1* (*PTCH1*) prevent binding of *SHH* to the transmembrane protein *PTCH1*, resulting in activation of the *SHH* signaling pathway and eventually tumor formation. The *SHH* pathway seems to be important in keratinocyte differentiation and proliferation, as Indian hedgehog (*IHH*), another member of the *HH* ligand family, plays an important role

here, and loss of IHH promotes the progression of benign papillomas to SCC.⁹ Vismodegib treatment could mimic loss of IHH, as both reduce SHH pathway activation. Therefore, an effect of vismodegib on keratinocyte differentiation seems plausible. Functional analysis might clarify the exact effect of vismodegib on keratinocyte differentiation and proliferation.

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Chapter 6

Discussion & valorization

BCC is the most common cancer worldwide and its incidence is still rising, by 3-10% annually. However, as there are few adequate skin cancer registries, the actual increase might be even higher. Although BCCs rarely metastasize and mortality is extremely low, they can cause significant morbidity due to locally aggressive behavior. Half of all BCC arise primarily on the face and (bald) scalp. Surgical excision, which is the current gold standard of treatment, has disadvantages, such as the risk of bleeding, infection, impaired healing and scarring, which can be disfiguring and cause patients to experience a significantly decreased quality of life.

The high incidence of BCC combined with the associated high costs of treatment and the attendant workload is causing a significant and mounting impact on the health-care system. Consequently, BCC is an important public health problem. For that reason, there is an urgent medical and societal need for a patient friendly and cheap (targeted) non-invasive treatment, and preferably one that can be performed by patients themselves. To develop such a treatment, a profound understanding of BCC pathogenesis is essential. The research described in this thesis aims to profile genetic and epigenetic alterations in both non-aggressive and locally advanced BCC in order to enhance our understanding of BCC pathogenesis. This will be described in **part one**. **Part two** discusses new topical and systemic drugs in the treatment of BCCs.

Part one: pathogenesis of BCC

Epigenetics and more in BCC

Epigenetics encompasses heritable changes in gene expression without changes in the DNA sequence. These changes can be present at the DNA (DNA methylation), RNA (RNA interference by non-coding RNAs), and protein (histone modifications, polycomb group proteins, and chromatin remodeling) level. The continuous interplay of these epigenetic regulations determines the organism's phenotype, which is referred to as the epigenome.^{1,2} The studies presented in this thesis focus on DNA-methylation, one of the most important and well-studied epigenetic alterations. DNA methylation consists of adding a methyl group to cytosine nucleotides, primarily when they are followed by a guanine, the so-called CpG dinucleotide. Aberrant DNA methylation patterns are seen in many cancers (o.a. colon, breast, kidney and prostate cancer)¹ and other human diseases, such as autoimmune and neurological disorders.³ In BCCs, however, only a few studies have so far looked at DNA methylation. Also, these studies focused on predefined tumor suppressor genes and did not provide evidence that any observed changes in methylation patterns were relevant for BCC carcinogenesis.⁴⁻⁹ Additional evidence shows that ultraviolet (UV) radiation A and UVB may alter epigenetic modifications¹⁰ and UV-signature mutations seem to preferentially occur at methylated C-phosphate-G (mCpG) dinucleotides.¹¹ As BCCs mainly occur at sun-exposed areas of the skin¹², they might be expected to show extensive methylation changes compared to non-diseased skin.

In a first exploration of promoter hypermethylation in BCCs, we assessed the promoter CpG island methylation status of nine candidate tumor suppressor genes (TSG). CpG islands

are regions with a higher frequency of CpG dinucleotides that are mostly found around the promoter region of the gene.¹³ As described in **chapter 2.1**, nested methylation specific polymerase chain reaction (MSP) was performed on bisulfite-modified genomic DNA of BCCs and healthy skin with primers specific for methylated DNA, respectively unmethylated DNA. Significantly more DNA methylation in BCCs was found in the gene promoters of *SHH*, *APC* and *SFRP5*, all key regulators of the SHH and WNT signaling pathways, and of *RASSF1A*. *RASSF1A* is a well-known tumor suppressor of which epigenetic inactivation is previously described in BCCs⁹, but also in malignant melanoma¹⁴ and Merkel cell carcinoma.¹⁵ Interestingly, healthy sun-exposed (SE) skin samples showed methylation patterns similar to sun-protected (SP) skin, suggesting that the methylation we observed is more tumor-specific than locus or patient specific, a finding that is in line with literature to date.¹⁶ It is of interest that methylation patterns in sun-exposed skin did not resemble those in BCC. Our findings therefore do not support a contribution of UVB exposure to the observed promoter hypermethylation of *SHH*, *APC*, *SFRP5* and *RASSF1A*. However, since the epigenetic changes that we found parallel genetic changes driving BCC growth, it seems likely that gene silencing by promoter hypermethylation is relevant to BCCs.

In this study we used nested MSP because it is highly sensitive and can detect 1 methylated allele in >50,000 unmethylated alleles. This makes it exquisitely suitable for methylation analysis of archived formalin-fixed paraffin-embedded (FFPE) DNA, as this generally yields small amounts of poor quality DNA.¹⁷ Since biopsy or excision tissue samples taken during regular care are commonly FFPE and stored in the archives of the pathology department, there is wealth of tissue available for research. On the down side, (nested) MSP is a 'yes or no' approach that does not provide quantitative results. Also, it can only be used to screen pre-defined candidate genes. Fortunately, novel techniques such as comprehensive probe-based, bisulfite whole-genome sequencing, and enrichment-based technologies¹⁸⁻²⁰ allow unbiased genomewide analysis of DNA methylation (the "methylome"). This is a major advantage compared to locus-specific, low-throughput approaches that are typically PCR based. However, the latter are still used for validation purposes.¹⁹

Chapter 2.2 describes the first analysis of the BCC methylome by genome-wide ^mCpG DNA enrichment sequencing (MethylCap-seq).²¹ MethylCap-seq is based on capture of methylated DNA on a genome-wide scale with the methyl-DNA binding domain (MBD) domain of the protein MeCP2. As only methylated DNA is subsequently sequenced, the complexity of the analysis is reduced and high sequence coverage can be obtained.²² MethylCap-seq is a robust, highly reproducible approach, especially for larger sample cohorts.²²

We identified 32979 methylated regions in 22121 genes, 13 of which were differentially methylated in sporadic BCCs compared to healthy skin, with a False Discovery Rate (FDR) of <0.1 (**chapter 2.2**). As methylation can regulate gene expression levels²³, we performed rRNA depleted, strand specific, total RNA sequencing (RNA-seq henceforth) and correlated these data to the methylome. This correlation provides information on the functional methylome: i.e. those methylation events that affect gene expression. Of note, there was little overlap

between the top-ranked differentially methylated loci and differentially expressed genes (FDR<0.1), even when preselecting for genes that might be differentially expressed in BCC. At present, we cannot explain this finding. The full spectrum of functions that DNA-methylation has remains to be delineated, as is its full impact on RNA expression levels. IFI30 was one of the hits that was both differentially methylated and differentially expressed, a finding that points to the importance of immune signaling in BCC. IFI30 is known to play a critical immunoregulatory role in melanoma by mediating antitumor immunity via CD4⁺ T-cells.²⁴

We compared the results of our first analysis in BCC with those obtained from methyl-Cap-seq and RNA analysis of a facial laBCC (**chapter 2.3**). We identified considerable agreements and differences between the non-aggressive and aggressive BCCs. The most striking difference was a dramatic increase in expression of matrix metalloproteinase 13 (MMP13) in the laBCC, which could explain the more aggressive behavior of this tumor.²⁵

Additional pathway analysis of the RNA-seq data was performed with SPIA software²⁶, showing that pathways activated in BCC are all involved in immune signaling: cytokine-cytokine receptor interaction signaling (KEGG hsa04060), chemokine signaling (Chemokine) (KEGG hsa04062) and Toll-like receptor signaling (KEGG hsa04620). Pathway enrichment analysis of the differentially methylated genes also revealed cytokine-cytokine receptor interaction as the most significant pathway. The importance of immune signaling in BCCs is further supported by results from our independent clinical trial, described in **chapter 4.1**. Previous work has also found increased expression of innate immune genes in squamous cell carcinomas (SCCs) and in BCCs.²⁷

Apparently, SHH signaling might not be the sole driver in sporadic BCCs. In fact, BCC pathogenesis seems to be rather complex, and this is further confirmed by the whole-exome sequencing of BCCs described by Jayaraman *et.al.*²⁸ Surprisingly, although BCC can be considered as the least malignant human cancer, they harbor the largest mutational burden compared to any other known human malignancy. The vast majority of mutations (75.7%) showed a clear UV signature. However, Jayaraman *et.al.* could not identify any novel genes as drivers in BCC. The authors hypothesized that the resulting high level of mutated proteins might increase the hosts' immune response, resulting in a less aggressive phenotype.²⁸ It is of interest that the incidence of nonmelanoma skin cancer (NMSC) in immunosuppressed patients, such as organ transplant recipients (OTR), is up to 250-fold higher than in the general population. For common cancers like gastrointestinal, lung, breast, bladder, prostate, and gynecological, this risk is only two to five-fold higher.^{29,30} In OTR, SCCs occur 4-10 times more often than BCCs and tend to behave in a more aggressive manner with increased metastatic potential, and higher recurrence rates compared to SCC in non-immunocompromised people.^{30,31} This increased risk of SCCs and also BCCs may be due to the higher rates of human papillomavirus (HPV) infection in immunosuppressed populations, however the most convincing evidence is available for the link between HPV and SCC. An etiologic association between HPV and BCC remains to be elucidated.³² In addition to viral oncogenic infections, decreased immune-surveillance and the direct effect of the immunosuppressive medications

used by OTR causes them to be more vulnerable to NMSC development.³² The influence of an immune response in BCC development was previously reported and shows that transplantation of human BCC biopsies is only successful in mice deficient of T, B and natural killer (NK) cells.³³ However, BCC nest are frequently, but not always, surrounded by an infiltrate consisting of T cells and only very few B lymphocytes and NK cells that do generally not invade the tumor nests.³⁴ Although complete tumor eradication is rare³⁰, higher number of T cells is seen in actively regressing BCCs.³⁴ Generally, BCCs express no or low levels of MHC class I (MHC-I) molecules and as a consequence there is an absence of infiltrating CD8⁺ effector T cells. Following treatment with topical imiquimod, a Toll-like receptor-7 antagonist, significantly higher expression levels of MHC-I accompanied by an increase of peritumoral CD8⁺ T cells were seen.³⁰ This pro-inflammatory Th1 initiated anti-tumor immune response has also been implicated in the well-established spontaneous regression of BCC after a biopsy.³⁵ Also, A Th2 anti-inflammatory response has been described in BCCs, which suggests a dynamic state in where pro- and anti-inflammatory immune response coexist and compete.³⁶ The exact mechanisms underlying an effective tumor specific immune response is unclear as there are still many missing links. In our study described in **chapter 4.1**, an extensive infiltrate was seen in the BCCs treated with topical diclofenac, in the majority of the cases even resulting in complete histological tumor regression. The nature of this immuneresponse will be analyzed in a future study in an attempt to unravel the role of the BCC immuneresponse.

DNA methylation in BCC and the future

As epigenetic alterations are potentially reversible, drugs have been developed to inhibit enzymes that catalyze epigenetic modifications. In the case of DNA methylation, these are the DNA methyltransferases (DNMTs), consisting of 3 main subtypes. DNMT1 maintains existing methylation during DNA replication, while DNMT3A and DNMT3B are required for *de novo* methylation. To date, the FDA has approved two demethylating drugs for the treatment of higher-risk myelodysplastic syndrome: 5-azacytidine (5-aza-CR; manufactured by Celgene as Vidaza) and 5-aza-2'-deoxycytidine (5-aza-CdR; manufactured by Eisai as Dacogen). However, these drugs have substantial side effects, which makes them less useful for BCC treatment, as the advantage of being a non-invasive treatment will not outweigh these side effects.³⁷ The systemic side effects could be avoided by using the 5-aza's topically. Unfortunately, they are both pharmacologically unstable agents, which impedes their topical formulation.

Whereas DNA methylation changes in cancer constitute a potential target for treatment, they could also be used to develop biomarkers for more accurate detection of cancer, a better insight in tumor prognosis, and even to predict treatment response.² In contrast to genetic biomarkers that detect the numerous different point mutations occurring throughout the length of the gene, DNA hypermethylation consistently affects a small promoter region, facilitating analysis. Also, DNA methylation alterations occur in higher percentages of tumors than genetic variations, resulting in a higher sensitivity.³⁷ The utility of biomarkers for BCC treatment can be debated, as these tumors are generally characterized by indolent growth, an excellent prognosis and easy accessibility

for assessment and treatment. However, given the large number of patients that will develop at least one or more subsequent BCC after the first BCC, a prognostic biomarker that could predict the individual risk of (recurrent) BCC could be extremely useful. It would enable classification of patients as having a low, an intermediate or a high risk of developing BCC. Using this information, follow-up schemes could be tailored to the patient's individual risk, thereby making care for BCC more efficient and cost-effective.

Beyond DNA methylation

In our work, we focused on DNA (cytosine) methylation, because it is the best-defined epigenetic change in malignancy. Hydroxymethylation of cytosine (5-hmC) is a more recently described modification, that affects the flexibility and stability of DNA differently from methylation. The 5-hmC level is strongly reduced in tumors of the brain, kidney, lungs, skin and liver, from which it was suggested that 5-hmC loss could serve as biomarker for the detection of cancers. However, the exact role of 5-hmC in the development of cancer remains unknown.^{38,39}

Additional epigenetic modifications that have been found to be involved in human cancers include histone modifications, chromatin remodeling and the activity of non-coding RNAs (ncRNAs).^{3,40} These different processes work together to establish and maintain the global and local condensed or decondensed chromatin states that eventually determine gene expression.² DNA is tightly compacted around histones to the nucleosome which is the basic unit of the chromatin.⁴⁰ These histones can be modified post-transcriptionally by (hyper) acetylation, methylation, phosphorylation and ubiquitination. Those different processes affect, among others, transcriptional regulation, DNA repair, DNA replication, alternative splicing, and chromosome condensation.³ Hyperacetylation of the lysines in histone tails is the best described modification and is facilitated by Histone Acetyltransferases (HATs). This results in a decondensed chromatin conformation that can be actively transcribed. On the other hand, histone deacetylases (HDACs) restore the condensed state, resulting in gene silencing by preventing binding of transcription factors and RNA polymerases.⁴⁰ In the case of tumor suppressor genes, this means that tumor development is facilitated. Thus, HDAC inhibition could be used to treat cancer. Indeed, Vorinostat (SAHA), a synthetic HDAC inhibitor (HDACi) was approved by the FDA in 2006 for the treatment of cutaneous T-cell lymphoma.⁴¹ Two more (romidepsin (Istodax) and belinostat (Beleodaq)) have followed in the past few years and many HDACis are in different stages of clinical trials for various hematological and solid tumors.⁴² Apart from the direct effect of HDCAis on acetylation resulting in re-expression of the gene, HDCAis can also target processes involved in tumor progression, cell cycle control, apoptosis, angiogenesis and cell invasion.⁴² Interestingly, HDACis have been shown to suppress SHH activity through Gli1, Gli2 and Gli3 in vitro.⁴³ A newly designed chimeric compound, NL-103, which integrates the functional inhibitory group of the HDACi vorinostat and SHH inhibitor vismodegib, downregulates the expression of Gli2 in a NIH3T3-12Gli mouse embryo fibroblast cell line. It was concluded that NL-103 can effectively inhibit SHH and that dual inhibition of HDACs and SHH signaling could help to overcome vismodegib⁴⁴, a phenomenon that is described in more detail in part two.

The dynamic process of chromatin remodeling is not only regulated by histone acetylation/deacetylation, but also through ATP-dependent protein complex formation, histone modification by polycomb proteins, and by interaction with ncRNAs.⁴⁰ ncRNAs are RNA fragments that are not translated into proteins of which several subtypes can be identified such as miRNA, siRNA, piRNA, XiRNA and long ncRNAs.⁴⁰ RNA interference (RNAi) is a part of a small regulatory RNA, including siRNA and miRNA, that regulates gene silencing. RNAi based therapies are already being tested in phase 1 studies as treatment in several solid tumors (brain, breast) and considered to be safe in use. These drugs have the benefit of being highly specific and they have only little side effects.⁴⁵ A comprehensive description of all possible epigenetic modification processes is beyond the scope of this thesis. But it is evident that the rise in knowledge on epigenetic modifications in human cancers like BCC can yield considerable diagnostic, therapeutic and even predictive opportunities in the (near) future.

mTOR signaling in BCC

Whereas most research on BCC has been focused on SHH and WNT signaling, it is important to think *out of the box* in order to unravel the pathogenesis of this tumor completely. As an example of such thinking, when looking for therapeutic targets we considered that BCCs are thought to be hair follicle tumors.⁴⁶ The hair follicle is considered as a moderately to severely hypoxic microenvironment, while the dermis is well oxygenated and the epidermis is only modestly hypoxic.⁴⁷ Therefore we hypothesized that hypoxia response pathways could be involved in the pathogenesis of hair follicle tumors such as BCCs. Hypoxia-inducible transcription factor 1 (HIF1) is a primary mediator of hypoxia-induced gene expression in human hair follicles.⁴⁸ HIF1 transcription can be regulated by mechanistic/mammalian target of rapamycin 1 (mTORC1) via 4E-binding protein 1 (4E-BP1), ribosomal protein S6 kinase-1 (S6K1) and signal transducer and activation of transcription 3 (STAT3).⁴⁹ mTORC1 in turn is stimulated by PI3K-Akt signaling pathways and once activated, it promotes several processes like angiogenesis, cell growth and tumorigenesis.⁵⁰ The importance of mTORC1 in the pathogenesis of different cancer types, including renal carcinoma and colon carcinoma is well recognized and has already resulted in the development of treatments that target mTORC-dependent signaling events.⁵¹ Interestingly, activated mTORC signaling is also associated with the development of benign skin tumors like angiofibromas in patients with tuberous sclerosis.⁴⁸

If HIF and mTORC1 pathways do contribute to the growth of hair follicle tumors, new opportunities for targeted therapy and diagnostics could emerge. In **chapter 3.1** we provide a systematic analysis of HIF and mTORC1 signaling in BCC and its benign counterpart, trichoepithelioma (TE). TEs strongly resemble BCC both on the macroscopic and microscopic level, but do not exhibit invasive growth. Differentiation between these two hair follicle-derived tumors is important because of their distinct biologic behavior and therapeutic approach. Immunohistochemical analysis of HIF1, mTORC1 and their most important target genes showed that both HIF and mTORC1 signaling seem to be active in BCC as well as in TE. However, there were no appreciable differences between these two tumors with respect to pathway activity. To date, immunohistochemical analyses of HIF, mTORC1 and their tar-

get genes does not provide a reliable diagnostic tool for the discrimination of BCC and TE.

In conclusion, we and others have shown that sporadic BCCs are tumors driven by a variety of derailed signaling systems, beyond SHH signaling. This observation is in line with the fact that sporadic BCC exhibit a mostly moderate response to targeted SHH inhibitors. There are thus several potential drug targets already, and doubtlessly many more remain to be discovered.

Part two: new topical and systemic treatment modalities for BCC

Vitamin D and NSAIDs as anticancer agents

Targeted non-invasive treatments: vitamin D

The primary aim of BCC treatment is complete tumor eradication. However, maximal preservation of function and cosmesis at the treatment site are very important aspects that must be considered when choosing an appropriate therapy. The treatment of choice largely depends on clinical and histopathological characteristics. The risk of recurrence should be carefully evaluated in order to avoid overtreatment of low-risk BCCs or under-treatment of high-risk BCCs.⁵² Conventional surgical excision is the gold standard in treatment of all BCCs, with success rates varying from 90-98% for primary (untreated) BCCs.^{53,54} For sBCCs, non-invasive treatment modalities such as photodynamic therapy (PDT) (photochemical), imiquimod (immune-modulating) and 5-fluorouracil (5-FU) cream (chemotherapeutic) are well studied and frequently prescribed.⁵⁵ Long term tumor-free survival rates in sBCCs are high, varying from 72.8-84.0% in PDT, 83.4-87.3% for Imiquimod and tumor-free survival rates of 80.1% were found after treatment with 5-fluorouracil.^{55,56} This illustrates that cure rates following surgical excision are still much higher. At the time of the clinical trial described in **chapter 4.1**, there were no pertinent data to support non-invasive treatments for nBCCs. Likewise, according to the Dutch 'evidenced-based guideline treatment of the basal cell carcinoma', non-invasive treatment modalities were reserved for sBCCs only. We concluded that there was still an opportunity for (the development of) alternative or additional non-invasive treatment modalities for sBCCs as well as nBCCs.

Current research in oncology is focusing on the development of targeted cancer treatments that are specifically aimed at key signaling pathways instead of the more generally used, non-specific cytotoxic agents. Interestingly, sometimes drugs already available for the treatment of one disease turn out to be effective against a different disease (including human cancers), which is known as drug repurposing. The active metabolite of vitamin D, 1 α ,25(OH)₂D₃ (calcitriol) is of particular interest in this regard. Preclinical research has indicated that this metabolite or other vitamin D analogues might have potential as anticancer agents because of their antiproliferative effects, activation of apoptotic pathways and inhibition of angiogenesis.⁵⁷ Vitamin D is known to be a steroid hormone crucial in calcium homeostasis and regulation of bone metabolism. It also has many other biological actions that are still not fully characterized.⁵⁸ In the skin, calcitriol is mainly synthesized under influence of UVB exposure.

For that reason, sun protection advice given by dermatologists in order to prevent skin aging and skin cancer is often disobeyed because patients fear becoming vitamin D deficient. Indeed, sunscreens do block the vitamin D production, however in daily practice, typically only little amounts of sunscreen are applied to the skin and vitamin D deficiency therefore is not normally an issue. Mean daily exposure during 15-30 minutes (depending on latitude and season) of the face, hands and underarms without any sun protection would generate the required amount of vitamin D in people with Fitzpatrick skin type I-III. However, dermatologists and cancer groups, including the Skin Cancer Foundation of the United States discourage such unprotected UV exposure, as all unprotected UV exposure contributes to cumulative DNA damage and thus to a further increased risk of skin cancer development. Their advice is to substitute vitamin D orally. In a time where vitamin D (deficiency) seems to be a real hype, it is important to realize that neither the influence of vitamin D insufficiency nor the health benefit of maintaining high serum vitamin D levels has been established for the general population.⁵⁹ Animal and cellular studies strongly suggest a role for vitamin D in the prevention and treatment of various human cancers. So far, however, clinical studies in most cancers have not yet delivered compelling evidence to support the use of vitamin D supplements in daily practice.⁶⁰

In keratinocytes, the vitamin D receptor (VDR) interferes in both SHH- and WNT signaling and that way seems to be crucial in the tumorigenic response.⁶¹ Also, normal keratinocytes and melanocytes respond to calcitriol with a reduction in proliferation and an increase in differentiation.⁶² There is some evidence to suggest that high vitamin D serum levels might protect against BCC development.⁶³ Conversely, an association with higher serum vitamin D levels and a higher risk of BCC has also been shown in two studies.^{64,65} This higher risk may well be explained by the fact that whereas UVB exposure does lead to higher vitamin D serum levels, it will also cause the DNA damage that results in skin cancer.⁶¹ Additionally, previous *in vitro* studies found that high doses of calcitriol can inhibit keratinocyte proliferation, while lower doses may stimulate proliferation.^{62,66} Apparently, the balance between the positive and negative effects of vitamin D is a very delicate one. In our study, described in **chapter 4.1**, topical calcitriol applied twice daily to sBCCs and nBCCs during 8 weeks did not have any effect. No histopathological tumor clearance was found, nor was there any effect on the proliferation marker Ki-67 and anti-apoptosis marker Bcl-2. However, based on our results we cannot completely rule out that vitamin D analogs could treat BCC. The lack of response we observed might have been due to calcitriol being unable to reach sufficiently high concentrations in the tumors.

Targeted non-invasive treatments: diclofenac

Nonsteroidal anti-inflammatory drugs (NSAIDs) are well-known and frequently used drugs with analgesic, antipyretic and anti-inflammatory effects. There are many different types of NSAIDs. The main class effect is the inhibition of cyclooxygenases 1 (COX-1) and 2 (COX-2), with a consequent decrease in prostaglandin synthesis. COX-1 is a housekeeping gene that is constitutively expressed and controls normal physiological functions such as maintenance of the gastric mucosa and platelet function, vascular homeostasis, as well as renal blood flow. In contrast, COX-2 is an immediate-early gene, and expression is generally be-

low detection levels.⁶⁷ COX-2 modulates cell proliferation, angiogenesis and suppression of apoptosis.⁶⁸ In BCCs, COX-2 is commonly highly expressed under influence of UV radiation and multiple other inflammatory stimuli, whereas in normal skin COX-2 is usually undetectable.^{67,68} Diclofenac is the most widely prescribed non-selective COX inhibitor worldwide.⁶⁹ Over the past three decades, epidemiologic, clinical, and experimental studies have established a chemopreventive effect of NSAIDs in various tissues. However NSAIDs are not used as chemopreventive agent because of incomplete efficacy and toxicities.⁷⁰ The effect of the oral selective COX-2 inhibitor Celecoxib on the development of BCCs was studied in a double-blinded, randomized controlled clinical trial in 60 patients with BCNS. After Celecoxib (200 mg, twice daily, 24 months) a trend towards reduction in BCC burden in the treatment group in all subjects was suggested.⁷¹ A recent meta-analysis did not find a statistically significant chemopreventive effect of NSAIDs on NMSC, not even after stratification for the different NSAIDs and for BCCs and SCCs.⁷² Thus, there is no convincing evidence to date that regular use of systemic NSAIDs could serve as chemopreventive agent in the development of BCCs. However, as the local bioavailability is expected to be higher after local application, and considering the fact that NSAIDs can target the WNT signaling pathway that is activated in BCCs⁷³, we assumed that topically applied diclofenac could be effective in the treatment of (low-risk) BCCs. Moreover, diclofenac both directly and indirectly induces apoptosis.^{74,75} In **chapter 4.1** we showed that application of diclofenac 3% gel with 2.5% hyaluronic acid for 8 weeks twice daily (under occlusion) to sBCC resulted in a complete histologic regression in 64.3% of all tumors. These results further support inflammation as a possible driver of BCC growth (see also **part one**). The histological clearance found was not as high as can be obtained with currently available non-invasive treatment modalities, however it should be noted that we made use of a diclofenac compound (Solaraze®) that is already FDA approved for the treatment of actinic keratosis (AK).⁷⁶ Previous dose-response studies for its specific use in BCC were not performed. Optimized dosage regimens might further improve efficacy. Apart from more targeted mode of action, further advantages of topical diclofenac are that it is cost-efficient, easy in use and that it has limited side effects. This makes topical diclofenac a promising drug for the treatment of (low-risk) BCCs.

Topical diclofenac as chemopreventative agent

Because of the promising results of topical diclofenac as treatment of sBCC and the ever larger number of patients affected, it is of interest to determine whether topical diclofenac could serve as a chemopreventative agent. About one third of the patients diagnosed with their first primary BCC will develop at least one subsequent BCC. This risk is highest within the first 6 months after diagnosis, but remains substantially high even after a follow-up period of 5 years. In particular patients with red hair, a higher socioeconomic status, and/or those with a BCC on their upper extremities have a higher risk of developing multiple BCCs.⁷⁷

Topically applied 3% diclofenac gel with 2.5% hyaluronic acid (the same formula used in the clinical trial in **chapter 4.1**) has already been used in the treatment of AK and resulted in complete clinical clearance of 41-60% of AKs after 3 months of treatment.⁷⁶ Topical diclofenac in

twice-daily doses is approved by the FDA for the treatment of AK over a period of 60-90 days and it is prescribed for this purpose in many European countries, although its use is not recommended by the 'Dutch guideline actinic keratosis'. Given the inhibitory effect of diclofenac on cell proliferation and the promising response rates both in AK and BCC, topical diclofenac could presumably help to reduce the risk of new or subsequent BCC development. The side effects are usually mild to moderate (e.g. erythema, pruritus and erosion) and resolve without sequelae (**chapter 4.1**). Therefore, it might be of interest to explore whether diclofenac could be used as a preventive additive to daily skin care creams. With a lower concentration or a reduced frequency of application, known side effects could be reduced whilst retaining efficacy.

Targeted non-invasive treatments and future perspectives of BCC management

In contrast to most cancers, skin tumors are readily accessible for clinical examination and tissue samples for (immuno)histochemical (IHC) evaluation can easily be obtained. It might be argued that accurate clinical follow-up instead histological examination could be considered when assessing new topical treatments in clinical trials. However, immunohistochemical stains on pre- and post-treatment tissue samples can provide insight in the mode of action or efficacy of new treatment modalities. In quantifying the expression levels of specific immunohistochemical markers, percentages of positive tumor cells need to be determined. Manual pathologist-based quantification (MQ) is still the most frequently used technique for IHC evaluation⁷⁸ and is considered as the gold standard. However, MQ is time-consuming and labor-intensive.⁷⁹ It is also a subjective approach that may have high intra- and inter-observer variability.⁸⁰⁻⁸² Larger studies with more immunohistochemical stains to be investigated inevitably generate a large number of slides to be assessed, rendering MQ impractical.

Automated, digital image analysis might offer a solution and has been used since the 1980s. Several different methods are available.⁸³ We used a so-called operator-dependent semi-automated quantification (SAQ) computerized thresholding technique (Leica Qwin version 3.5.1, Leica Microsystems, Cambridge, UK), which is an extensively evaluated method, especially for mammographic density.^{84,85} SAQ is time saving, provides easily accessible data and generates good reproducible results, even by generally inexperienced young researchers (**chapter 4.2**). However, we found large discrepancies between MQ and SAQ, that point to the need for studies in which consensus evaluation by more than one pathologist is compared with SAQ measurements. Evidence from a well-designed study that SAQ is a reproducible and accurate method for assessing immunohistochemical stains would be very relevant for research. Studies assessing changes in expression values in pre- versus post-treatment tissue samples are especially suitable for SAQ, as the change in expression is less susceptible for random errors.

In **chapter 4.1**, two pathologists manually assessed all pre- and post-treatment tissue samples. In contrast to the more promising results in sBCCs, in nBCCs we did not detect significant changes in proliferation, apoptosis or total tumor clearance after treatment with diclofenac, calcitriol or a combination of both. The difference in response between nBCCs and

sBCCs is in agreement with reports from other studies investigating non-invasive therapies. sBCCs spread from the epidermis into the papillary dermis, which makes the tumors more accessible to topical treatment. In contrast, nBCCs show deep, compact, and nodular growth⁸⁶ presumably leading to insufficient penetration of topically applied drugs. Only recently, high 3-year success rates (81.8%) were found for nBCCs treated with imiquimod for 12 weeks, 7 days a week.⁵⁴ This is an intensified treatment schedule compared to the presently licensed schedule of 5 days a week for 6 weeks. Imiquimod has several antitumor activities. The major biological effect of imiquimod is the activation of nuclear factor-kappa B (NF- κ B) through toll-like receptors 7 (TLR7) and 8 (TLR8). Induction of NF- κ B results in the production and secretion of pro-inflammatory cytokines and chemokines, stimulating a strong Th1-mediated antitumoral cellular immune response.⁸⁷ Additionally, imiquimod directly stimulates antitumoral activation of dendritic cells (DCs). DCs are important (tumor) antigen presenting cells in the skin and they are thought to be crucial in generating an aggressive immune response against cutaneous cancers.⁵¹ Interestingly, a recent study in a *Patched1* deficient murine BCC cell line suggests that imiquimod may actually serve as a targeted therapy by directly inhibiting SHH signaling downstream of SMO. Imiquimod can cause GLI phosphorylation and subsequent reduction in GLI target genes.⁸⁸ The fact that imiquimod, apart from its immunomodulating effect, also seems to directly inhibit SHH signaling, could explain why it is the most effective topical treatment currently available.⁵⁵ Indeed (pre-) clinical evidence has suggested that simultaneously targeting SHH and other signaling pathways may have a synergistic effect.⁸⁹ In **chapter 4.1** we hypothesized that combining diclofenac gel and calcitriol ointment would enhance their individual efficacy. In our clinical trial, the combination did not show the expected synergistic effect. The observed clinical effect of the “combination therapy” arm was probably attributable to diclofenac, as there was no effect at all in the calcitriol arm. Since calcitriol ointment was applied five minutes after application of diclofenac gel, dilution or suboptimal absorption could explain the observed reduced efficacy compared to diclofenac monotherapy in sBCCs. Of note, in the combination therapy group, there were four non-responding tumors that appeared to have a nodular BCC component although they were initially diagnosed as sBCC on the biopsy. Since we also found that nodular BCCs did not respond to topical therapy, treatment failure in these patients was probably related to the histological subtype. Due to the sample size of 16 tumors per arm, those 4 non-responders with a different histological subtype had a large impact on the overall treatment outcome. Nevertheless, the promising results of topical diclofenac in sBCCs suggest new possibilities for the management of BCCs. Further optimization with dose-response trials and studies combining topical diclofenac with available (targeted) non-invasive therapies are needed to test whether topicals can obtain cure rates comparable to those of the gold standard, surgery. Based on our present understanding, treatment with diclofenac gel and imiquimod cream is a promising combination that we are currently investigating.

Non-invasive treatments in general can be expected to have several advantages, making them worth pursuing. They will generally give a better cosmetic outcome, with an increased quality of life for patients as a result.⁵⁴ Patients will be able to treat themselves at home, which makes them less dependent on hospital care. A second advantage of this home-

based treatment is that the workload for dermatologists will decrease, resulting in lower overall healthcare costs. Since the skin cancer epidemic is yet to peak, the workload for dermatologists is expected to increase. General practitioners might be able to help in this increase in demand. However, according to a self-administered questionnaire filled in by 268 general practitioners, over 50% of the general practitioners felt the need to know more about skin cancer care.⁹⁰ Intensified training programs on recognition and management of skin cancer are therefore essential to facilitate treatment by non-specialists. General practitioners are already used to take biopsies of suspicious lesions, but they generally do not perform therapeutic surgical excisions of BCCs (yet). Also, in general, they are not familiar with prescribing non-invasive treatments. Good collaborations between the general practitioners and dermatologists are necessary to improve care of the patient with (pre-) skin cancer, and is essential in order to reduce health care costs. The department of dermatology (MUMC) already collaborates with general practitioners in the so called '1.5th line of care' or 'stadspolieklinieken', where dermatologists visit the general practitioner one day a week in order to facilitate dermato(onco-)logical care by reducing the number of unnecessary referrals to the hospital. Another way to make current dermatological practice more efficient is the employment of specialized nurse practitioners and physician assistants, who could participate in the management of skin cancer. Of course, oncological care of the skin should then be thoroughly implemented in their educational training. With the appropriate knowledge, these paramedics could not only educate patients about skin cancer prevention, but they would also be able to recognize (pre-) skin malignancies and potentially even treat AK and low-risk BCCs.⁹¹ Of note, high risk BCC, SCC and malignant melanoma should always be treated by a dermatologist, as they are associated with higher morbidity and even mortality. However, paramedics could serve as a first point of call for BCC patients and help manage further care. It should be stressed that paramedics should always collaborate with a dermatologist.

Targeted SMO-inhibitors in BCC

Vismodegib and (acquired) resistance

BCC is characterized by aberrant activation of the SHH pathway, mostly due to inactivating mutations in the tumor suppressor gene *PTCH1* or activating mutations in the *SMO* oncogene.⁹² SHH-inhibitors specifically target SMO, but GLI or other downstream pathway components may be targeted in the near future.⁹³ Vismodegib (Erivedge™, also known as GDC-0449) is an orally active synthetic SMO inhibitor and was approved by the FDA in 2012 for the treatment of laBCC and mBCC.⁹⁴ In July 2015, a second SMO inhibitor, sonidegib (Odomzo™, also known as LDE255), was approved by the FDA for laBCC not amenable to curative surgery or radiation therapy.⁹⁵ As the mode of action of these Hedgehog Pathway Inhibitors (HPIs) is identical, similar efficacy rates and adverse events are seen (class effect).^{95,96} Recently, pre-planned interim results of the largest international, open label trial to the safety and efficacy of vismodegib in laBCC and mBCC (STEVIE study) were published.⁹⁷ After at least 12 months of treatment, complete and partial response was seen in 32% of laBCC and 33% of mBCC patients. Twenty-seven percent of all patients treated had stable disease and progressive

disease was seen in 3% of the patients. The mean duration of treatment was 36.3 weeks for laBCCs and 52.0 weeks for mBCCs. Treatment was discontinued in no less than 80% of all patients treated in this study. Almost all patients reported one or more adverse events (99%), of which the most important events were muscle spasms, alopecia and dysguesia (loss of taste). These adverse events were the most important reason for treatment discontinuation. The authors concluded that the safety profile described in the STEVIE study was consistent with previous analyses of vismodegib in patients with laBCC and mBCC.⁹⁷ The department of dermatology of the MUMC participated in this STEVIE trial and included 6 patients.

Initial response to vismodegib followed by secondary tumor progression has been observed and is suggested to occur in 20% of the patients within the first year of treatment.⁹⁸⁻¹⁰² The mechanisms underlying resistance to vismodegib in BCCs may clinically be divided into primary resistance (no response to treatment) or secondary (acquired) resistance (progression after an initial response).¹⁰³ We purposely speak of a clinical classification, because it is still unclear whether the non-responding tumor cells are already present in the primary tumor and grow out to visible tumor due to selection, or whether HPIs may even induce mutations that lead to resistance. Acquired resistance to vismodegib has also been described in medulloblastoma, caused by a heterozygous *SMO* mutation that appeared in the metastatic medulloblastoma during treatment.¹⁰⁴ In this thesis, two patients with laBCCs who had developed respectively primary and secondary resistance to vismodegib are outlined. **Chapter 5.1** describes two heterozygous mutations ((p.Trp281Leu and p.Val321Met) in newly developed tumor tissue after 20 weeks of treatment in a laBCC patient who initially had a good clinical response to vismodegib. Neither *SMO* mutation was found in the laBCC biopsy specimen obtained before initiation of vismodegib therapy, or in the biopsy specimens of responding sclerotic skin during therapy. Analysis of a buccal swab revealed no mutations at all. It is of particular interest that the same novel somatic *PTCH1* mutation was detected in both the primary and the resistant BCC, and was absent in clinically and histologically responding tissue. This observation implies that cells from the original tumor had survived. Thus, even though the resistant tumors arose from the same, clonal primary tumor, a clonal origin of the resistant BCC nests is precluded, a finding that supports tumor heterogeneity. Interestingly, the *SMO* codon 321 that we found was previously described as a critical binding site for vismodegib binding¹⁰², but codon 281 has never been related to vismodegib resistance. It appears that resistance to vismodegib in BCC can be caused by several different acquired *SMO* mutations that interfere with drug binding, a finding that is also described by Sharpe *et.al.*⁹² Also, in a few resistant cases, recurrent copy number variants in the downstream effectors *SUFU* and *Gli2* were detected. There is a complex interplay between mutual and heterogeneous resistance mechanisms to *SMO* inhibition in BCCs, even within the same tumor.⁹² The fact that sporadic BCC is a very heterogeneous tumor is confirmed by whole-exome sequencing of the genetic landscape of BCC, showing that BCCs harbor the most mutations of all known human cancers.²⁸ From this point of view, it could be suggested that the use of oral *SMO* inhibitors like vismodegib should be reserved for BCNS patients only, given the fact that these BCC are presumed to be obligatory and solely SHH driven, in contrast with sporadic BCC that appears to be more multifactorial (see **part one** of this discussion).

A better understanding of the tumorigenesis of sporadic BCC and the mechanisms underlying vismodegib resistance is important for the development of alternative treatment strategies. Sequential mutation analysis on pre-treatment and recurrent tumor tissue may help to anticipate on the type of resistance (either primary or secondary) in order to actively alter therapy. Then, treatment schedules and combination therapies can be customized to both patient and tumor characteristics, in an example of so-called personalized medicine. Overall, possible therapeutic approaches are sequential or rotational therapy with non-treatment periods ('drug holidays') in order to obtain a better tolerability for side effects, combination therapies, or alternating different therapies. Combining different systemic therapies in laBCC or mBCC may have the advantage of synergistic or additional therapeutic effects, lower dosage application and, possibly, the prevention of (acquired) resistance.¹⁰⁵ In addition, systemic itraconazole and nicotinamide might be promising agents in BCC treatment.^{106,107}

Vismodegib, SCC and future analyses

Chapter 5.2 discusses a patient with a 15-year history of a recurrent infiltrative BCC of the nose and right cheek, who was included in the STEVIE study and treated with vismodegib. No clinical improvement was observed and we even feared for progression into SCC as impressive hyperkeratosis developed after 7 weeks of treatment. Formation of SCC within the initial BCC has been reported in several case studies describing patients treated with vismodegib, as has development of SCC and keratoacanthoma on other body sites.¹⁰⁸⁻¹¹³ In our patient, histologic examination of tissue specimens acquired during surgery performed 5 months after initiation of vismodegib treatment, revealed residual BCC cells with positive staining for staining Ber-Ep4 (BCC marker). No signs of basosquamous carcinoma or SCC were found. Also, there were epidermal cysts that stained negatively for Ber-Ep4, excluding the possibility that these were BCC remnants that had survived of the original tumor. A possible explanation for the emergence of the benign squamous neoplasms could be the effect of vismodegib on keratinocyte differentiation. Vismodegib can mimic the loss of Indian hedgehog (IHH), a member of the HH ligand family the loss of which was shown to promote the progression of benign papillomas to SCC.¹¹⁴ In the case of SCCs emerging upon SMO inhibition, it has been hypothesized that a phenotypic switch from BCC to SCC, caused by new mutations (*NOTCH1/2* and *KMT2C*), could occur as a mechanism of tumor escape.¹¹⁵ Secondly, SMO inhibition might promote carcinogenesis by selecting tumor cells driven by other molecular pathways, like RAS/MAPK signaling. Activation of RAS/MAPK signaling can override the oncogenic addiction of the tumor to SHH signaling and thereby enabling proliferation of resistant tumors with enhanced metastatic behavior.¹¹⁶ However, further functional analysis is needed if the complex effects of vismodegib on keratinocyte differentiation and proliferation. Unfortunately, we were not able to perform any mutational analyses in our patient, mostly because no pre-treatment fresh frozen tissue samples had been obtained.

In daily dermatological and pathological practice, tissue samples acquired for diagnostic considerations are generally formalin-fixed paraffin-embedded (FFPE). DNA extracted from FFPE is fragmented and can also contain sequence artifacts arising from DNA dam-

age that can be difficult to distinguish from true mutations, especially in highly heterogeneous tumors such as BCC. These sequence variants are more often seen in FFPE than in fresh frozen tissue.¹¹⁷ One of the potential techniques to overcome this problem is the use of molecular inversion probes (MIPs). MIPs provide a practical, highly sensitive method for the detection of low-frequency mutations and subclonal variations in FFPE, requiring low amount of sample input, while per-sample costs are very low.¹¹⁸ This technique makes it possible to retrospectively perform pre- and post-treatment analysis. Such approaches will help to further unravel mechanisms of resistance, not only to vismodegib in BCC, but also to other small molecule inhibitors used to treat human malignancies such as melanoma.

Vismodegib and its place in daily dermatological practice

Systemic targeted treatments such as vismodegib are associated with potential toxicities and the number of patients reporting adverse events is considerably high, making its use limited to laBCCs and mBCCs. However an international multicenter clinical trial to the efficacy and safety of two different vismodegib regimens in patients with multiple BCC (MIKIE) (NCT01815840, clinicaltrials.gov) is currently ongoing. This study already anticipates on the fact that this group of patients will require a long-term treatment by introducing rotational therapy in which 'drug-holidays' of 8 weeks are implemented in an attempt to minimize adverse events. It can be speculated that also the occurrence of resistance may be diminished following rotational therapy. The interim results of the STEVIE study showed a median time to first onset of the most common adverse events of 2.83 months for muscle spasms, 5.55 months for alopecia, and 6.51 months for dysgeusia.⁹⁶ The side effects are cumulative and tend to get worse with increasing duration of the treatment. Temporary treatment stops e.g. every 3 months will lower or even prevent the burden of adverse events and is specially desirable in patients with multiple BCCs and BCNS. The possibility to use vismodegib in alternating treatment schedules in order to support a longer maintenance on treatment would provide an extra indication for FDA approval for this indication. However, experts in the field already implement drug-holidays, especially in BCNS patients, in which the treatment regimen is personalized to the patient and mainly patient driven rather than doctor driven. Interesting is a phase II, single-armed, multicenter trial (the NICCI trial), assessing the utility of vismodegib as neo-adjuvant treatment prior to surgery in laBCC and rBCC.¹¹⁹ Although currently still off-label, limited case reports already describe results of neo-adjuvant treatment of laBCCs with vismodegib.^{98,120,121} Neo-adjuvant therapy with vismodegib was effective in reducing the surgical defect, leading to less scarring and morbidity. However, long term follow-up is lacking and BCC remnants were found during Mohs surgery in the majority of the cases after three to six months of treatment with vismodegib.^{98,120,121} One should be aware of the fact that reduction of BCC nests following vismodegib not necessarily occurs from the periphery to the center of the tumor. Thus when performing Mohs surgery of only the center of the clinically remaining tumor, (subclinical) scattered tumor nests in the former tumor area are probably missed. This inevitably results in inadequate treatment and a high risk of recurrence. If therapy is optimized possibly by combination therapy, and the percentage of resistance for therapy is decreased, we do agree that planning patients for surgical excision

may reduce the chance of recurrence and could offer interesting further treatment options. Currently, the use of vismodegib as systemic treatment is limited to laBCCs and mBCCs because of potential toxicities which do not outweigh the disadvantages of available treatments of regular low-risk BCCs. In these low risk tumors, topical SMO inhibitors would have a broader applicability, however the results of their effectiveness in humans are rather disappointing. Topical CUR61414 had little or no effect on proliferation and apoptosis of BCCs, assessed by Ki-67 and cleaved caspase-3 immunohistochemical stains respectively, probably due to low penetration or rapid clearance of the active component.¹²² LDE225 0.75% cream is a different topical SMO inhibitor and although clinical regression is seen in almost all BCCs of eight BCNS patients after 4 weeks of treatment, histological examination of post-treatment biopsy samples of all tumors revealed that tumor nests were still present. A major advantage of topical application is the excellent safety profile with no local or systemic side effects.¹²³ Topical SMO inhibitors could emerge as an attractive non-invasive alternative for low-risk BCCs. However, future studies are needed to improve their efficacy and provide confident evidence for their use in daily practice.

The development and availability of small-molecule (targeted) inhibitors as vismodegib offer a new area in the management of BCC. As the dermatologist is the specialists with the most experience in treatment of different forms of skin cancer and the only specialist that has accessibility to all available treatment options, the dermatologist should always be involved in setting the indication for such treatments. In any case a multidisciplinary team in which the dermatologist, medical oncologist, plastic surgeon, radiotherapist and ENT-specialist closely cooperate should be involved. As laBCC/mBCC is generally a disease of the elderly, comorbidities and polypharmacy are not uncommon. The management of these patients requires a more holistic approach. Some have argued that the prescription and treatment with vismodegib should be restricted to medical oncologists. However, most dermatologists are experienced in prescribing systemic drugs and even cytostatic drugs and they are capable of dealing with possible side effects and adverse events. Treatment should be supervised by the physician who is experienced in the patient's ill-health and it is likely that the physician who decides to start vismodegib is the main therapist. In mBCCs, this could be the medical oncologist, but in laBCCs treatment by the dermatologists seems more logical. A good collaboration between all members of the multidisciplinary team is a key point. As the number of patients is limited, it is important to gain and share experiences with all team members and discuss cases regularly.

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Chapter 7

Summary/Samenvatting

Summary

A **general introduction** to this thesis is given in **chapter 1**. This chapter provides a brief overview of the current knowledge on basal cell carcinoma (BCC), the most common cancer worldwide, with emphasis on BCC epidemiology, pathogenesis, clinical presentation, diagnosis and treatment. The content of this thesis is divided into two parts. **Part one** focuses on the pathogenesis of BCC and the occurrence of epigenetic modifications in particular. New topical and systemic treatments for both non-aggressive BCC (naBCC) and locally advanced BCC (laBCC) are evaluated and described in **part two**. At the end, the aims of this thesis are discussed.

The genetic background of BCC has been studied extensively, while its epigenetic makeup has received comparatively little attention. Epigenetic modifications are hereditary alterations by mechanisms other than changes in the DNA sequence, of which DNA methylation is the most widely studied. These epigenetic alterations are thought to occur at least as frequent as genetic mutations in the development of cancer by silencing of crucial tumor suppressor genes. We hypothesized that DNA-methylation might also contribute to BCC tumorigenesis and could even affect gene expression (**part one**).

In a first exploratory study (**chapter 2.1**), we analyzed the promoter methylation status of ten candidate (tumor suppressor) genes that are associated with Sonic Hedgehog (SHH) signaling, WNT signaling and tumor growth. Methylation-specific PCR (MSP) was used in a large series of 112 BCCs and 124 healthy control samples. We found significantly more methylation in BCCs vs. healthy skin samples for the following gene promoters: *SHH*, adenomatous polyposis coli (*APC*), secreted frizzled-related protein 5 (*SFRP5*) and Ras association domain family 1A (*RASSF1A*). As promoter methylation may result in gene silencing, we performed reverse transcription and quantitative reverse transcription PCR (RT-PCR), which showed reduced mRNA levels of these four genes for APC and SFRP5 in BCCs (n=6) vs. normal skin (n=6). Significant down regulation of SHH, APC and RASSF1A could be confirmed on protein level as well by immunohistochemical staining. Increased canonical WNT activity was visualized by β -catenin staining, showing nuclear β -catenin in only 28/101 (27.7%) of BCC. Absence of nuclear β -catenin in the majority of the samples may be due to high levels of membranous E-cadherin (in 94.1% of the samples). We detected no considerable changes in methylation status between sun-protected and sun-exposed skin, suggesting that the methylation pattern we detected is tumor, rather than locus or patient-specific. In conclusion, we provide evidence suggesting promoter hypermethylation-mediated silencing of key players within the SHH and WNT pathways is frequent in BCCs, which is consistent with their known constitutive activation in BCCs. However, as BCCs are highly heterogeneous, it seems likely that aberrant epigenetic regulation of additional genes could contribute to BCCs development. With MSP it is possible to analyze predefined genes only. Therefore, in **chapter 2.2**, we performed genome-wide methylated DNA enrichment sequencing (MethylCap-seq) on 5 sporadic BCC and 5 healthy skin samples. These results were integrated with genome wide RNA-sequence data (RNA-seq henceforth), constituting the functional methylome. Fifteen differentially methylated or expressed loci in the BCC

samples were identified, among which *PTCH1* (part of SHH pathway), *PTPRN2* (methylation marker in squamous lung cell carcinoma and *IFI30* (playing a critical immunoregulatory role in melanoma). To further place the RNA expression patterns in a functional context, we performed pathway analysis of the RNA-seq data with a SPIA software tool. This *in silico* pathway analysis firmly implicated cytokine signaling, chemokine signaling and Toll-like receptor signaling in BCC growth and pathway activity, which we confirmed with immunohistochemical staining. Thus, we identified significant epigenetic modulation of gene expression in BCC and uncovered immunomodulatory signaling as a driver in BCC development.

In **chapter 2.3** we compared the MethylCap-seq and RNA-seq data acquired in **chapter 2.2** with those of a laBCC. This comparison provides the first evidence that there are substantial differences in gene methylation and expression levels between laBCC and naBCC. We found a significantly higher level of MMP13 expression in laBCC. This finding might explain why laBCC are much more aggressive than naBCC, because MMP13 is an enzyme that degrades extracellular matrix and bone. We also suggest that MMP13 might be used as a biomarker to distinguish BCC that are potentially aggressive and should be treated and followed up more rigorously to prevent local recurrence or evolution into a laBCC.

In our attempts to gain better insight into BCC pathogenesis, we realized that thinking 'out of the box' is crucial. As hair follicles are hypoxic microenvironments and BCCs are considered hair follicle tumors, we hypothesized that hypoxia-induced signaling pathways could be involved in BCCs as they are in other human malignancies. Hypoxia-inducible factor 1 (HIF1) and mechanistic/mammalian target of rapamycin (mTOR) are key players in the hypoxia pathways. In addition, the differentiation between BCCs and trichoepitheliomas (TEs), benign neoplasms that strongly resemble BCC, can be hard. Therefore we decided to compare these two tumors in **chapter 3.1**. We used immunohistochemical staining of formalin-fixed paraffin-embedded BCCs (n=45) and TEs (n=35) samples to assess activity of HIF1, mTORC1 and their most important target genes. Among the 45 BCCs and 35 TEs examined, most expression levels of several HIF1a and mTORC1 targets were comparable between BCCs and TEs. However, CAIX, Glut1 and PHD2 expression levels were significantly higher in TEs when only samples with at least 80% expression were included. From these results we concluded that HIF and mTORC1 signaling seems active in both BCCs and TEs. However, there are no appreciable differences between the two with respect to pathway activity. At this moment immunohistochemical analyses of HIF, mTORC1 and their target genes does not provide a reliable diagnostic tool for the discrimination of BCCs and TEs.

Current cancer research focuses ever more on the development of targeted treatments that specifically suppress key signaling pathways, as opposed to the more generally used nonspecific cytotoxic agents. **Part two** of this thesis provides novel insights in the usefulness of both topical and systemic targeted treatments in naBCCs and laBCCs respectively.

Non-invasive treatments for low risk BCCs have several advantages over conventional surgical excision, such as lower healthcare costs, a decrease in the workload for dermatologists, a better cosmetic outcome and a higher patient preference. Photodynamic therapy (PDT) (photochemical), imiquimod (immune-modulating) and 5-fluorouracil cream (chemotherapeutic) are available non-invasive treatments for low risk BCC that are well studied and already frequently prescribed. Apart from imiquimod, however, they all seem to have a mode of action of targeting non-specific signaling pathways activated in BCC. We hypothesized that higher efficacy might be expected when signaling pathways activated in BCC are specifically targeted, herewith potentially reaching efficacy rates of surgical excision. Non-steroidal anti-inflammatory drugs (NSAIDs) and vitamin D derivatives such as calcitriol can interfere in the SHH and WNT pathways, as well as in apoptosis signaling. From this, we hypothesized in **chapter 4.1** that topically applied diclofenac sodium 3% gel (Solaraze®), calcitriol 3µg/g ointment (Silkis®) and a combination of both would result in a decrease in proliferation and an increase in apoptosis in superficial (sBCCs) and nodular (nBCCs). In a phase II, single blind, randomized controlled intervention trial, patients with a primary, histologically proven sBCC (n=64) or nBCC (n=64) were randomly assigned to receive either topical diclofenac, calcitriol, a combination of both or no topical treatment (control group). For eight weeks, patients applied the vehicle twice a day and covered it with an occlusive sheet. At the end of the treatment period, all tumors were excised and histologically examined. The primary outcome measure was the post-treatment expression of Ki-67 (proliferation marker) and Bcl-2 (apoptosis marker). Histological clearance, adverse events, application-site reactions, patient compliance and patient satisfaction were secondary outcomes. We were able to confirm a significant decrease in Ki-67 and Bcl-2 expression in sBCCs treated with diclofenac and for Ki-67 after combination therapy. In addition, we found complete histological tumor regression in 64.3% of sBCCs after diclofenac and 43.8% of sBCCs after combination therapy, compared to 0.0% of controls. We found no tumor regression in nBCCs, and no appreciable changes in either Ki-67 or Bcl-2 expression levels, probably due to insufficient penetration of the drugs at the tumor site. Application-site reactions were reported frequently in the diclofenac groups, though most reactions were of mild to moderate severity and similar as seen in other non-invasive therapies for sBCCs, such as imiquimod and 5-fluorouracil cream. Repetitive occlusion as used in our trial may have enhanced the severity of the skin reactions. Despite the side effects, the compliance was high, ranging from 91.82% to 98.18%. From our results we conclude that, although surgical excision remains the gold standard for all BCCs, topical diclofenac may be a promising new treatment for low risk sBCCs. Because of its different mode of action by targeting several molecular pathways activated in BCCs, the expected major benefit of diclofenac gel is that is effective in BCC's non-responsive to non-specific or SHH-targeting agents. Additionally, as simultaneously targeting SHH and other signaling pathways may have a synergistic effect, we speculate that combining diclofenac with imiquimod (currently the most effective non-invasive treatment in sBCCs) might be promising.

Immunohistochemical (IHC) analysis as assessed in **chapter 4.1** is being used more often to gain insight in the underlying mode of action or efficacy of new, targeted therapies for BCCs. Manual pathologist-based quantification (MQ) is still the gold standard in the assessment of IHC slides, however, it is time-consuming and labor-intensive as well as subject to high intra- and inter-observer variability. Digital image analysis techniques offer an objective approach with a high sensitivity for change and are expected to have an increased reproducibility. However, comparative studies that test the assumption of high reproducibility of digital BCC image analyses are lacking. In **chapter 4.2** we used semi-automated quantification (SAQ) for the evaluation of 122 sBCCs and nBCCs and found reasonable high inter-observer agreements with 95% limits of agreement between -9.2% and 10.8% for Ki-67 and between -12.2% and 20.0% for Bcl-2. Two trained, but previously inexperienced, investigators performed SAQ independently. However, large discrepancies were seen between SAQ and MQ (performed by one experienced dermato-pathologist), especially at higher expression levels (more than 20% positive tumor cells) of Ki-67 and Bcl-2, which could partly be explained by systematic error. Our findings point to the need for a further study wherein consensus evaluation by more than one pathologist will be compared with SAQ measurements.

When naBCC are neglected or inadequately treated, they have the potential to develop into a laBCC. In 2012, the U.S Food and drug administration (FDA) approved vismodegib, an orally active synthetic SMO inhibitor, for the treatment of laBCC and mBCC. However, primary (no initial response to treatment) or secondary/acquired resistance (progression after an initial response) are emerging problems. **Chapter 5.1** describes a 68-year old woman with a laBCC of the left shoulder who developed tumor regrowth 20 weeks after an initially good response to vismodegib. Mutation analysis of *PTCH1* and *SMO* was performed on primary tumor tissue as well as on clinically responsive skin and tumors that arose during treatment. *PTCH1* mutations were found in the primary tumor and in two newly developed tumor nodules, while they were absent in clinically and histologically responding tissue. This finding implies that cells from the original tumor had survived. Additionally, the recurrent tumor nodules harbored two SMO mutations (p.Trp281Leu and p.Val321Met) which were neither found in the primary BCC nor in the responding skin. Thus, even though the resistant tumors arose from the same, primary tumor, they were not clonal. Both SMO mutations may probably cause resistance for SMO inhibitors. Currently, classification of resistance as being primary or secondary is based on clinical characteristics. A patient with presumably primary resistance to vismodegib is discussed in **chapter 5.2**. A 52-year old man was treated with vismodegib for a recurrent BCC of the nose and right cheek, that had previously been inadequately treated. After 7 weeks of treatment, hyperkeratosis had increased and comedo-like lesions had developed on the nose. No improvement was observed and a transition into a squamous cell carcinoma (SCC) was feared. The Sequential skin biopsies showed epidermal cysts, but no residual BCC or newly formed SCC. Because of the possibility of a sample error, Mohs' micrographic surgery was performed, showing infiltrative BCC but no SCC, which was confirmed by immunohistochemical stains for Ber-Ep4. Formation of pseudocyst-like structures and SCC within the initial BCC following vismodegib treatment has been reported previously in limited cases only. Also, the development of SCC and keratoacanthoma on other body

sites has been described in literature. It is therefore important to be aware of the possibility of occurrence of SCC during vismodegib treatment. Sequential mutation analysis on pre-treatment and recurrent tumor tissue may be helpful to anticipate on the resistance (either primary or secondary) in order to actively alter therapy. Accordingly, treatment schedules and combination therapies can be customized to both patient and tumor characteristics.

Samenvatting

In het **eerste hoofdstuk** wordt een **algemene inleiding** tot dit proefschrift gegeven. Het hoofdstuk schetst in het kort de huidige kennis van het basaalcelcarcinoom (BCC), wereldwijd de meest voorkomende vorm van kanker. De nadruk ligt hierbij op epidemiologie, pathogenese, klinische presentatie, diagnose en behandeling. Inhoudelijk bestaat dit proefschrift uit twee delen. Het **eerste deel** is gericht op de pathogenese van BCC, en dan met name op de epigenetische modificaties. In het **tweede deel** worden nieuwe topicale en systemische behandelingen voor zowel niet-agressieve (naBCC) als lokaal agressieve BCC (laBCC) besproken en geëvalueerd. Tot slot worden de doelstellingen van dit proefschrift beschreven.

De genetische achtergrond van BCC is uitvoerig onderzocht, maar aan de epigenetische achtergrond is verhoudingsgewijs minder aandacht besteed. Epigenetische modificaties zijn erfelijk bepaald door andere mechanismen dan veranderingen in de DNA sequentie, waarvan DNA methylering het meest onderzocht is. Verondersteld wordt dat deze epigenetische veranderingen minstens zo vaak optreden bij de ontwikkeling van kanker als genetische mutaties, dit door inactivatie van cruciale tumorsuppressorgenen. Onze hypothese is dat DNA-methylering mogelijk ook bijdraagt aan de tumorgenese van het BCC en zelfs de genexpressie kan beïnvloeden (**deel 1**).

In een eerste experimentele studie (**hoofdstuk 2.1**) hebben we de promotor methyleringsstatus van tien kandidaat (tumorsuppressor) genen geanalyseerd. Deze genen zijn geassocieerd met de Sonic Hedgehog (SHH) signalering, de WNT signaaltransductieroute en tumorgroei. Er is gebruikgemaakt van methylering-specifieke PCR (MSP) bij de analyse van 112 BCC's en 124 gezonde controlemonsters. In de volgende promotorgenen is er significant meer methylering gevonden in BCC's dan in de gezonde controlegroep: *SHH*, adenomateuze polyposis coli (*APC*), gesecreteerde frizzled gerelateerd eiwit 5 (*SFRP5*) en Ras geassocieerde domein familie 1A (*RASSF1A*). We hebben een reverse transcriptie en kwantitatieve reverse transcriptie PCR (RT-PCR) uitgevoerd omdat methylering van een promotor kan leiden tot inactivatie van een gen. Van de hiervoor genoemde vier genen werd er in APC en SFRP5 een verminderde concentratie mRNA in BCC's (n=6) gezien ten opzichte van normale huid (n=6). Een significante downregulatie van SHH, APC en RASSF1A zou bevestigd kunnen worden zowel op eiwitniveau als door middel van immunohistochemische kleuringen. Door aankleuring van β -catenine werd in slechts 28/101 (27,7%) van de BCC's verhoogde canonieke WNT-activiteit zichtbaar. Nucleair β -catenine was in de meerderheid van de samples afwezig; dit kan te verklaren zijn door de hoge concentraties membraanous E-cadherine (94,1% van de samples). We hebben geen noemenswaardige veranderingen in het methyleringspatroon tussen de aan zon blootgestelde huid en de voor zon beschermde huid gevonden. Dit suggereert dat het vastgestelde methyleringspatroon eerder bij een tumor past dan dat het locus- en patiëntspecifiek is. We leveren dus bewijs aan dat doet vermoeden dat inactivatie van hoofdrolspelers in de SHH en WNT signaaltransductieroutes door middel van hypermethylering van een promotor frequent voorkomt in BCC's. Dit komt overeen met de reeds bekende constitutieve activatie van deze signaaltransductieroutes in

BCC's. BCC's zijn echter zeer heterogeen, waardoor het waarschijnlijk lijkt dat een abnormale epigenetische regulatie van additionele genen kan bijdragen aan de ontwikkeling van BCC's. Met behulp van MSP is het alleen mogelijk om vooraf bepaalde genen te analyseren. In **hoofdstuk 2.2** hebben we dan ook gekeken naar genomwijde 'methylated DNA enrichment sequencing' (MethylCap-seq) op vijf sporadische BCC's en op vijf gezonde huidmonsters. Deze resultaten zijn geïntegreerd met genomwijde RNA-sequentie data (RNA-seq) die het functionele methyloom vormen. Er zijn vijftien differentieel gemethyleerde of tot expressie gebrachte loci in de BCC-monsters geïdentificeerd, waaronder *PTCH1* (deel van de SHH signaaltransductie), *PTPRN2* (methylering marker in plaveiselcelcarcinoom van de long) en *IFI30* (speelt een cruciale rol in de immunoregulatie van melanomen). Om de RNA expressiepatronen in een functionele context te kunnen plaatsen, hebben we met de SPIA software de signaaltransductieroutes van de RNA-seq data geanalyseerd. Deze *in silico* signaaltransductierouteanalyse liet duidelijk cytokine signalering, chemokine signalering en Toll-like receptor signalering zien in BCC-groei en signaaltransductieactiviteit. Dit laatste hebben wij door middel van immunohistochemische kleuringen bevestigd. We hebben dus significante epigenetische modulatie van genexpressie in BCC geïdentificeerd en ontdekt dat de immunomodulerende signalering een aanjager is in de ontwikkeling van BCC's.

In **hoofdstuk 2.3** hebben we de verkregen MethylCap-seq en RNA-seq data uit **hoofdstuk 2.2** vergeleken met die van een laBCC. Deze vergelijking levert het eerste bewijs dat er substantieel verschil is in de hoeveelheid gen methylering en expressie tussen laBCC en naBCC. We hebben een significant hogere concentratie MMP13 expressie gevonden in laBCC. Deze bevinding zou kunnen verklaren waarom laBCC een veel agressievere vorm is dan naBCC. MMP13 is namelijk een enzym dat de extracellulaire matrix en het bot afbreekt. Wij suggereren dat MMP13 als biomarker gebruikt zou kunnen worden om in potentie agressieve BCC's te herkennen. Op deze BCC's zouden dan een rigoureuzere behandeling en follow-up kunnen worden toegepast om een lokaal recidief of de ontwikkeling tot een laBCC te voorkomen.

Bij onze pogingen om meer inzicht te krijgen in de pathogenese van het BCC hebben we ons gerealiseerd dat 'out of the box' denken van cruciaal belang is. Een haarfollikel is een hypoxisch micromilieu en er wordt gedacht dat BCC's haarfollikeltumoren zijn. Onze hypothese is dan ook dat door hypoxie geïnduceerde signaaltransductieroutes een rol kunnen spelen bij BCC's. De activiteit van deze signaaltransductieroutes wordt ook in andere humane carcinomen gezien. Hypoxia-inducible factor 1 (HIF-1) en mechanistic/mammalian target of rapamycin (mTOR) zijn hoofdrolspelers in de hypoxische signaaltransductieroutes. Het onderscheid tussen BCC en een trichoepitheliom (TE), een goedaardige tumor die veel op het BCC lijkt, kan moeilijk zijn. In **hoofdstuk 3.1** hebben we daarom deze twee tumoren met elkaar vergeleken. We hebben immunohistochemische kleuringen gebruikt om de activiteit van HIF1, mTORC1 en hun belangrijkste doelgenen aan te tonen op formaline gefixeerde paraffine preparaten van BCC (n=45) en TE (n=35). De meeste expressieniveaus van de verschillende HIF1a en mTORC1 targets zijn vergelijkbaar tussen de onderzochte 45 BCC's en 35 TE's. Als alleen samples met een expressie van

tenminste 80% worden meegerekend, is in de TE's de expressie van CAIX, Glut1 en PHD2 significant hoger. Hieruit kunnen we concluderen dat HIF en mTORC1 signalering in zowel BCC's als TE's actief is. Er zijn echter geen noemenswaardige verschillen gevonden tussen de twee groepen ten aanzien van de activiteit van de signaaltransductieroute. Op dit moment lijken de immunohistochemische analyses van HIF, mTORC1 en hun doelgenen geen zinvol diagnosticum voor het onderscheid tussen BCC's en TE's.

De huidige onderzoeken naar kanker richten zich steeds meer op de ontwikkeling van doelgerichte behandelingen, die specifieke hoofdrolspelers van een signaaltransductieroute onderdrukken, in tegenstelling tot de huidige meer generieke en niet-specifieke cytotoxische middelen. In **deel 2** van dit proefschrift wordt nader ingegaan op de bruikbaarheid van zowel systemische als topicale doelgerichte behandelingen in respectievelijk naBCC en laBCC.

Non-invasieve behandelmethoden voor BCC's met een laag risico hebben verschillende voordelen boven conventionele chirurgische excisie: lagere kosten, daling van de werkdruk voor dermatologen, een beter cosmetisch resultaat en een hogere patiënttevredenheid. Photodynamische therapie (PDT) (fotochemisch), imiquimod (immuunmodulerend) en 5-fluorouracil crème (cytostaticum) zijn goed onderzochte en reeds vaak voorgeschreven non-invasieve behandelopties voor weinig risicovolle BCC's. Op imiquimod na, lijken ze allemaal aan te grijpen op niet-specifieke signaaltransductieroutes die geactiveerd zijn in BCC. Onze hypothese is dat er een hogere effectiviteit van topicale behandelingen bereikt kan worden, als de topicale middelen specifiek op de geactiveerde signaaltransductieroutes van BCC aangrijpen. Op deze manier kan mogelijk de effectiviteit van chirurgische ingreep worden benaderd. Niet-steroïde ontstekingsremmers (NSAID's) en vitamine D-derivaten, zoals calcitriol, kunnen interfereren met de SHH, WNT en apoptose signaaltransductieroutes. Op basis hiervan is onze hypothese in **hoofdstuk 4.1** dat topicale diclofenac sodium 3% gel (Solaraze®), calcitriol 3µg/g crème (Silkis®) en een combinatie van beide leiden tot een daling van de proliferatie en een stijging van de apoptose in superficiële BCC (sBCC) en nodulaire BCC (nBCC). In een fase II, geblindeerd, gerandomiseerd en gecontroleerd onderzoek werden patiënten met een primair, histologisch bevestigd sBCC (n=64) of nBCC (n=64) gerandomiseerd in ofwel topicale diclofenac, calcitriol of een combinatie van beide ofwel geen topicale behandeling (controlegroep). Patiënten hebben gedurende acht weken tweemaal daags de crème aangebracht en vervolgens bedekt met afsluitende folie. Aan het eind van de behandelperiode zijn alle tumoren geëxideerd en histologisch onderzocht. De primaire uitkomstmaat was de meting van de expressie van Ki-67 (proliferatiemarker) en Bcl-2 (apoptose marker) na de behandeling. Histologische remissie, bijwerkingen, lokale huidreacties, compliantie en patiënttevredenheid waren secundaire uitkomstmaten. We hebben een significante daling van Ki-67 en Bcl-2 expressie gevonden in sBCC's die behandeld werden met diclofenac. Bij patiënten die behandeld zijn met de combinatietherapie is een significante daling van Ki-67 gevonden. Verder werd volledige histologische regressie van de sBCC's in 64,3% en 43,8% van de patiënten die behandeld werden met respectievelijk diclofenac en de combinatietherapie tegen 0,0% in de controlegroep. Bij de nBCC's hebben we geen tumorregressie en ook geen

noemenswaardige veranderingen in de Ki-67 of Bcl-2 expressie gevonden. Dit is mogelijk te verklaren door onvoldoende penetratie van de medicijnen op de plek van de tumor. Lokale huidreacties kwamen frequent voor in de groepen met diclofenac. De meeste reacties waren mild tot matig van ernst en vergelijkbaar met die van andere non-invasieve behandelingen voor sBCC's, zoals imiquimod en 5-fluorourcil creme. De herhaaldelijke occlusie zoals wij in onze trial hebben gebruikt, heeft mogelijk tot heftiger huidreacties geleid. Ondanks de bijwerkingen was de compliantie hoog, tussen 91,82% en 98,18%. Uit onze resultaten kunnen we concluderen dat, ondanks het feit dat chirurgische excisie voor alle BCC's de gouden standaard blijft, topicale diclofenac een veelbelovende nieuwe behandelmethode kan zijn voor laag risico sBCC's. Diclofenac gel heeft door het andere werkingsmechanisme effect op verschillende in BCC's geactiveerde moleculaire signaaltransductieroutes. Het grote voordeel dat we hiervan verwachten, is dat diclofenac juist effectief is in BCC's die niet reageren op de niet-specifieke behandelingen of de behandelingen die alleen op de SHH-sigtaaltransductie aangrijpen. Als bij een behandeling van BCC's zowel de SHH als andere signaaltransductieroutes het doel zijn, kan dit een synergetisch effect hebben. We speculeren dan ook dat de combinatie van diclofenac met imiquimod (op dit moment de meest effectieve vorm van non-invasieve behandeling in sBCC's) veelbelovend kan zijn.

De immunohistochemische kleuringen (IHK) die in **hoofdstuk 4.1** zijn beoordeeld, worden gebruikt om meer inzicht te krijgen in het onderliggende werkingsmechanisme en de effectiviteit van nieuwe, doelgerichte behandelopties in BCC's. Handmatige kwantificatie (HK) door een patholoog wordt voor de beoordeling van IHK nog steeds als gouden standaard gezien. Dit is echter tijdrovend en arbeidsintensief en heeft een hoge intra- en interobserver variabiliteit. Digitale coupe analysetechnieken bieden een objectieve benadering met hoge sensitiviteit voor verandering en hebben waarschijnlijk een hogere reproduceerbaarheid. Er zijn nog geen vergelijkende studies bekend die deze verwachte reproduceerbaarheid van digitale coupeanalyses voor BCC's bevestigen. In **hoofdstuk 4.2** hebben we 122 sBCC's en nBCC's beoordeeld door middel van semi-automatische kwantificatie (SAK). Hierbij hebben we toereikend hoge interobserver overeenstemming gevonden met 95%-betrouwbaarheidsintervallen tussen -9,2% en 10,8% voor Ki-67 en tussen -12,2% en 20,0% voor Bcl-2. De SAK is onafhankelijk uitgevoerd door twee daartoe opgeleide maar nog onervaren onderzoekers. Hierbij zijn grote discrepanties gezien tussen de SAK en HK (verricht door één ervaren dermato-patholoog), met name bij hogere expressie van Ki-67 en Bcl-2 (meer dan 20% van de tumorcellen is positief). Dit is gedeeltelijk te verklaren door een systematische fout. Onze bevindingen geven aanleiding voor verder onderzoek waarin de HK, die door meer dan één wordt verricht, wordt vergeleken met de uitkomsten van de SAK.

Een naBCC heeft de potentie om zich te ontwikkelen tot een laBCC indien niet of onvoldoende behandeld. In 2012 heeft de Amerikaanse 'Food and drug administration' (FDA) vismodegib, een oraal actieve synthetische SMO inhibitor, goedgekeurd voor de behandeling van laBCC en gemetastaseerd BCC (mBCC). Primaire (geen reactie op de behandeling) en secundaire/verkregen (progressie na initiële respons) resistentie is bij deze behandeling een probleem. In **hoofdstuk 5.1** wordt een casus beschreven van een 68-jarige vrouw met een laBCC op de

linkerschouder. De tumor reageerde initieel goed op vismodegib maar na 20 weken begon deze opnieuw te groeien. Er zijn mutatieanalyses verricht van *PTCH1* en *SMO* op de primaire tumor, de klinisch reagerende huid en de gedurende de behandeling groeiende tumoren. De *PTCH1* mutaties werden gevonden in de primaire tumor en in de twee nieuw ontwikkelde tumornoduli, terwijl de mutatie afwezig was in de klinisch en histologisch reagerende huid. Dit impliceert dat tumorcellen van de oorspronkelijke tumor de behandeling overleefd hebben. Daarnaast werden bij de twee recidief noduli twee *SMO* mutaties (p.Trp281Leu en p.Val321Met) gevonden. Deze mutaties waren niet terug te vinden bij de primaire tumor, noch bij het gereageerde weefsel. Hoewel de resistente tumoren vanuit de primaire tumor groeiden, waren ze er dus geen klonen van. Beide *SMO* mutaties zouden de oorzaak kunnen zijn voor de resistentie voor de *SMO* inhibitors. Op dit moment is de classificatie primaire of secundaire resistentie gebaseerd op het klinische beeld. In **hoofdstuk 5.2** wordt een patiënt besproken met vermoedelijk primaire resistentie voor vismodegib. Bij een 52-jarige man werd een recidief BCC van de neus en rechterwang behandeld met vismodegib, nadat het eerder inadequaat behandeld was. Na een behandelperiode van zeven weken was er een toename van de hyperkeratose te zien en ontwikkelde er zich verruceuze afwijkingen op de neus. Ondanks de behandeling werd er geen verbetering gezien en de angst bestond dat de tumor zich had ontwikkeld tot een plaveiselcelcarcinoom (PCC). In de daaropvolgende biopsieën werden epidermale cysten gezien, maar er waren geen aanwijzingen voor een recidief BCC of nieuw ontwikkeld PCC. Gezien de kans op een steekproeffout werd besloten Mohs' micrografische chirurgie te verrichten. Dit liet een infiltratief BCC maar geen PCC zien. Dit werd door middel van immunohistochemische kleuringen van Ber-Ep4 bevestigd. De ontwikkeling van pseudocysten en PCC binnen het initiële BCC bij behandeling met vismodegib is eerder in slechts een beperkt aantal casus besprekingen beschreven. De ontwikkeling van PCC en keratoacanthomen op andere locaties van het lichaam is ook in de literatuur beschreven. Het is dus belangrijk om alert te zijn op de mogelijke ontwikkeling van een PCC tijdens de behandeling met vismodegib. Sequentiële mutatieanalyses op onbehandeld en recidief tumorweefsel kan zinvol zijn om te anticiperen op resistentie (primaire of secundaire) en actief de behandeling hierop aan te passen. Op deze manier kunnen behandelingschema's en combinatietherapieën worden toegespitst op zowel de patiënt als de tumorkenmerken.

Chapter 8

Curriculum vitae

Grants and awards

List of publications

Oral & poster presentations

Dankwoord

Curriculum vitae

Tjinta Brinkhuizen werd geboren op 15 november 1982 in Maastricht. Hier groeide ze samen met haar zusje en haar ouders op. In 2002 behaalde ze haar gymnasium diploma aan het Trichter College te Maastricht, waarna ze startte met de opleiding moleculaire levenswetenschappen (nu biomedische wetenschappen) aan de transnationale Universiteit Limburg, dit omdat ze was uitgeloot voor geneeskunde. In 2003 werd ze alsnog ingeloot en startte ze met de opleiding geneeskunde. Tijdens de co-schappen ontstond haar passie voor de dermatologie. Deze werd verder bevestigd gedurende haar semi-arts stage in het laatste jaar van haar opleiding tot basisarts aan de afdeling dermatologie van het Maastricht Universitair Maastricht Centrum (MUMC). In dit laatste jaar werd ook de interesse voor wetenschappelijk onderzoek gewekt. Zij schreef een onderzoeksvoorstel voor een persoonsgebonden beurs van KWF kankerbestrijding, welke werd toegekend. Hierdoor kon ze aansluitend aan het behalen van haar artsexamen in oktober 2009 beginnen als arts-onderzoeker op de afdeling dermatologie van het MUMC. Onder begeleiding van promotoren prof.dr.M.A.M. van Steensel en prof.dr.P.M. Steijlen en co-promotor mevr.dr.K. Mosterd werkte zij aan verschillende studies op het gebied van epigenetische modificaties en nieuwe behandelingsmodaliteiten van het basaalcelcarcinoom, leidend tot deze dissertatie. Daarnaast is ze betrokken geweest bij studies naar het Birt-Hogg-Dubé syndroom en infantiele hemangiomen. In oktober 2012 is zij met veel enthousiasme gestart met de opleiding tot dermatoloog en combineert ze haar klinische taken met haar onderzoekswerkzaamheden. Medio 2017 zal zij deze opleiding afronden. Met haar onderzoek won ze in 2010 de prijs voor beste voordracht op het dermato-oncologiecongres in Spa (België). In oktober 2014 won ze de eerste prijs op het Pélerin wetenschapssymposium (MUMC), waarvoor ze eerder in 2012 ook genomineerd was. In 2013 won ze de NVDV reisbeurs voor het IID congres in Edinburgh, Schotland.

Grants and Awards

Grant

Personal research grant Dutch Cancer Society (KWF):

Sum €243.000,-

Awards

October 2014

Winner Pélerin scientific award, MUMC, Maastricht, the Netherlands

Oral presentation: *Topical diclofenac 3%, vitamin D or a combination of both as treatment for basal cell carcinoma.*

Reward €5000,-

January 2013

Travel grant for IID 2013 by Dutch Society for Dermatology and Venereology.

Reward €750,-

October 2012

Pélerin scientific award, MUMC, Maastricht, the Netherlands

Oral presentation: *Basal cell carcinoma 'out of the box'.*

Project selected for oral presentation, €1000,-

June 2010

Dermato-oncology congress, Spa, Belgium

Oral presentation: *Basal cell carcinoma, Genetics, Epigenetics and future possible treatments.*

Best presenting resident Dermatology, *€500,-*

List of publications

Brinkhuizen T, Frencken KJA, Nelemans PJ, Hoff MLS, Kelleners-Smeets NWJ, zur Hausen A, van der Horst MPJ, Rennspiess D, Winnepenninckx WJL, van Steensel MAM, Mosterd K. *The effect of topical diclofenac 3% and calcitriol 3 µg/g on superficial basal cell carcinoma (sBCC) and nodular basal cell carcinoma (nBCC): A phase II, randomized controlled trial.* J Am Acad Dermatol. 2016 April.

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Frencken KJ*, Hacking MN*, **Brinkhuizen T**, Abdul Hamid MA, Martens H. *Soft yellowish papules on the neck: a clinicopathological challenge.* Clin Exp Dermatol. 2016 Mar;41(2):218-20.

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Dahlqvist J, Klar J, Tiwari N, Schuster J, Törmä H, Badhai J, Pujol R, van Steensel MA, **Brinkhuizen T**, Gijzen L, Chaves A, Tadini G, Vahlquist A, Dahl N. *A single-nucleotide deletion in the POMP 5' UTR causes a transcriptional switch and altered epidermal proteasome distribution in KLICK genodermatosis*. *Am J Hum Genet*. 2010 Apr 9;86(4):596-603.

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Oral presentations on conferences

The effect of topical diclofenac 3% and calcitriol 3µg/g on superficial and nodular basal cell carcinoma: a phase II, randomized controlled trial.

11th European Association of Dermato Oncology, Marseille, France, October 2015.

Acquired resistance to the Hedgehog pathway inhibitor vismodegib due to smoothened mutations in treatment of locally advanced basal cell carcinoma.

16th Annual scientific meeting Dutch Association for Experimental Dermatology, Lunteren, the Netherlands, February 2015.

Topical diclofenac 3%, vitamin D or a combination of both as treatment for basal cell carcinoma.

Pélerin scientific meeting (MUMC), Maastricht, the Netherlands, October 2014.

The functional methylome of Basal Cell Carcinoma and beyond.

Singapore International Conference on Skin Research, Biopolis, Singapore, March 2014

Topical diclofenac 3%, vitamin D or a combination of both as treatment for basal cell carcinoma.

15th Annual scientific meeting Dutch Association for Experimental Dermatology, Lunteren, the Netherlands, January 2014.

Basal Cell Carcinoma 'Out of the Box'.

Pélerin scientific meeting (MUMC), Maastricht, the Netherlands, October 2012.

An exceptional mutilating basal cell carcinoma.

Annual scientific meeting Dutch Association for Dermatology and Venereology, Maastricht, the Netherlands, June 2012.

Basal Cell Carcinoma, Genetics, Epigenetics and future possible treatments.

World Congress of Dermatology, Seoul, South Korea, May 2011.

Basal Cell Carcinoma, Genetics, Epigenetics and future possible treatments.

12th Annual scientific meeting Dutch Association for Experimental Dermatology, Lunteren, the Netherlands, January 2011.

SMO inhibitors and Basal Cell Carcinoma.

Scientific meeting Dermatology departments 'Limburg', Maastricht, the Netherlands, November 2010.

Basal Cell Carcinoma, Genetics, Epigenetics and future possible treatments.

2nd World Congress on Genodermatology, Maastricht, the Netherlands, November 2010.

Basal Cell Carcinoma, Genetics, Epigenetics and future possible treatments.

European Academy of Dermatology and Venereology, Gothenburg, Sweden, October 2010.

Basal Cell Carcinoma, Genetics, Epigenetics and future possible treatments.

Congress Dermato-oncology, Spa, Belgium, May 2010.

Basal cell carcinoma, Genetics, Epigenetics and future possible treatments.

Conference New developments in nonmelanoma skin cancer, Integral Cancer Centre Limburg, Sittard, the Netherlands, March 2010.

Poster presentations

The effect of topical diclofenac 3% and calcitriol 3µg/g on superficial and nodular basal cell carcinoma: a phase II, randomized controlled trial.

45th annual meeting of the European Society for Dermatological Research, Rotterdam, the Netherlands, September 2015.

A comparison of semi-automated versus pathologist-based manual quantification of immunohistochemical stains in basal cell carcinoma.

Resident scientific meeting (MUMC), Maastricht, the Netherlands, May 2015.

Comprehensive analysis of the basal cell carcinoma DNA methylome using MBD-enriched genomic sequencing.

14th Annual scientific meeting Dutch Association for Experimental Dermatology, Lunteren, the Netherlands, February 2013.

Comprehensive analysis of the basal cell carcinoma DNA methylome using MBD-enriched genomic sequencing.

Grow Science day, Maastricht, the Netherlands, November 2012.

Topical Diclofenac and Vitamin D as treatment for superficial Basal Cell Carcinoma.

The Dasil 1st annual congress, St. Julians, Malta, November 2012.

Aberrant promoter methylation in Basal Cell Carcinoma.

13th Annual scientific meeting Dutch Association for Experimental Dermatology, Lunteren, the Netherlands, February 2012.

Aberrant promoter hypermethylation in Basal Cell Carcinoma.

Epigenetic Mechanisms in Health & Disease. Brussels, Belgium, June 2010.

Aberrant promoter hypermethylation in Basal Cell Carcinoma.

American Association for Cancer Research 101st Annual Meeting, Washington, US, April 2010.

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