The hedgehog pathway in basal cell carcinoma

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The hedgehog pathway in basal cell carcinoma

Target for diagnostics and therapy

Marieke Reinders

The hedgehog pathway in basal cell carcinoma

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The hedgehog pathway in basal cell carcinoma

Target for diagnostics and therapy

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Voor mijn ouders

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Chapter 1

General introduction

General introduction

Insight into the pathogenesis of rare genetic disorders increases our understanding of the pathogenesis of common diseases. For example, in dermatology the discovery of filaggrin mutations in ichthyosis provided a key to unravel the pathogenesis of eczema.¹ Genetic studies on patients with basal cell naevus syndrome (BCNS) eventually led to the identification of inactivating mutations in the human homolog of the *Drosophila patched-1* gene (*PTCH1*), the genetic defect underlying this syndrome. At the same time, it turned out that sporadic basal cell carcinoma (BCC) has the same genetic signature as BCNS.^{2,3} Increased knowledge of the genetic basis of cancer helps us to understand its complex nature. This information can lead to the development of targeted cancer treatments. The genetic basis of BCC is the leading thread of this thesis. The first part focuses on the underlying genetics of BCNS. The second part gives an overview of targeted therapy for BCC with the hedgehog pathway inhibitor vismodegib and the mechanism of the development of resistance for this therapy.

Basal cell carcinoma

BCC is the most common cancer among Caucasians, with an increasing incidence of 3-10% per year.^{4,5} BCCs classically appear as slow-growing, translucent, elevated lesions on the sun-exposed skin (Figure 1.1). In the Netherlands 37,500 new patients were diagnosed with BCC in 2014. Ageing of the population is not the only cause of this rising number, since especially the number of young women with BCC is growing.⁴ Changing sun bathing behaviour plays an important role, as the major risk factor for BCC is exposure to ultraviolet (UV) radiation. It is estimated that one in five to six Dutch inhabitants will develop at least one BCC during lifetime.⁶ About 30% of these patients will develop more than one BCC.⁷ The majority of BCCs is easy to treat by conventional excision or Mohs' micrographic surgery. Non-invasive treatments, like topical imiquimod cream, 5-fluorouracil cream and photodynamic therapy, are an option in case of superficial tumour growth.⁸ Radiotherapy is a good alternative when patients are ineligible for surgery.⁹ Longstanding untreated or undertreated tumours can develop into a locally advanced BCC. In these cases, curative treatment with surgery or radiotherapy may be impossible because of technical restrictions or mutilating and functional side effects. BCC is a slow-growing tumour with a very low risk of metastasis (0.003-0.55%). Metastasised BCC however has a poor prognosis with a median survival of 8-10 months, if left untreated.¹⁰ Treatment with conventional chemotherapy or radiotherapy has been described in case reports and series, but no clinical trials are available and despite treatment, prognosis seems to remain poor.¹¹ Aggressive locoregional treatment with primary surgery or radiotherapy, or both, may be effective in patients with only locoregional disease.¹²

Figure 1.1. Typical basal cell carcinoma on the right cheek.

Hedgehog signalling pathway

The *PTCH1* gene is the human homolog of the *Drosophila patched-1* gene and is located on chromosome 9q22.3. It encodes a 1447-amino acid transmembrane glyco-protein, which is part of the hedgehog (Hh) signalling pathway. The Hh pathway is a key regulator in embryonic development and tumorigenesis controlling cell differentiation, tissue polarity and cell proliferation. The function of the PTCH1 protein is inhibition of the transmembrane protein smoothened (SMO). In the presence of active Hh ligands, binding to the PTCH1 receptor leads to release of this inhibition, allowing SMO to signal downstream and activate the glioma-associated oncogene (GLI) family of transcription factors. GLI transcription factors are inhibited in part by suppressor of fused (SUFU), which controls their nuclear entry and transcriptional effects (Figure 1.2a).

Figure 1.2. Hedgehog signalling pathway in a) embryonic development b) basal cell carcinoma.¹³

Based on its role in preventing cells from uncontrolled proliferation, *PTCH1* is considered as a tumour suppressor gene. *SMO* on the other hand acts as an oncogene. Deregulation of the Hh signalling pathway seems to be the primary event in the pathogenesis of BCC (Figure 1.2b). About 85% of sporadic BCCs carry mutations in genes from the Hh pathway, including *PTCH1* in 70%, activating *SMO* mutations in 10-20% and *SUFU* in about 8%.¹³⁻¹⁵ Aberrant activation of the Hh pathway has been shown in a variety of other human cancers, including malignant glioma, medulloblastoma, leukaemia, and cancer of the breast, lung, pancreas, and prostate.¹⁶

Basal cell naevus syndrome

BCNS (MIM #109400), also known as Gorlin syndrome, is a rare autosomal dominant disorder characterized by the development of multiple BCCs at a young age, maxillary keratocysts and cerebral calcifications.¹⁷ Other characteristic symptoms are palmoplantar pits, rib deformities and typical phenotypical features like macrocephaly and frontal bossing. Of the affected individuals, 3-5% develops a medulloblastoma in early childhood. Diagnosis of BCNS is based on the presence of one major criterion and genetic confirmation, two major criteria, or one major and two minor criteria (Table 1). Diagnostic criteria were first established by Evans et al., modified by Kimonis et al., and revised by Bree in 2011.¹⁸⁻²⁰ The incidence of BCNS is estimated at 1 in 50,000 to 256,000.²¹ In 1996 *PTCH1* was firstly reported as a candidate gene for BCNS. Two different heterozygous mutations in the *PTCH1* gene were identified in two patients with BCNS.³ Now we know that in up to 85% of BCNS patients a germline mutation in the *PTCH1* gene is found.²² Unilateral or segmental BCNS, caused by postzygotic mosaicism, has been described as well.^{23,24} Also, there are few reports of germline mutations in other participants of the Hh pathway like *PTCH2* and *SUFU*.²⁵⁻²⁷ To date, no genotype-phenotype correlation has been demonstrated in BCNS.^{28,29} Patients with a germline mutation in *SUFU* have a slightly higher risk of developing medulloblastoma in childhood.³⁰

The developmental defects in BCNS are likely to be due to haploinsufficiency²⁸, while tumours are believed to develop according to the two-hit theory described by Knudson.³¹ The first hit in BCNS is a germline mutation in the *PTCH1* gene. The remaining healthy allele is lost by loss of heterozygosity or somatic mutation. In BCNS this second hit is usually UV-induced, leading to BCC development.³²

Knowing the exact underlying genetic cause of the disease in an individual BCNS patient is important for providing adequate genetic counselling. It is important to diagnose BCNS at an early age, so an adequate screening program can be established and information about sun-protective behaviour can be provided to limit the burden of BCCs. The development of numerous BCCs is a major problem in patients with BCNS. The number of BCCs varies between BCNS patients, with a

mean lifetime number of tumours of more than 250.³³ Most tumours are easy to treat with surgery or non-invasive therapy. However, treatment can be difficult and has significant psychological impact.³⁴ Radiotherapy is contraindicated because it induces new skin tumours.

Table 1. Diagnostic criteria for basal cell naevus syndrome (Bree et al.²⁰). Two major criteria, one major criterion and two minor criteria, or one major and genetic confirmation are required for diagnosis.

Major criteria	Minor criteria
Multiple BCCs or one BCC in a person younger than 20 years	Bifid, fused or splayed ribs
Odontogenic keratocysts	Other specific skeletal and radiologic abnormalities (i.e. pectus excavatum, scoliosis, hemivertebrae, Sprengel's deformity, syndactyly of digits, bony bridging of the sella turcica, flame-shaped lucencies of phalanges)
Palmar or plantar pits	Macrocephaly
Lamellar calcification of the falx cerebri	Cleft lip or palate
Medulloblastoma in early childhood	Ovarian or cardiac fibroma
First-degree relative with BCNS	Lymphomesenteric cysts
	Ocular anomalies (i.e. congenital cataract, coloboma, glaucoma, hypertelorism)

Cutaneous mosaicism

A mosaic refers to a composite of different materials, for example a design made by putting together differently coloured pieces of stone. A mosaic organism is composed of two or more genetically distinct cell populations, derived from a genetically homogeneous zygote.³⁵ In the animal and plant kingdom there are several examples of mosaicism (Figure 1.3). In fact all female individuals are mosaics because of lionisation (inactivation) of the X-chromosome. In dermatology, mosaicism is a common phenomenon as well. Patterns of mosaicism can be easily studied on the skin because it is accessible to visual examination. In 1901 dermatologist Alfred Blaschko first described different linear patterns in epidermal and sebaceous nevi.³⁶ This system of lines, in which cells divide along prerequisite patterns during embryogenesis, is now referred to as Blaschko lines (Figure 1.4)^{36,37}. Mosaic skin diseases may follow Blaschko lines, but additional patterns of cutaneous mosaicism have been described by dermatologist Rudolph Happle (Figure 1.5).³⁵

Several autosomal dominant disorders manifest themselves in mosaic patterns on the skin. Type 1 segmental mosaicism refers to the situation in which a *de novo* postzygotic mutation occurs somewhere during embryogenesis, only affecting cells from the mutant progenitor, resulting in mixed healthy and affected cell populations. Depending on the mutation load and the tissues involved, an individual with postzygotic mosaicism may develop clinical signs to a certain degree or none at all. Gonadal cells can also be affected in type 1 segmental mosaicism, meaning that an individual with this condition can give birth to a child with a germline mutation. Type 2 segmental mosaicism refers to an autosomal dominant disorder which is accompanied by a postzygotic mutation that affects the healthy allele, resulting in pronounced cutaneous symptoms of the disease in a mosaic pattern (Figure 1.6).³⁸

Figure 1.3. Mosaicism in an apple (picture provided by the Dutch Fruit Growers Organisation).

Figure 1.4. Lines of Blaschko.³⁷

Figure 1.5. Patterns of cutaneous mosaicism according to dr. R. Happle.³⁵

Figure 1.6. Postzygotic mosaicism in autosomal dominant disorders.³⁸

Hedgehog pathway inhibitors

In the 1950s sheep ranchers in the western United States experienced episodic outbreaks of one-eyed lambs. It turned out that ewes that grazed on corn lily (*Veratrum californicum*) during a certain period of their pregnancy gave birth to lambs with midline defects, like cyclopia and a proboscis-like nasal structure. In 1968 the responsible substance for these birth defects was isolated from the plant and named cyclopamine.³⁹ In 1978 the Hh signalling pathway was discovered in the fruit fly (*Drosophila melanogaster*). Inactivation of this pathway caused the *Drosophila* larvae to be covered with spiny dendricles, resembling a hedgehog, hence its name. Mouse embryos lacking Sonic hedgehog gene function develop congenital abnormalities in the brain and spinal cord, axial skeleton and the limbs, featuring holoprosencephaly (failure of the forebrain to develop into hemispheres) and cyclopia.⁴⁰ Eventually it was found that loss of Hh signalling in human embryonic development can also result in holoprosencephaly.^{41,42} Because of the similarities between the cyclopamine induced developmental abnormalities in sheep and the clinical presentation of holoprosencephaly in humans, it was suggested that cyclopamine is capable of inhibiting the Hh pathway.⁴³ Taipale et al. demonstrated in vitro in primary murine fibroblasts lacking functional PTCH (PTCH^{-/-}) that cyclopamine inhibits the Hh pathway by antagonizing SMO.⁴⁴ Apart from its role in embryonic development, the Hh pathway is activated in several forms of cancer, including BCC. Eventually, synthetic cyclopamine-like SMO antagonists, so called Hh inhibitors, have been developed as a targeted cancer treatment. In 2009 the results of a phase 1 trial to assess the safety and efficacy of Hh pathway inhibitor vismodegib in the treatment of locally advanced and metastatic BCC were published, followed by phase 2 trials.^{45,46} In 2012, the FDA registered the Hh inhibitor vismodegib for the treatment of metastasised and locally advanced BCC ineligible for surgery or radiotherapy.⁴⁷ In 2014 vismodegib was approved in the Netherlands for this indication. Only few patients with BCC are eligible for treatment with Hh inhibitors. In 2015, 25 patients were treated with vismodegib in the Netherlands. Treatment with vismodegib is effective in the majority of patients. However, side effects are often a reason for discontinuation of the therapy and the development of tumour resistance is a major problem.⁴⁸ Some patients with BCNS benefit from maintenance treatment with Hh inhibitors. However, treatment is still off-label and discontinuation of treatment because of side effects is even more common in this group of patients.^{49,50}

Figure 1.7. Corn lily (*Veratrum californicum*).³⁹

Figure 1.8. Cyclopic lamb fed *Veratrum californicum* on day 14 of gestation.³⁹

Aims of this thesis

1. To create a database of all *PTCH1* mutations detected in the Netherlands.
2. To learn more about the clinical and genetic characteristics in patients with BCNS, to eventually improve patient customized health care.
3. To understand mechanisms of resistance to hedgehog inhibitors in BCC.
4. To share experiences of treatment of locally advanced and metastasised basal cell carcinoma with vismodegib.

Outline of this thesis

Part one

Basal cell naevus syndrome

In **chapter 2** we established a locus-specific database for the *PTCH1* gene using the Leiden Open Variation Database (LOVD). We gathered 111 mutations from 142 patients in whom *PTCH1* mutation analysis was performed in either the VU University Medical Centre (VUMC) or the Maastricht University Medical Centre (MUMC) between 1995 and 2015. All these mutations were included in this public database together with previously published mutations worldwide.

In **chapter 3** we describe the clinical characteristics of patients who carry a *PTCH1* mutation. We looked for a genotype-phenotype correlation in our population and provided a review of the literature on this topic.

In **chapter 4** we present a patient with BCNS caused by low grade postzygotic mosaicism of a *PTCH1* mutation. Low grade mosaicism is not always detectable with standard genetic tests but has important implications for preventive measures and genetic counselling.

In **chapter 5** we describe two patients with mosaic BCNS and no *PTCH1* mutation in blood. Genetic analysis of their BCCs led to detection of postzygotic *PTCH1* mosaicism.

Part two

Treatment of locally advanced and metastasised basal cell carcinoma with vismodegib

In **chapter 6** we describe the first Dutch patient treated with vismodegib for BCC. An overview of vismodegib treatment for BCC is given in **chapter 7**. Three different patient cases from three academic hospitals are presented. Indications, efficacy, adverse events and future perspectives of vismodegib are discussed. Our goal was to inform dermatologists, general practitioners, and other specialities about this new treatment modality for locally advanced and metastasised BCC.

In **chapter 8** we describe a patient with locally advanced BCC who develops resistance to vismodegib after a complete clinical response. We performed mutational analysis on primary tumour tissue and resistant tumour tissue to explain the mechanism of development of drug resistance in this patient.

In **chapter 9** we present a patient with locally advanced BCC who showed primary resistance to vismodegib together with the development of hyperkeratosis and epidermal cysts. This case illustrates how difficult the clinical assessment of tumour response can be and stresses the importance of repeated skin biopsies in the event of doubts. Furthermore, the development of epidermal cysts can give us information about the mechanism of action of hedgehog inhibitors.

Chapter 10 provides a summary, discussion and valorisation of this thesis, together with some future perspectives. In **chapter 11** a Dutch summary of the thesis is given.

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Part one

Basal cell naevus syndrome

Chapter 2

New mutations and an updated database
for the *patched-1 (PTCH1)* gene.

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Molecular Genetics and Genomic Medicine, Mar 2018

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Abstract

Background Basal cell naevus syndrome is an autosomal dominant disorder characterized by multiple basal cell carcinomas, maxillary keratocysts and cerebral calcifications. BCNS most commonly is caused by a germline mutation in the *patched-1* gene. *PTCH1* mutations are also described in patients with holoprosencephaly.

Methods We have established a locus-specific database for the *PTCH1* gene using the Leiden Open Variation Database (LOVD). We included 117 new *PTCH1* variations, in addition to 331 previously published unique *PTCH1* mutations. These new mutations were found in 141 patients who had a positive *PTCH1* mutation analysis in either the VU University Medical Centre or Maastricht University Medical Centre between 1995 and 2015.

Results The database contains 331 previously published unique *PTCH1* mutations and 117 new *PTCH1* variations.

Conclusion We have established a locus-specific database for the *PTCH1* gene using the Leiden Open Variation Database. The database provides an open collection for both clinicians and researchers and is accessible online at <http://www.lovd.nl/PTCH1>.

Introduction

Basal cell naevus syndrome (BCNS, MIM#109400) or Gorlin syndrome is a rare autosomal dominant disorder characterized by multiple basal cell carcinomas (BCCs), maxillary keratocysts and cerebral calcifications.¹ The incidence of BCNS is estimated at 1 in 50,000 to 256,000.² Diagnostic criteria for BCNS were first established by Evans et al.³, modified by Kimonis et al.⁴, and revised in 2011 by Bree et al.⁵ Diagnosis is based on two major criteria, one major criterion and two minor criteria or one major criterion and genetic confirmation (Table 1).

Most frequently occurring symptoms are multiple BCCs, maxillary keratocysts, palmoplantar pits and calcification of the falx cerebri. About 60% of patients have a typical phenotype with macrocephaly, frontal bossing, coarse facial features and hypertelorism.^{3,4} 3-5% of patients develop medulloblastoma in childhood.⁶

In 1996 the *patched-1* (*PTCH1*) gene (MIM#601309) was first reported as a candidate gene for BCNS. Two different heterozygous mutations in the *PTCH1* gene were identified in two patients with Gorlin syndrome.⁷ Another disorder that is caused by a germline mutation in the *PTCH1* gene is holoprosencephaly-7 (MIM#610828), a structural anomaly of the brain in which there is failed or incomplete separation of the forebrain early in gestation. In addition, the vast majority of sporadic BCCs have somatic mutations in *PTCH1*.^{8,9}

Table 1. Diagnostic criteria for basal cell naevus syndrome (Bree et al.⁵). Two major criteria, one major criterion and two minor criteria, or one major and genetic confirmation are required for diagnosis.

Major criteria	Minor criteria
Multiple BCCs or one BCC in a person younger than 20 years	Bifid, fused or splayed ribs
Odontogenic keratocysts	Other specific skeletal and radiologic abnormalities (i.e. pectus excavatum, scoliosis, hemivertebrae, Sprengel's deformity, syndactyly of digits, bony bridging of the sella turcica, flame-shaped lucencies of phalanges)
Palmar or plantar pits	Macrocephaly
Lamellar calcification of the falx cerebri	Cleft lip or palate
Medulloblastoma in early childhood	Ovarian or cardiac fibroma
First-degree relative with BCNS	Lymphomesenteric cysts
	Ocular anomalies (i.e. congenital cataract, coloboma, glaucoma, hypertelorism)

The *PTCH1* gene

PTCH1 (NCBI Reference Sequence NM_000264.3) is the human homolog of the *Drosophila patched-1* gene and is located on chromosome 9q22.3. It contains 24 exons with the transcriptional start site in exon 1 and the termination site in exon 23. *PTCH1* encodes a 1447-amino acid transmembrane glyco-protein, which is part of the hedgehog (Hh) pathway. The Hh pathway is a key regulator in embryonic development and tumorigenesis controlling cell differentiation, tissue polarity and cell proliferation.

The function of the *PTCH1* protein is inhibition of the transmembrane protein smoothened (SMO). Extracellular Hh ligands can bind to the *PTCH1* receptor, releasing this inhibition, allowing SMO to signal downstream and activate GLI transcription factors. Based on this role in preventing cells from uncontrolled proliferation, *PTCH1* is seen as a tumour suppressor gene. SMO on the other hand acts as an oncogene.¹⁰

The typical congenital features of BCNS seem to occur due to haploinsufficiency,¹¹ while tumours in BCNS are believed to develop according to the two-hit hypothesis described by Knudson.^{12,13} In the latter either both alleles of the gene harbour a mutation, or one mutated allele is accompanied by allelic loss of the remaining wild type allele. Recent mouse model studies show that haploinsufficiency of *PTCH1* may be sufficient for the development of medulloblastoma and rhabdomyosarcoma, so tumour formation not always follows the two-hit hypothesis.^{14,15} With DNA sequencing analysis of the *PTCH1* gene, mutation detection frequency ranges from 50% to 85% in individuals with typical findings of BCNS.¹⁶ Mosaic presentations of BCNS can occur.^{17,18}

The *PTCH1* database

We have established a database for *PTCH1* using the Leiden Open Variation Database (LOVD) version 3.0.¹⁹ The purpose of this database is to assemble molecular variants of the *PTCH1* gene in a standardized format. The database provides an open collection for both clinicians and researchers containing published and unpublished *PTCH1* mutations.

For each mutation, information is provided at the molecular level: DNA change, predicted protein change, RNA change, exon, type of mutation, reported pathogenicity, technique used and source of material, and phenotype information if available. The Sequence Variant Nomenclature of all mutations (new and published) is updated according to the latest guidelines of the Human Genome Variation Society (HGVS) version 15.11 and based on NCBI Reference Sequence NM_000264.3.

Analysis of the database

The database lists 331 previously published unique *PTCH1* mutations.¹⁹ In addition, we included 117 new *PTCH1* variations (Table 2). These mutations were found in 141 patients who had a positive *PTCH1* mutation analysis in either the VU University Medical Centre (VUMC) or Maastricht University Medical Centre (MUMC) between 1995 and 2015. Mutation analysis was performed using Sanger sequencing and Multiplex Ligation-dependent Probe Amplification. Polymorphisms and patients from the same family were excluded. Of the 117 different *PTCH1* variations, 110 mutations are classified as pathogenic or probably pathogenic according to the guidelines of the Associations for Clinical Genetic Science and the Dutch Society of Clinical Genetic laboratory Specialists and the American College of Medical Genetics and Genomics (ACMG).^{21,22} A number of 23 *PTCH1* mutations are reported by previous studies and 79 are novel.

Of the 110 new mutations 38% (42/110) are frameshift (33 small deletions, 8 small duplications, 1 small indel), 26% (29/110) nonsense, 13% (14/110) missense and 15% (16/110) are splicing mutations. The remaining 8% (9/110) are large genomic *PTCH1* deletions and duplications. The mutations were found in all coding exons (1-23) and no hotspot was found.

The majority of patients were from the Netherlands (68%, 76/110). The age of DNA test ranged from 0-70 years, with a mean age of 26.5 years. Motivations for genetic testing written on the application forms were: 1) clinical suspicion of BCNS (47.4%); 2) clinical diagnosis of BCNS (32.6%); 3) first degree family member with BCNS (16.8%); and 4) family members with symptoms of BCNS (3.2%). In total four individuals were prenatally screened for BCNS, because of a first-degree family member or a clinical suspicion based on ultrasonography.

Database availability

The data is accessible to the public at <http://www.lovd.nl/PTCH1>. Contributors will have to register for a login and password.

TABLE 2 New *PTCH1* mutations

Case identifier	Members affected	Gender	Age of diagnosis	Exon/ Intron	DNA variant	Protein change	RNA change	Classification
Nonsense mutations								
BCNS1	1	M	67.7	2	c.205A>T	p.(Lys69*)	r.(?)	5
BCNS2	1	F	24.4	2	c.279C>G	p.(Tyr93*)	r.(?)	5
BCNS3	1	F	27.9	2	c.294C>A	p.(Cys98*)	r.(?)	5
BCNS4	2	M	38.9	3	c.403C>T	p.(Arg135*)	r.(?)	5
BCNS5	3	F	26.1	3	c.403C>T	p.(Arg135*)	r.(?)	5
BCNS6	3	M	11.0	3	c.466C>T	p.(Gln156*)	r.(?)	5
BCNS7	1	M	14.1	5	c.707G>A	p.(Trp236*)	r.(?)	5
BCNS8	2	U	56.6	8	c.1081C>T	p.(Gln361*)	r.(?)	5
BCNS9	1	F	36.7	8	c.1093C>T	p.(Gln365*)	r.(?)	5
BCNS10	1	M	8.4	8	c.1119C>G	p.(Tyr373*)	r.(?)	5
BCNS11	1	F	1.1	8	c.1198C>T	p.(Gln400*)	r.(?)	5
BCNS12	1	F	8.3	10	c.1379G>A	p.(Trp460*)	r.(?)	5
BCNS13	2	U	31.0	10	c.1380G>A	p.(Trp460*)	r.(?)	5
BCNS14	1	M	0.0	12	c.1691T>G	p.(Leu564*)	r.(?)	5
BCNS15	1	M	31.1	13	c.1804C>T	p.(Arg602*)	r.(?)	5
BCNS16	1, mosaicism	F	23.0	13	c.1810G>T	p.(Glu604*)	r.(?)	5
BCNS17	1	F	33.0	14	c.1975C>T	p.(Gln659*)	r.(?)	5
BCNS18	2	F	24.4	14	c.2098C>T	p.(Gln700*)	r.(?)	5
BCNS19	1	F	25.1	14	c.2170G>T	p.(Glu724*)	r.(?)	5
BCNS20	2	F	56.7	15	c.2308C>T	p.(Arg770*)	r.(?)	5
BCNS21	1	M	42.4	15	c.2359G>T	p.(Glu787*)	r.(?)	5
BCNS22	1	F	11.5	15	c.2446C>T	p.(Gln816*)	r.(?)	5
BCNS23	1	M	19.7	15	c.2557C>T	p.(Gln853*)	r.(?)	5
BCNS24	1	M	4.3	16	c.2619C>A	p.(Tyr873*)	r.(?)	5
BCNS25	1	M	25.8	16	c.2619C>A	p.(Tyr873*)	r.(?)	5
BCNS26	1	M	14.1	16	c.2619C>G	p.(Tyr873*)	r.(?)	5
BCNS27	1	F	6.2	18	c.3027C>A	p.(Tyr1009*)	r.(?)	5
BCNS28	2	F	47.9	18	c.3027C>G	p.(Tyr1009*)	r.(?)	5
BCNS29	1, de novo	F	14.1	18	c.3058C>T	p.(Gln1020*)	r.(?)	5
Missense mutations								
BCNS30	1, de novo	F	28.8	4	c.591G>C	p.(Trp197Cys)	r.(?)	5
BCNS31	1	M	11.7	5	c.689C>G	p.(Thr230Arg)	r.(?)	4
BCNS32	1	M	31.1	6	c.890T>C	p.(Leu297Pro)	r.(?)	4
BCNS33	2	M	7.4	10	c.1439C>T	p.(Ser480Leu)	r.(?)	4
BCNS34	1	M		10	c.1450G>A	p.(Gly484Arg)	r.(?)	4
BCNS35	1	M	36.9	11	c.1526G>A	p.(Gly509Asp)	r.(?)	5
BCNS36	1	M	58.4	11	c.1526G>A	p.(Gly509Asp)	r.(?)	5
BCNS37	3	M	15.1	11	c.1526G>A	p.(Gly509Asp)	r.(?)	5
BCNS38	1	F	40.3	11	c.1555G>C	p.(Ala519Pro)	r.(?)	4
BCNS39	1	U	39.2	12	c.1712G>C	p.(Arg571Pro)	r.(?)	4

(Continues)

TABLE 2 (Continued)

Case identifier	Members affected	Gender	Age of diagnosis	Exon/ Intron	DNA variant	Protein change	RNA change	Classification
BCNS40	1	M	38.9	14	c.2250G>C	p.(Lys750Asn)	r.sp!?	4
BCNS41	1	M	46.7	15	c.2414T>G	p.(Ile805Arg)	r.(?)	4
BCNS42	1	M	22.6	15	c.2447A>G	p.(Gln816Arg)	r.(?)	4
BCNS43	1	F	52.1	18	c.2917C>A	p.(Gln973Lys)	r.(?)	4
Splice site mutations								
BCNS44	1	F	17.1	1i	c.202-2A>G	p.?	r.sp!?	4
BCNS45	2	F	23.5	2i	c.394+1G>A	p.?	r.sp!?	4
BCNS46	2	M	17.7	2i	c.394+1G>C	p.?	r.sp!?	4
BCNS47	1	F	13.6	4i	c.566_584+8del	p.?	r.sp!?	4
BCNS48	1	M	12.3	4i	c.655-1G>A	p.?	r.sp!?	4
BCNS49	1	F	21.5	5i	c.747-2A>G	p.?	r.sp!?	4
BCNS50	4	M	2.6	6i	c.946-1G>T	p.?	r.sp!?	4
BCNS51	2	F	22.3	8i	c.1216-2A>G	p.?	r.sp!?	4
BCNS52	2	F	33.1	9i	c.1347+1G>A	p.?	r.sp!?	4
BCNS53	2	M	32.3	9i	c.1348-1G>C	p.?	r.sp!?	4
BCNS54	1	M	24.0	10i	c.1504-1G>C	p.?	r.sp!?	4
BCNS55	1	M	59.7	10i	c.1504-2A>T	p.?	r.sp!?	4
BCNS56	2	M	27.1	12i	c.1729-1G>C	p.?	r.sp!?	5
BCNS57	1	M	69.4	14i	c.2250+1G>T	p.?	r.sp!?	4
BCNS58	1	F	28.2	14i	c.2251-2A>G	p.?	r.sp!?	4
BCNS59	1	M	7.1	15i	c.2561-2A>G	p.?	r.sp!?	4
Small deletions or duplications								
BCNS60	1, de novo	M	5.4	1	c.114del	p.(Leu39Cysfs*41)	r.(?)	5
BCNS61	2	M	31.0	2	c.254_255del	p.(Arg85Thrfs*4)	r.(?)	5
BCNS62	1	F	49.7	2	c.258_259del	p.(Leu87Ilefs*2)	r.(?)	5
BCNS63	1	M	28.5	2	c.258_259del	p.(Leu87Ilefs*2)	r.(?)	5
BCNS64	1	M	52.0	2	c.262_266del	p.(Phe88Thrfs*50)	r.(?)	5
BCNS65	1	M	30.9	2	c.385_386dup	p.(Trp129Cysfs*9)	r.(?)	5
BCNS66	1	M	14.7	3	c.479_482del	p.(Gln160Profs*10)	r.(?)	5
BCNS67	1	M	10.7	3	c.572_575dup	p.(Met192Ilefs*61)	r.(?)	5
BCNS68	1	F	63.8	5	c.724del	p.(Gln242Serfs*8)	r.(?)	5
BCNS69	1	F	25.8	6	c.770_771delinsGGTTTGG	p.(Thr257Argfs*14)	r.(?)	5
BCNS70	1	F	43.8	6	c.842del	p.(Met281fs*2)	r.(?)	5
BCNS71	1	F	11.4	7	c.1040_1049del	p.(Val347Alafs*17)	r.(?)	5
BCNS72	1	F	35.1	8, 14	c.[1114del;2183C>T]	p.[(Met372Cysfs*60); p.(Thr728Met)]	r.(?)	5;3
BCNS73	1	F	23.9	9	c.1279del	p.(Leu427Trpfs*5)	r.(?)	5
BCNS74	1	M	35.1	10	c.1348_1350del	p.(Leu450del)	r.(?)	4
BCNS75	1	M	55.5	10	c.1366dup	p.(Thr456Asnfs*41)	r.(?)	5
BCNS76	2	F	13.3	10	c.1415_1429del	p.(Ala472_Leu476del)	r.(?)	4
BCNS77	1	M	47.9	11	c.1508dup	p.(Leu503fs*)	r.(?)	5
BCNS78	1	M	2.7	13	c.1767_1769del	p.(Leu590del)	r.(?)	4

(Continues)

TABLE 2 (Continued)

Case identifier	Members affected	Gender	Age of diagnosis	Exon/ Intron	DNA variant	Protein change	RNA change	Classification
BCNS79	1	F	8.3	14	c.1852del	p.(Cys618Alafs*5)	r.(?)	5
BCNS80	1	M	43.0	14	c.1925dup	p.(Pro643Thrfs*11)	r.(?)	5
BCNS81	1	F	16.4	14	c.2011dup	p.(His671Profs*10)	r.(?)	5
BCNS82	1	M	15.5	14	c.2178_2179insA	p.(Cys727Metfs*11)	r.(?)	5
BCNS83	1	M	14.9	14	c.2179del	p.(Cys727Valfs*19)	r.(?)	5
BCNS84	1	M	42.2	16	c.2612_2615del	p.(Asn871Ilefs*31)	r.(?)	5
BCNS85	1	F	9.7	17	c.2748del	p.(Ser917Alafs*7)	r.(?)	5
BCNS86	1	F	1.2	17	c.2793del	p.(Val932Serfs*30)	r.(?)	5
BCNS87	1	F	14.5	17	c.2833_2843del	p.(Arg945Glyfs*10)	r.(?)	5
BCNS88	1	M	52.4	18	c.3050del	p.(Phe1017Serfs*32)	r.(?)	5
BCNS89	2	M	40.3	18	c.3056_3059del	p.(Glu1019Glyfs*29)	r.(?)	5
BCNS90	2	F	21.0	18	c.3107_3108del	p.(Leu1036Cysfs*108)	r.(?)	5
BCNS91	3	F	0.6	18, 23	c.[3135del;4048C>T]	p.[(Phe1046Serfs*3; (Arg1350Trp)]	r.(?)	5;3
BCNS92	1	M	15.8	18	c.3139_3142del	p.(Leu1047*)	r.(?)	5
BCNS93	1	M	13.8	18	c.3139del	p.(Leu1047fs*11)	r.(?)	5
BCNS94	1	F	23.1	18	c.3150del	p.(Trp1051fs*7)	r.(?)	5
BCNS95	1	M	33.2	19	c.3233_3239del	p.(Leu1078Profs*7)	r.(?)	5
BCNS96	1	U	9.3	19	c.3251_3272del	p.(Val1084Alafs*2)	r.(?)	5
BCNS97	1	M	44.7	20	c.3364_3365del	p.(Met1122Valfs*22)	r.(?)	5
BCNS98	1	M	10.9	20	c.3364_3365del	p.(Met1122Valfs*22)	r.(?)	5
BCNS99	1	F	54.4	20	c.3375del	p.(Val1126Serfs*13)	r.(?)	5
BCNS100	1	F	41.8	21	c.3497dup	p.(Asn1166Lysfs*18)	r.(?)	5
BCNS101	1	U	6.7	21	c.3525_3526del	p.(Leu1175Phefs*8)	r.(?)	5
Large deletions or duplications								
BCNS102	1	F	48.5	_1_24_	c.(?-188)_(*3411_?)del	p.0?	r.0?	5
BCNS103	1	M	47.2	_1_24_	c.(?-188)_(*3411_?)del	p.0?	r.0?	5
BCNS104	1	F	14.2	_1_24_	c.(?-188)_(*3411_?)del	p.0?	r.0?	5
BCNS105	1	M	36.6	_1_2i	c.(?-188)_ (394+1_395-1) del	p.?	r.(?)	5
BCNS106	1	F	38.0	2i_12i	c.(394+1_395-1)_ (1728+1_1729-1)(2)	p.?	r.(?)	4
BCNS107	1, de novo	F	14.9	2i_16i	c.(394+1_395-1)_ (2703+1_2704-1)(2)	p.?	r.(?)	4
BCNS108	1	F	37.1	2i_24_	c.(394+1_395-1)_ (*3411_?)del	p.?	r.(?)	5
BCNS109	1	F	19.9	14i_16i	c.(2250+1_2251-1)_ (2703+1_2704-1)del	p.?	r.(?)	5
BCNS110	4	M	35.0	16i_19i	c.(2703+1_2704-1)_ (3306+1_3307-1)del	p.?	r.(?)	5
Probably nonpathogenic								
BCNS111	1	F	27.3	7i	c.1067+5G>C	p.?	r.spl?	2
BCNS112	1	F	51.2	13	c.1792A>T	p.(Met598Leu)	r.(?)	2

(Continues)

TABLE 2 (Continued)

Case identifier	Members affected	Gender	Age of diagnosis	Exon/ Intron	DNA variant	Protein change	RNA change	Classification
BCNS113	1	M	70.3	14	c.2173C>T	p.(Pro725Ser)	r.(?)	2
BCNS114	1	F	33.7	18	c.3155C>T	p.(Thr1052Met)	r.(?)	2
BCNS115	1	M	14.0	18	c.3155C>T	p.(Thr1052Met)	r.(?)	2
BCNS116	2	F	43.3	18	c.3155C>T	p.(Thr1052Met)	r.(?)	2
BCNS117	2	M	29.9	21	c.3487G>A	p.(Gly1163Ser)	r.(?)	2

NCBI Reference transcript *PTCH1*: NM_000264.3. All variants were detected in a heterozygous state (if two variants were present, they are presumed to be on the same allele, in trans). Variant nomenclature is according to HGVS guidelines. Classification is according to ACMG, ACGS, and VKGL criteria; class 5: pathogenic, class 4: likely pathogenic, class 3: uncertain significance, class 2: likely benign, class 1: benign.

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Chapter 3

A genotype-phenotype study in basal cell naevus syndrome: a Dutch multicentre retrospective cohort study and review of the literature

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Abstract

Background Basal cell naevus syndrome is an autosomal dominant disorder most commonly caused by a germline mutation in the *patched-1* (*PTCH1*) gene. It is characterized by multiple basal cell carcinomas, odontogenic keratocysts, palmoplantar pits and cerebral calcifications.

Objectives To assess whether there is a genotype-phenotype correlation in a Dutch population of patients with BCNS and to review the literature on this topic.

Methods All patients who had *PTCH1* mutation analysis for diagnostic purposes in the Netherlands were recorded. Detailed clinical information from patients with a *PTCH1* mutation was collected from their medical files. Subsequently, patients with germline mutations were assessed for BCNS diagnostic criteria described by Kimonis et al. and the revised criteria by Bree et al. A heat-map was created to enable visualization of a possible genotype-phenotype correlation.

Results In 141 of 673 (21.0%) patients a pathogenic *PTCH1* mutation was detected. Detailed clinical information could be obtained from 83 patients of whom, 95.2% and 96.4% fulfilled the old and revised diagnostic criteria for the diagnosis of BCNS, respectively. A heat map showed no correlation between the location or type of *PTCH1* mutation and the presence of clinical symptoms.

Conclusion By using heat maps to demonstrate a genotype-phenotype correlation in our population of patients with BCNS, the earlier reported absence of a clear association was corroborated.

Introduction

Basal cell naevus syndrome (BCNS), also known as Gorlin syndrome, is a rare autosomal dominant disorder (MIM#109400). It was first described by Gorlin and Goltz in 1960.¹ The estimated prevalence of BCNS reported in the literature is 1 in 56,000-256,000.² Characteristic symptoms include basal cell carcinomas (BCCs), odontogenic keratocysts (OKCs), calcification of the falx cerebri, palmoplantar pits and skeletal malformations.² The underlying cause is a heterozygous germline mutation in the *patched-1* (*PTCH1*) gene, located on chromosome 9q22-31.^{3,4}

The different symptoms that can be present in BCNS are explained by the dual function of the PTCH1 protein. By activating the hedgehog (Hh)-pathway, the PTCH1 protein has an important function in embryonic development. It regulates fate and patterning in the neural tube development and plays an important role in osteoblast differentiation.⁵ The various congenital malformations such as rib anomalies, macrocephaly and skeletal malformations, can be explained by this function and require only monoallelic inactivation of the *PTCH1* gene to become manifest.⁶ The other important function of *PTCH1* is its role as tumour suppressor in the Hh-signalling pathway. The pro-oncogenic Hh-pathway is involved in cell proliferation and differentiation, and PTCH1 inhibits activation of the pathway by repressing the activity of smoothened. However, a bi-allelic inactivated *PTCH1* gene leads to development of tumours according to the two-hit hypothesis of Knudson.⁷ Tumours, cysts, hamartomas and other described proliferations will only develop if a second hit in the normal remaining allele occurs (e.g. loss of heterozygosity). In BCNS this second hit occurs usually later in life resulting in BCCs, OKCs, medulloblastomas, ovarian fibromas and other less frequently occurring tumours. Mutations in other components of the Hh-pathway such as *SUFU* and *PTCH2* have also been found to be responsible for the development of BCNS.⁸⁻¹¹

Diagnostic criteria for BCNS were first proposed in 1993 by Evans et al. and modified by Kimonis et al. in 1997 (Table 1).^{12,13} BCNS mainly is a clinical diagnosis based on the presence of two major, or one major and two minor criteria. Additional mutation analysis can be used to confirm the diagnosis on a molecular level. The mutation detection rate of *PTCH1* reported in the literature lies between 40 and 85%.^{9,14-17} Bree et al. revised the diagnostic criteria in 2011 and integrated genetic test results, allowing to make this diagnosis with only one major criterion and the presence of a *PTCH1* mutation (Table 2). Furthermore, medulloblastoma was designated as a major criterion instead of a minor, because of the relevance in early diagnosis. Although the overall incidence of medulloblastoma in BCNS is only 1-2%, studies showed that 5-20% of all children that developed a medulloblastoma under the age of three had BCNS.^{18,19} Labelling medulloblastoma as an indicator for BCNS may therefore lead to earlier recognition of this syndrome. Further modifications of the diagnostic criteria, such as the switch of rib anomalies from a major to minor criterion and the addition of cardiac fibroma, lymphomesenteric cysts and ocular abnormalities to the minor criteria are shown in table 1 and 2.

Previous studies have tried to demonstrate a genotype-phenotype correlation in BCNS.^{14,20-22} To date, no clear correlation was detected between clinical features of BCNS patients, the number of major symptoms, age of onset of BCCs and OKCs and the nucleotide position or type of *PTCH1* mutation. In this study, we evaluated 83 Dutch patients with a confirmed *PTCH1* mutation. We searched for possible genotype-phenotype correlations in this cohort and reviewed the literature on this topic.

Table 1. Diagnostic criteria for BCNS by Kimonis et al.¹³ Presence of two major, or one major and two minor criteria are required for diagnosis.

Major criteria	Minor criteria
>2 BCCs or one BCC in a person younger than 20 years	Macrocephaly
Odontogenic keratocysts	Congenital malformation (cleft lip/palate, frontal bossing, coarse face, hypertelorism)
≥3 palmar or plantar pits	Bridging of the sella turcica, vertebral anomalies, modeling defects of the hands and feet, or flame-shaped lucencies of the hands or feet
Bilamellar calcification of the falx cerebri	Other skeletal abnormalities (i.e. Sprengel deformity, pectus deformity, marked syndactyly of the digits)
Bifid, fused or splayed ribs	Ovarian fibroma
First-degree relative with BCNS	Medulloblastoma

Table 2. Revised diagnostic criteria for BCNS by Bree et al.²³ Two major criteria, one major criterion and two minor criteria, or one major and genetic confirmation are required for diagnosis.

Major criteria	Minor criteria
Multiple BCCs or one BCC in a person younger than 20 years	Bifid, fused or splayed ribs
Odontogenic keratocysts	Other specific skeletal and radiologic abnormalities (i.e. pectus excavatum, scoliosis, hemivertebrae, Sprengel's deformity, syndactyly of digits, bony bridging of the sella turcica, flame-shaped lucencies of phalanges)
Palmar or plantar pits	Macrocephaly
Lamellar calcification of the falx cerebri	Cleft lip or palate
Medulloblastoma in early childhood	Ovarian or cardiac fibroma
First-degree relative with BCNS	Lymphomesenteric cysts
	Ocular anomalies (i.e. congenital cataract, coloboma, glaucoma, hypertelorism)

Materials and methods

A multicentre retrospective cohort study was conducted at the Maastricht University Medical Centre + (MUMC+), and the VU University Medical Centre (VUMC) the Netherlands. All blood samples sent to the two diagnostic DNA laboratories for *PTCH1* standard mutation analysis by Sanger sequencing and multiplex ligation-dependent probe amplification in the period 1999-2015 were listed. During this period, the two laboratories processed requests from all hospitals in the Netherlands for *PTCH1* mutation analysis, in addition to patients from various hospitals in Belgium, Norway, Finland, Portugal and Sweden.²⁴ For all Dutch patients with a *PTCH1* mutation, classified as pathogenic or probably pathogenic, detailed clinical information was retrieved from their medical records. Foreign patients were excluded because of practical difficulties.

A heat map was created in Microsoft Excel using conditional formatting to investigate a genotype-phenotype correlation. A heat map is a two-dimensional representation of data that provides an immediate visualization of information. It is based on colour encoding and on meaningful reordering of the rows and columns. Using suitable colour scales, clustering can help to see structure and detect patterns.²⁵ A red colour is given if the symptom is present, a green colour if the symptom is absent and a black colour if it is unknown. In this study, we used two different heat maps. In the first heat map the mutations are sorted according to their location within the gene, plotted against the clinical symptoms (BCCs, odontogenic keratocysts, palmoplantar pits, calcification of the falx cerebri, skeletal abnormalities and macrocephaly). In the second heat map, the mutations are sorted according to the type of mutation (frameshift or missense) against the clinical symptoms, since loss-of-function mutations are known to have more destructive effect on protein function (especially for a tumour suppressor), while missense mutations may have more subtle effects.¹⁴ Additionally, the different domains in the *PTCH1* gene (N-terminal region, 2 extracellular loops, intracellular loop, sterol-sensing domain and C-terminal region) in which the mutation is located were included in the heat maps. Because symptoms requiring a second hit (BCCs and OKCs) become evident later in life and may not be present in younger patients, age could have been a potential bias. Separate analyses were performed successively excluding patients under 12 and 18 year.

Results

From 1999 until 2015 *PTCH1* variant analysis was performed in 673 patients. In 141 (21.0%) of these individuals a *PTCH1* variant was detected and 117 different *PTCH1* variations were found. Of those, 110 were classified as pathogenic or probably pathogenic.^{24,26} After excluding foreign applications and non-pathogenic mutations, a pathogenic *PTCH1* mutation was detected in 95 patients from the Netherlands (average population of 16,330,500). Clinical data could not be obtained for 12 patients, so 83 patients from 77 families were included for further analysis: 37 males and 46 females (ratio 1:1.2). The age of diagnosis ranged from 0-69 years, with a mean age of 25.2 years. Reasons for genetic testing written on the application forms were: 1) clinical suspicion of BCNS (37.3%); 2) clinical diagnosis of BCNS (37.3%); 3) first degree family member with BCNS (20.4%); and 4) family members with symptoms of BCNS (3.6%). In total four individuals were tested within the context of prenatal screening, because of a first-degree family member with BCNS or a clinical suspicion based on a second trimester ultrasound scan.

The type of mutations detected in this study were nonsense in 32.5% (27/83), frameshift in 32.5% (27/83), missense in 12.0% (10/83), splicing in 13.3% (11/83) or a whole *PTCH1* gene deletion in 3.6% (3/83). The remaining 6.0% (5/83) of mutations were in-frame duplications and deletions.

The mean age of diagnosis of the first BCC was 23.3 years. Compared to nonsense and frameshift mutations, the mean age at diagnosis of the first BCC is slightly higher in case of missense mutations (26.3 years vs 23.2 years). The clinical symptoms found in our cohort of BCNS patients are shown in table 3.

Comparing the diagnostic criteria

Of all 83 included individuals, 80 (96.4%) fulfilled the revised criteria by Bree et al (table 2). The remaining three patients did not meet the diagnostic criteria, but actually did have a *PTCH1* mutation. One four-year-old patient with a nonsense mutation fulfilled four minor criteria (rib anomalies, macrocephaly, cardiac fibroma and ocular abnormalities), a three-year-old patient with an inframe deletion had one minor criterion (Sprengel deformity) and one 39-year-old patient with a missense mutation had two minor criteria (polydactyly and ocular anomalies). Based on the clinical criteria of Kimonis et al (table 2) the diagnosis of BCNS would have been made in 79 (95.2%) out of 83 patients. Of the remaining four patients, the three-years-old patient and 39-years-old patient mentioned above did not meet the Kimonis criteria. In the other two patients *PTCH1* mutation analysis was performed prenatally because of family members with BCNS.

Genotype-phenotype correlation

Of all recorded clinical symptoms, only BCCs, OKCs, palmoplantar pits, calcification of the falx cerebri, skeletal abnormalities and macrocephaly were used for analysis. Other clinical symptoms, such as rib anomalies and ocular anomalies were excluded, because (specific) data were missing for many cases. To create robust heat maps, patients with less than two known symptoms were excluded. In total 78 patients were analysed.

In the first heat map, that compares the localization of the mutation with the presence of clinical symptoms (Figure 1), we could not visualize specific clustering of symptoms in an exon or part of the *PTCH1* gene. Furthermore, no phenotype similarities could be found within families. In the second heat map, based on the type of mutation (Figure 2), including only missense (10) and truncating (49) (frameshift and nonsense) mutations, we could not identify an association between the type of mutation and clinical symptoms. Excluding patients younger than the age of 12 and 18 did not change our findings. Of note, the missense mutations were predominantly located in the transmembrane domains and specifically in the sterol-sensing domain (SSD).

Table 3. The frequency of clinical symptoms in our Dutch cohort of BCNS patients.

Major criteria	Number of affected patients (n/total number*) (%)	
1. Multiple BCCs or BCC prior to 20 years of age	53/71	74.6
2. OKCs of the jaws prior to 20 years of age	61/74	82.4
3. Palmar and plantar pitting	42/59	71.2
4. Lamellar calcification of the falx cerebri	21/40	52.5
5. Medulloblastoma	1/83	1.2
6. First degree relative with BCNS	37/64	57.8
Minor criteria		
1. Rib anomalies (bifid, fused, splayed)	25/41	61.0
2. Other specific skeletal malformations and radiologic changes (i.e., vertebral anomalies, scoliosis, pectus deformity, Sprengel deformity, short fourth metacarpals, postaxial polydactyly)	43/54	79.6
3. Macrocephaly	44/51	86.3
4. Cleft/lip palate	5/29	17.2
5. Ovarian/cardiac fibroma	13/54	24.1
Ovarian fibroma/cysts	10/54	18.5
Cardiac fibroma	3/54	5.6
6. Lymphomesenteric cysts	1/83	1.2
7. Ocular abnormalities (i.e., hypertelorism, strabismus, congenital cataracts, glaucoma, coloboma)	32/41	78.0
Other malignancies (squamous cell carcinoma, adenocarcinoma abdominal cavity, brain tumour, melanoma, meningioma, iris tumour)	6/83	7.2

* Note that the denominator is not constant due to missing data

Figure 1. Heatmap. Genotype versus clinical symptoms (Basal cell carcinomas (BCCs), odontogenic keratocysts (OKCs), palmoplantar pits (PP), calcification of the falx cerebri (CFC), skeletal abnormalities (SA) and macrocephaly (MC)) in 78 individuals. Clinical symptoms are shown in red (present), green (absent) and black (unknown). Domains are noted as N-terminal region, ECL: Extracellular loop, ICL: Intracellular loop, SSD: Sterol-sensing domain and TRANS: Transmembrane domain.

Exon	Domain	Change in DNA	Change in protein	Type mutation	BCCs	OKCs	PPP	CFC	SA	MC
2	N-terminal	c.205A>T	p.(Lys69*)	nonsense						
2	N-terminal	c.254_255del	p.(Arg85Thrfs*4)	frameshift						
2	N-terminal	c.279C>G	p.(Tyr93*)	nonsense						
2	N-terminal	c.294C>A	p.(Cys98*)	nonsense						
2	ECL	c.385_386dup	p.(Trp129Cysfs*9)	frameshift						
3	ECL	c.403C>T	p.(Arg135*)	nonsense						
3	ECL	c.403C>T	p.(Arg135*)	nonsense						
3	ECL	c.403C>T	p.(Arg135*)	nonsense						
3	ECL	c.466C>T	p.(Gln156*)	nonsense						
3	ECL	c.466C>T	p.(Gln156*)	nonsense						
3	ECL	c.479_482del	p.(Gln160Profs*10)	frameshift						
5	ECL	c.707G>A	p.(Trp236*)	nonsense						
6	ECL	c.770_771delinsGGTTTGG	p.(Thr257Argfs*14)	frameshift						
6	ECL	c.842del	p.(Met281fs*2)	frameshift						
7	ECL	c.1040_1049del	p.(Val347Alafs*17)	frameshift						
8	ECL	c.1081C>T	p.(Gln361*)	nonsense						
8	ECL	c.1093C>T	p.(Gln365*)	nonsense						
8 and 14	ECL	c.[1112del;2183C>T]	p.[(Met372Cysfs*60);p.(Thr728Met)]	frameshift						
9	ECL	c.1279del	p.(Leu427Trpfs*5)	frameshift						
12	SSD	c.1691T>G	p.(Leu564*)	nonsense						
12	SSD	c.1691T>G	p.(Leu564*)	nonsense						
13	ICL	c.1804C>T	p.(Arg602*)	nonsense						
13	ICL	c.1810G>T	p.(Glu604*)	nonsense						
14	ICL	c.1852del	p.(Cys618fs)	frameshift						
14	ICL	c.2098C>T	p.(Gln700*)	nonsense						
14	ICL	c.2170G>T	p.(Glu724*)	nonsense						
14	ICL	c.2178_2179insA	p.(Cys727Metfs*11)	frameshift						
14	ICL	c.2179del	p.(Cys727fs)	frameshift						
15	ECL	c.2308C>T	p.(Arg770*)	nonsense						
15	ECL	c.2359G>T	p.(Glu787*)	nonsense						
15	ECL	c.2446C>T	p.(Gln816*)	nonsense						
15	ECL	c.2557C>T	p.(Gln853*)	nonsense						
17-19	ECL	c.(2703+1_2704-1).(3306+1_3307-1)del	p.?	?						
17-19	ECL	c.(2703+1_2704-1).(3306+1_3307-1)del	p.?	?						
17-19	ECL	c.(2703+1_2704-1).(3306+1_3307-1)del	p.?	?						
17-19	ECL	c.(2703+1_2704-1).(3306+1_3307-1)del	p.?	?						
16	ECL	c.2619C>A	p.(Tyr873*)	nonsense						
16	ECL	c.2619C>A	p.(Tyr873*)	nonsense						
17	ECL	c.2748del	p.(Ser917Alafs*7)	frameshift						
17	ECL	c.2824del	p.(Arg942Glyfs*20)	frameshift						
18	ECL	c.3027C>A	p.(Tyr1009*)	nonsense						
18	ECL	c.3056_3059del	p.(Glu1019Glyfs*29)	frameshift						
18	ECL	c.3058C>T	p.(Gln1020*)	nonsense						
18	TRANS	c.3107_3108	p.(Leu1036Cysfs*108)	frameshift						
18	TRANS	c.3107_3108	p.(Leu1036Cysfs*108)	frameshift						
18 and 23	TRANS	c.[3135del;4048C>T]	p.[(Phe1046Serfs*3);(Arg1350Trp)]	frameshift						
18 and 23	TRANS	c.[3135del;4048C>T]	p.[(Phe1046Serfs*3);(Arg1350Trp)]	frameshift						
18	TRANS	c.3150del	p.(Trp1051fs*7)	frameshift						
20	TRANS	c.3364_3365del	p.(Met1122Valfs*22)	frameshift						
4	ECL	c.591G>C	p.(Trp197Cys)	missense						
10	SSD	c.1450G>A	p.(Glu484Arg)	missense						
11	SSD	c.1526G>A	p.(Gly509Asp)	missense						
11	SSD	c.1526G>A	p.(Gly509Asp)	missense						
11	SSD	c.1526G>A	p.(Gly509Asp)	missense						
11	SSD	c.1526G>A	p.(Gly509Asp)	missense						
11	SSD	c.1555G>C	p.(Ala519Pro)	missense						
14	TRANS	c.2250G>C	p.(Lys750Asn)	missense						
15	ECL	c.2414T>G	p.(Ile805Arg)	missense						
15	ECL	c.2447A>G	p.(Gln816Arg)	missense						

Figure 2. Heat map. Type of mutation (truncated and missense) versus clinical symptoms (Basal cell carcinomas (BCCs), odontogenic keratocysts (OKCs), palmoplantar pits (PP), calcification of the falx cerebri (CFC), skeletal abnormalities (SA) and macrocephaly (MC)) in 59 individuals. Clinical symptoms are shown in red (present), green (absent) and black (unknown). Domains are noted as N-terminal region, ECL: Extracellular loop, ICL: Intracellular loop, SSD: Sterol-sensing domain and TRANS: Transmembrane domain.

Discussion

The goal of this study was to assess whether there is a genotype-phenotype correlation in BCNS. Detection of a genotype-phenotype correlation in BCNS patients would enable to predict phenotype severity and offer proper surveillance programs.

This study is one of the largest studies conducted to detect a genotype-phenotype correlation in BCNS patients. In our Dutch cohort, we did not find a correlation based either on the location or on the type of *PTCH1* mutation. Even within families or individuals with a shared *PTCH1* mutation, there was a high phenotypic variability. These findings are consistent with previous studies (Table 4).^{14,20-22}

A recently published study by Evans et al has shown a number of key clinical features that may be predictive for the presence and type of a pathogenic germline *PTCH1* variant.²⁷ In this study, patients with missense variants in *PTCH1* were diagnosed later and were less likely to develop at least ten BCCs, multiple OKCs, and bifid ribs than those with other *PTCH1* variants. Our study shows comparable results as patients with a missense variant in *PTCH1* were diagnosed later (31.7 years) compared to patients with frameshift or nonsense mutations (24.6 years), were less likely to develop rib anomalies (10% vs. 32% respectively) and the mean age at diagnosis of the first BCC is slightly higher (26.3 years vs 23.2 years respectively).

Notably, the found missense mutations in our study are predominantly located in the sterol-sensing domain (SSD) (Figure 2). This is in line with the findings of Lindström et al. who analyzed the distribution pattern of germline *PTCH1* mutations in BCNS patients and also demonstrated that missense mutations were predominantly found in the transmembrane domains of the SSD.²⁸ Generally, SSDs are functional important regions and thought to function as a regulatory domain linking vesicle trafficking and protein localization in processes such as cholesterol homeostasis and cell signalling.²⁹⁻³¹ Probably, missense mutations only have important consequences for the function of the *PTCH1* protein, when located in the SSD. A recent study showed that the presence of common genetic variants, that are associated with an increased risk of developing sporadic BCC, like MC1R and TERT-CLPTM1L, are also associated with an earlier age of onset of BCCs in patients with BCNS.³²

Table 4. Clinical studies on genotype-phenotype correlations in BCNS patients.

Study	Population	Method	Statistical analysis	Conclusion
Wicking et al, 1997 (14)	N=28	<i>PTCH1</i> mutation type vs age at onset of BCCs, age at onset of OKCs and the number of major features	Linear regression analysis	No correlation
Jones et al, 2011(20)	N=158	Presence or absence of <i>PTCH1</i> mutation and <i>PTCH1</i> mutation type vs age of onset of BCCs and total numbers of BCCs	Kaplan-meier curves	No correlation
Tanioka et al, 2004 (21)	N=8	<i>PTCH1</i> mutation type vs clinical features	-	No correlation
Torrelo et al, 2014 (22)	N=2	Comparing <i>PTCH1</i> mutation in two young patients with acral BCC	-	No correlation
Evans et al, 2017 (28)	N=182	<i>PTCH1</i> and <i>SUFU</i> mutation type vs clinical features	Chi-squared test	<i>PTCH1</i> missense mutations result in later diagnosis and less BCCs and OKCs (<10). <i>SUFU</i> mutations result in more medulloblastomas, meningiomas and ovarian fibromas and less OKCs

BCNS: Basal cell naevus syndrome, BCC: Basal cell carcinomas, OKC: Odontogenic keratocysts.

Due to the retrospective nature of this study, specific clinical information was sometimes missing. Symptoms were obtained from clinical files that have been listed according to the old diagnostic criteria. Symptoms that were not part of the old criteria, but are added in the new criteria such as cardiac fibroma and lymphomesenteric cysts, are probably underdocumented. In our study the mutation detection rate is low (21%). A possible explanation for this is that genetic testing is relatively easily accesible in The Netherlands. The threshold to request genetic testing to exclude BCNS cases is probably low. On top of that standard genetic tests do not detect genomic rearrangements or deep intronic variants causing cryptic splicing in *PTCH1*.³³ And mutations could be located in other components of the Hh pathway. Moreover, mutation loads under 5%, that can be present in low-grade mosaicism, are impossible to detect with Sanger sequencing. With the currently available techniques the estimated mutation prevalence would probably be higher.

Whole Exome Sequencing (WES), that is more and more used to diagnose heterogeneous disease phenotypes, enables the sequencing of all genes. The method is however still costly and has a limited sequencing depth. In case of a high clinical suspicion of BCNS it is more efficient to look specifically at components of the Hh pathway. The use of a next-generation sequencing (NGS) based gene panel, specifically developed for the analysis of genes involved in the Hh pathway is not only more sensitive, but also less expensive. In case of suspected mosaicism, NGS of target genes (i.e. *PTCH1*) has sufficient sequence read depth to reliably detect and quantify the degree of mosaicism. Notably, BCNS is not exclusively caused by mutations in the *PTCH1* gene, other genes of the Hh signalling pathway, such as *SUFU* and *PTCH2*, should be part of the NGS gene panel.⁸⁻¹¹

Conclusion

In a large cohort of Dutch BCNS patients, a high genotypic and phenotypic variability was found with no evidence for a genotype-phenotype correlation.

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Chapter 4

Postzygotic mosaicism in basal cell naevus syndrome

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Abstract

Basal cell naevus syndrome (BCNS) is an autosomal dominant disorder most commonly caused by a germline mutation in the *Drosophila* homolog of *patched-1* (*PTCH1*) gene. Here we describe a patient with clinical signs of BCNS, caused by postzygotic mosaicism of a *PTCH1* gene mutation. We performed Restriction Fragment Length Polymorphism analysis and Droplet Digital Polymerase Chain Reaction to determine the degree of mosaicism in different tissues of this patient. Our case shows that a relatively low-grade mosaicism can lead to clinical signs reminiscent to those caused by a germline mutation. This finding has important implications for genetic counselling and therefore is pivotal to recognize for dermatologists as well as for clinical geneticists and clinical laboratory geneticists.

Introduction

Basal cell naevus syndrome (BCNS, MIM #109400), also known as Gorlin syndrome, is an autosomal dominant disorder characterized by multiple basal cell carcinomas (BCCs), maxillary keratocysts and cerebral calcifications.¹ The incidence of BCNS is estimated at 1 in 50,000 to 256,000.² Diagnostic criteria for BCNS were first established by Evans et al., modified by Kimonis et al. and revised by Bree and Shah in 2011.³⁻⁵ Diagnosis is based on two major criteria, one major criterion and two minor criteria or one major criterion and genetic confirmation (Table 1, Chapter 1). The cause of BCNS generally is a germline mutation in the *Drosophila* homolog of *patched-1* (*PTCH1*) gene that encodes the PTCH1 protein.^{6,7} Postzygotic mosaicism has been described in BCNS.^{8,9} In postzygotic mosaicism a mutation occurs somewhere during embryogenesis, only affecting cells from the mutant progenitor, resulting in mixed healthy and affected cell populations. Depending on the mutation load and the tissues involved in the mosaicism, an individual with postzygotic mosaicism may develop clinical signs to a certain degree or none at all.¹⁰ Type 1 and type 2 mosaicism as a cause of segmental distribution of clinical symptoms in BCNS have been described.^{9,11} Germline cells can be affected by genetic mosaicism as well, referred to as gonadal mosaicism. In theory, when the mutation load in gonadal cells is lower than the regular 50% in classic germline BCNS patients, the recurrence risk of BCNS in the patient's offspring probably is lower than 50%.

Case

A 22-year-old woman was referred to our outpatient clinic with a BCC in the medial canthus of the right eye (Figure 1A). During total body skin examination, we detected two additional BCCs below her right eye and palmar pits on both hands (Figure 1B). An orthopantomography was negative for odontogenic keratocysts and a MRI of the head showed no cerebral calcifications. Based on the presence of two major clinical criteria, BCNS was diagnosed.

Figure 1A. Patient with a BCC in the medial canthus of the right eye (arrowhead) and two additional small BCCs (arrows) and **B.** Multiple palmar pits (arrows).

Sanger sequencing analysis of the protein-coding exons and flanking introns of the *PTCH1* gene (primer sequences on request) on DNA extracted from blood (Wizard Genomic DNA purification Kit, Promega) detected a barely observable mutation in exon 13, c.1810G>T (NCBI RefSeq: NM_000264.3). Although Sanger sequencing is not a quantitative method, the mutation seemed to have a remarkably lower level than one would expect in case of a germline mutation (Figure 2A). Based on this finding we hypothesized that a postzygotic mutation in the *PTCH1* gene, resulting in genetic mosaicism, was responsible for the clinical presentation of our patient. The mutation results in the alteration of a glutamic acid resulting in a stop at position 604 (p.(Glu604*)) of the PTCH1 protein. This premature termination codon likely results in a non-functional shorter protein. This mutation has not been detected in databases (dbSNP, ExAC) nor described in literature before, although a similar mutation (p.(Arg602*)) has been described in a family with BCNS.¹²

In postzygotic mosaicism, the percentage of affected cells can vary between different tissues and may be segmentally distributed. To determine the degree of mosaicism in other tissues, we isolated DNA from urine derived urothelial cells and buccal mucosa cells of the patient (Wizard Genomic DNA purification Kit, Promega). On these samples we performed Restriction Fragment Length Polymorphism (RFLP) analysis with restriction enzyme *Bfal* (digesting the mutant allele, figure 2B). In addition, we performed Droplet Digital PCR (QX200 ddPCR, Bio-Rad Laboratories, Hercules, CA, USA) on DNA of the same tissues and BCC tumour cells of this patient (DNA isolation with Maxwell 16 FFPE plus LEV DNA Purification Kit, Maxwell 16 MDX, Promega) to determine the degree of mosaicism (Figure 2C). ddPCR technology makes use of standard TaqMan probe-based assays (Custom Taqman Assays, Catalog #: 4331349, ID nr: AHN1XWW, Life Technologies Europe). The results are summarized in Table 2. Our patient has a low-grade mosaicism of approximately 16%, based on the average of the different degrees of mosaicism determined by ddPCR.

Table 2. Tissues analysed for degree of genetic mosaicism. Percentages of DNA mutation load with *Bfal*-RFLP and Droplet Digital PCR. Assessment of the tumour sample with RFLP was not feasible because the PCR amplicon size (556 bp) was too large for analysis on fragmented formalin-fixed, paraffin-embedded tissue DNA.

Sample	<i>Bfal</i> RFLP	Droplet Digital PCR
Blood	6.4 %	13.5 %
Urine derived endothelial cells	14.7%	17.3 %
Buccal mucosa	8.7 %	17.0 %
Tumour	-	36.1 %

Figure 2. Analyses of genetic mosaicism in *PTCH1* using different techniques **A.** The *PTCH1* sequence trace across the mutation. Top panel: control trace, lower panel: patient trace with c.1810G>T mutation (arrow) from leucocyte isolated DNA. **B.** RFLP analysis with Bfal of wild type DNA (top) and patient DNA isolated from blood (bottom). Fragment size is depicted horizontally in base pairs (bp), signal intensity vertically (Genemarker software V2.4.0, SoftGenetics LLC). The blue peaks represent the FAM-labelled PCR products; orange peaks fragment size standards (600 LIZ Size Standard). Percentage mosaicism was 6.4% calculated by mutant peak (348 bp) surface area divided by total peak surface area (348+556 bp). **C.** Representative dot plot picture of results from ddPCR on DNA from the patient's blood. Fluorescence intensity for 6-FAM TaqMan probe is displayed vertically (channel 1) and VIC TaqMan probe horizontally (channel 2). Left upper quadrant (Ch1+Ch2) contains positive droplets (blue) for the mutant DNA fragments, right lower quadrant (Ch1 Ch2+) has positive droplets for wild type DNA fragments (green), right upper quadrant (Ch1+Ch2+) contains positive droplets for one or more wild type as well as mutant DNA fragments (orange) and the left lower quadrant (Ch1 Ch2) has empty negative droplets (black). Percentage mosaicism was 13.5% calculated from positive mutant (blue, proportion orange) droplets divided by the total number of DNA containing (blue, orange, green) droplets using a Poisson distribution (QuantaSoft software, Bio-Rad Laboratories).

Discussion

Our findings confirm that a low-grade postzygotic mosaicism of a *PTCH1* gene mutation can cause a clinical presentation fulfilling the diagnostic criteria for BCNS. Phenotypic variability between BCNS patients is common, even within families.¹³ Such variability exists between patients with genetic mosaicism as well. Therefore the phenotype may be difficult to predict, even when the degree of mosaicism is known. Segmental arrangement of symptoms has been described in mosaic BCNS. In this patient we did not observe this phenomenon, she also developed a BCC on the left cheek during dermatological follow-up. However, a segmental distribution could become apparent later in life.

Previously it has been suggested that in patients with segmental Darier disease the percentage of affected gonadal cells may be comparable to that of other tissues.¹⁴ A lower level of mosaicism can very well translate into a lower recurrence risk of BCNS for future offspring. Mutation analysis in gonadal cells (oocytes and spermatocytes) is extremely challenging. In our patient we could estimate the degree of gonadal mosaicism somewhere between 13 and 17%, based on the percentages found with ddPCR in the different tissues. If the degree of gonadal cells with the *PTCH1* gene mutation is below 50%, transmission to the offspring probably will be lower than 50%, maybe even as low as 13%. Providing a more precise recurrence risk will be beneficial for genetic counselling. Only then, patients can make an adequate informed decision about prenatal diagnosis or preimplantation genetic diagnosis.

Different techniques can be used to determine the degree of mosaicism. With standard Sanger sequencing it may be difficult to detect low-grade mosaicism and impossible to quantify the degree of mosaicism. We used quantitative techniques, RFLP and ddPCR, leading to different results. RFLP analysis showed a lower degree of mosaicism compared to ddPCR. This difference may be due to less accurate calculation of the peak-surface-area in RFLP and lack of a complete digestion control in the *Bfal* RFLP. ddPCR does not have these limitations, since it makes use of massive partitioning of the sample. This way, thousands of individual data points are produced, rather than a single result, generating a powerful tool for statistical analysis. We therefore think that ddPCR will provide a better measure of the actual degree of mosaicism than RFLP.

As a control we performed ddPCR on patient's BCC tumour tissue DNA. In theory, the tumour sample has to consist of cells containing the c.1810G>T *PTCH1* mutation along with an acquired second somatic mutation or loss of heterozygosity (LOH) to inactivate the tumour suppressor function of *PTCH1*. Mutation load in the tumour therefore is expected to be 50% or more, respectively. ddPCR however, detected the mutation only in 36% of tumour DNA. This is probably due to the presence of a certain percentage of normal tissue, since we did not microdissect the tumour from the tissue. Histologically the percentage tumour in the sample was estimated to be 60-70%. Based on these findings LOH is probably less likely than a second somatic mutation.

In conclusion, our results show that a low-grade postzygotic mosaicism of a *PTCH1* mutation can cause a clinical picture fulfilling the diagnostic criteria of BCNS. This is an important finding for dermatologists as well as for clinical geneticists and clinical laboratory geneticists. It underlines the importance to perform genetic tests in patients suspected of BCNS, even when they do not meet the clinical criteria, as it has implications for genetic counselling. Standard techniques are not always suitable to pick up low-grade postzygotic mosaicism, which makes additional quantitative techniques essential. When initial genetic tests in BCNS patients suggest an underlying genetic mosaicism, we recommend to determine the degree of mosaicism as accurately as possible, for example with ddPCR, for adequate genetic counselling.

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Chapter 5

Genetic profiling of basal cell carcinomas detects
postzygotic mosaicism in basal cell naevus syndrome

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Abstract

Basal cell naevus syndrome is associated with germline mutations in the *PTCH1* gene. Also, postzygotic mosaicism can cause BCNS. Here we describe two patients, one with multiple basal cell carcinomas and one with clinically BCNS, who had no *PTCH1* mutation in DNA extracted from blood. In both patients, we performed genetic analysis on different BCCs, revealing the presence of a shared *PTCH1* mutation in all tumours. Our findings show that in patients with symptoms of BCNS and initial absence of a *PTCH1* mutation in blood, genetic profiling of BCCs can detect postzygotic mosaicism.

Introduction

Basal cell naevus syndrome (BCNS, MIM #109400), also known as Gorlin syndrome, is an autosomal dominant disorder most commonly caused by a germline mutation in the *patched-1* (*PTCH1*) gene. Characteristic features of this syndrome include multiple basal cell carcinomas (BCCs), odontogenic keratocysts, palmoplantar pits and cerebral calcifications.¹ Diagnosis is based on two major criteria, one major criterion and two minor criteria or one major criterion and genetic confirmation using the revised criteria of Bree and Shah.² Several cases of unilateral or segmental BCNS have been reported in the past.³⁻⁵ Recently we described a patient with a low-grade postzygotic mosaicism in *PTCH1* as the cause of this syndrome.⁶ Another case of postzygotic mosaic BCNS caused by an activating mutation in *SMO*, has been published.⁷ It has been argued though, if this case represents a mosaic manifestation of Gorlin syndrome or actually could be diagnosed with Happle-Tinschert syndrome,⁸ as the same activating mutation was detected in the original case of this syndrome.⁹ In type 1 segmental mosaicism or postzygotic mosaicism a mutation occurs somewhere during embryogenesis, only affecting cells from the mutant progenitor, resulting in mixed healthy and affected cell populations. Depending on the mutation load and the tissues involved, an individual with postzygotic mosaicism could develop limited clinical signs or may be asymptomatic. Type 2 segmental mosaicism in BCNS has been described as well.¹⁰ This type of mosaicism refers to an autosomal dominant disorder which is accompanied by a postzygotic mutation that affects the healthy allele, resulting in pronounced cutaneous symptoms of the disease in a mosaic pattern. In this paper, we present two patients with symptoms of BCNS, who had no *PTCH1* mutation in blood with Sanger sequencing, where subsequent genetic profiling of their BCCs led to detection of postzygotic mosaicism.

Case 1

A 41-year old woman was referred to our outpatient clinic because of a history of multiple BCCs exclusively on the right side of her body (Figure 1a and b). No other symptoms of BCNS were observed. Hundreds of BCCs were treated with excision, Mohs' micrographic surgery and photodynamic therapy. The patient was carrier of a *BRCA2* gene mutation and her medical history mentioned left-sided breast cancer which was treated with mastectomy, radiotherapy and tamoxifen.

Figure 1. Photograph of female patient (case 1) showing right-sided distribution of multiple basal cell carcinomas and scars after excision on the face (A) and on the body (B).

Sanger sequencing of the coding exons of the *PTCH1* gene (BigDye v1.1 sequencing kit, 3730 DNA Analyzer, Applied Biosystems, primer sequences on request) on DNA extracted from blood (Wizard Genomic DNA purification Kit, Promega) detected no mutation. Because of the unilateral distribution of BCCs we suspected postzygotic mosaicism. Mutation analysis with single molecule Molecular Inversion Probes (smMIPs) and Next Generation Sequencing (NGS, Illumina NextSeq) targeting the coding exons of *PTCH1*, *PTCH2*, *SUFU* and *SMO* (NCBI RefSeqs resp. NM_000264.4, NM_003738.4, NM_016169.3, NM_005631.4) of four different BCCs on the right shoulder and upper arm revealed a shared exon 14 *PTCH1* mutation in all tumours, designated c.2197_2198del (Table 1). In addition, the split half of the tumours were used for

RNA analysis of *PTCH1*. In two of the BCCs a second somatic mutation was detected in *PTCH1* (c.3054G>A in BCC1 and c.1603-1G>A in BCC3, also detected in cDNA analysis, the latter affecting splicing, table 1). In addition, we analysed multiple samples of visibly normal skin on both ends of the fusiform excision specimen of a BCC, fibroblasts grown from one of these ends, subcutaneous healthy tissue underneath the tumour and a biopsy of normal skin on the left, unaffected side of the body (Figure 2). Mutation analysis on BCC surrounding skin samples showed variable DNA mutation loads. No mutation was found with smMIPs-NGS in the skin biopsy from the left side of the body or in blood. These findings identified type 1 segmental mosaicism of *PTCH1* as the cause of her multiple BCCs.

Figure 2. Segmental distribution of the *PTCH1* mutation in the skin, potentially in a Blaschkoid pattern. The imaginary black lines represent the affected skin without a clinical phenotype, but harbouring a heterozygous *PTCH1* mutation, following the lines of Blaschko. The location of a BCC is boxed and magnified on the right. Detected mutation loads in 1. Excision end 1: 1.0%, 2. Excision end 2: 9.0%, 3. Healthy subcutaneous tissue underneath the BCC: 0.6%, 4. Fibroblast culture grown from excision end 2: 0% and 5. Skin biopsy on the left unaffected side of the body (see also table 1).

Table 1. DNA mutation load in different BCCs and visibly normal skin samples.

Case 1						
	<i>PTCH1</i> mutation	smMIP mutation load (%)	Mutation on RNA ^a (cDNA)	2 nd <i>PTCH1</i> mutation	2 nd <i>PTCH1</i> smMIP mutation load (%)	2 nd <i>PTCH1</i> mutation on RNA ^a (cDNA)
blood	c.2197_2198del	0				
BCC1	c.2197_2198del	16.3	r.2197_2198del heterozygous	c.3054G>A, p.(Trp1018*)	14.7	r.3054G>A heterozygous
BCC2	c.2197_2198del	9.5	r.2197_2198del heterozygous			
BCC3	c.2197_2198del	14.4	r.2197_2198del heterozygous	c.1603-1G>A	11.7	r.1603del heterozygous ^b
BCC4	c.2197_2198del	3.4	r.2197_2198del heterozygous			
1) Excision end 1	c.2197_2198del	1.0				
2) Excision end 2	c.2197_2198del	9.0				
3) subcutis underneath BCC	c.2197_2198del	0.6				
4) Fibroblasts grown from end 2	c.2197_2198del	0				
5) biopsy left body side	c.2197_2198del	0				
Case 2						
blood	c.2460C>G	1.0				
BCC1	c.2460C>G	20.2	r.2460C>G hemizygous	LOH or NMD		
BCC2	c.2460C>G	15.7	r.2460C>G heterozygous	c.1603-1_1603delinsAA	11.2	r.1603_1607del heterozygous ^b

LOH: Loss of heterozygosity, NMD: Nonsense mediated RNA decay, ^a: zygosity calling is not quantitative but an estimation on the distribution of the Sanger sequencing peaks, ^bfor splicing effect see supplemental figure.

Case 2

A 36-year old woman was referred to our outpatient clinic with a very large basosquamous carcinoma on her forehead, which was treated with radiotherapy 9 years earlier. On physical examination, a thoracic scoliosis was noticed and several BCCs, diffusely distributed on the body, together with palmo-plantar pits were detected. Based on the clinical criteria we diagnosed her with BCNS.¹⁰ Two mandibular odontogenic cysts were found. A cranial CT scan showed a skin tumour on the forehead with extensive bone destruction and invasion into dura and cranial part of the superior sagittal sinus. Curative surgery could not be achieved and after DIEP (deep inferior epigastric perforator) flap reconstruction, she was treated with radiation therapy (60-70 Gy). The remaining BCCs were treated with Mohs' micrographic surgery, conventional excision and 5-fluorouracil crème.

Despite a clinical diagnosis of BCNS, Sanger sequencing and smMIP-NGS of the *PTCH1* gene on DNA extracted from blood identified no mutation. Mutation analysis on RNA (Qiagen RNA isolation kit, iScript cDNA Synthesis Kit, BioRad, primer sequences on request) from 2 different BCCs on the right shoulder and left upper arm showed a shared mutation in exon 15 of *PTCH1*, designated c.2460C>G (p.(Tyr820*)). This mutation was also found with smMIP-NGS on DNA isolated from the split half of the two BCCs. The results are indicative for type I postzygotic mosaicism. Re-interpretation of the smMIP-NGS data on blood by visual inspection detected the mutation in 1.0% of the sequence reads, a percentage close to the detection limit with the used coverage depth (< 1000x). One of the tumours was hemizygous for the mutation on cDNA analysis, due to either loss-of-heterozygosity (LOH, chromosomal deletion or allele specific methylation) of the other allele or to nonsense mediated decay (NMD) caused by an intragenic *PTCH1* mutation (exon-deletion/ -duplication or splicing). In the other tumour, a second splice site mutation was identified on DNA, whose functional effect was also detected with cDNA analysis (r. 1603_1607del, table 1).

Discussion

Postzygotic mosaicism as the cause of BCNS can be difficult to detect in DNA from blood with standard Sanger sequencing techniques, as this method's mutation detection limit is approximately 5%. NGS of a targeted gene panel is more sensitive and has sufficient sequence read depth to reliably detect and quantify the degree of mosaicism. In the present patients, a postzygotic mutation could not be demonstrated in blood with Sanger sequencing, but in patient 2 the smMIP-NGS technique did detect the *PTCH1* mutation in blood in a very low percentage.

Some forms of segmental mosaicism of dominant mutations can easily be recognized when the presence of the mutation leads to visible skin lesions, such as in mosaic ichthyosis.¹¹ Cutaneous mosaicisms follow different patterns. Lesions mostly follow the lines of Blaschko, in broad or narrow bands, or appear in a checkerboard pattern.¹² In patients with BCNS, skin with only one affected *PTCH1* allele has a normal appearance. Only a second hit of the other *PTCH1* allele (through loss-of-heterozygosity (LOH) or somatic gene mutation), initiates the development of a basal cell carcinoma. As the presence of a BCC is a marker for affected skin, genetic mosaicism of a larger cell population in the skin, can be demonstrated when different BCCs share the same *PTCH1* mutation. Our first patient clinically had a clear unilateral distribution of BCCs, already indicating genetic mosaicism. The results of the genetic analysis of the different tissue samples suggest a unilateral segmental distribution of the mutated skin following the lines of Blaschko (Figure 1 and 2). The mosaicism probably is restricted to the cells of the epidermis, as fibroblasts did not harbour the mutation and subcutaneous tissue underneath the tumour area only in a very low degree. The existence of a Blaschkoid pattern in mosaic BCNS is supported by two earlier reports of BCNS patients with a type 2 segmental *PTCH1* mosaicism and a type 1 segmental mosaicism of *SMO*, showing a comparable pattern.^{7,10} The second patient had no segmental distribution of symptoms, but the presence of an identical *PTCH1* mutation in two BCCs, a second somatic *PTCH1* mutation in one of the tumours and the low percentage of the mutation in blood strongly supports our conclusion that *PTCH1* mosaicism is the cause of the disease.

Type 1 segmental mosaicism as the cause of BCNS or a partial BCNS phenotype could be more common than previously assumed. It is plausible that these patients not always fulfil the diagnostic criteria of BCNS and even may only present with multiple BCCs at a young age. A segmental distribution of the BCCs is not always visible. Yet, it is important to be informed about the presence of mosaic BCNS as these patients may have other health problems and radiotherapy is contra-indicated for the treatment of their BCCs. Also patients with type 1 segmental genetic mosaicism have a slightly elevated risk of giving birth to a child with a heterozygous *PTCH1* mutation, probably depending on the presence and degree of gonadal mosaicism. In patients with symptoms of BCNS and absence of a mutation in blood, it is worthwhile to search for a shared mutation in the different BCCs with an NGS based gene panel, to demonstrate genetic mosaicism. This information can be important to ensure proper surveillance programs, to choose the right therapy and to provide adequate genetic counselling.

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Part two

Treatment of locally advanced and metastatic
basal cell carcinoma with vismodegib

Chapter 6

Vismodegib in metastasised
basal cell carcinoma

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Abstract

Background The majority of basal cell carcinomas is easy to cure with surgery or non-invasive treatments. However, large and long-standing tumours can be locally very destructive and in rare cases even metastasise. Hedgehog pathway inhibitors such as vismodegib constitute a new and promising therapy for metastatic or locally advanced basal cell carcinoma.

Case description We describe a patient with metastasised basal cell carcinoma and basal cell naevus syndrome who, in the context of a study, was successfully treated with vismodegib. The main undesirable effects in our patient were muscle cramps, loss of taste, nausea and hair loss.

Conclusion Basal cell carcinoma is potentially a locally destructive skin tumour that only very rarely metastasises. Hedgehog pathway inhibitors such as vismodegib can be administered in a selected group of patients with basal cell carcinoma.

Inleiding

Het basaalcelcarcinoom is de meest voorkomende vorm van huidkanker en bij de meeste patiënten eenvoudig curatief te behandelen. Chirurgische behandeling heeft de voorkeur, maar ook fotodynamische therapie, fluorouracil- of imiquimodcrème en radiotherapie worden toegepast. Hoewel het basaalcelcarcinoom langzaam groeit, kunnen grote, lang bestaande tumoren lokaal zeer destructief zijn en in zeldzame gevallen zelfs metastaseren. Een nieuwe, veelbelovende behandeling bij patiënten met gemetastaseerde of niet-resectabele basaalcelcarcinoom is de groep van 'hedgehog'-signaaltransductieremmers, waar vismodegib deel van uit maakt. Wij beschrijven een patiënt met gemetastaseerd basaalcelcarcinoom én het basaalcelnaevussyndroom die in studieverband behandeld werd met vismodegib.

Ziektegeschiedenis

Patiënt A, een 54-jarige vrouw, bezocht de polikliniek Dermatologie met een grote tumor op de behaarde hoofdhuid (Figuur 1). Haar medische voorgeschiedenis vermeldde het basaalcelnaevussyndroom, waarvoor zij in verschillende ziekenhuizen behandeld was. Ruim 10 jaar geleden was bij haar al eens een basaalcelcarcinoom op de behaarde hoofdhuid geëxcideerd; enkele jaren later had zij een recidief ontwikkeld, waarvoor zij micrografische chirurgie volgens Mohs had ondergaan. Ook elders op het lichaam waren basaalcelcarcinomen geëxcideerd of behandeld met 5-fluorouracilcrème. De laatste jaren had zij echter het zorgcircuit gemeden. Bij lichamelijk onderzoek zagen wij verspreid over haar lichaam nog tientallen basaalcelcarcinomen (Figuur 2).

Histopathologisch onderzoek van de tumor op de hoofdhuid toonde het beeld van een basaalcelcarcinoom met een sprieterige groeiwijze. Een CT- (Figuur 3) en een MRI-scan van het hoofd lieten zien dat de tumor doorgroeide tot in het schedelbot, mogelijk met invasie van de dura. Een cytologische punctie van een vergrote lymfeklier rechts in de hals was positief voor een niet-kleincellig carcinoom, passend bij een metastase van het basaalcelcarcinoom.

Tijdens een multidisciplinaire oncologiebespreking werd besloten tot resectie van de tumor met verwijdering van het onderliggende schedelbot en lymfeklierdissectie van de betrokken halsregio. Peroperatief bleek echter dat de dura uitgebreid geïnvadeerd was, waardoor radicale resectie van de tumor niet mogelijk was. De hals bevatte 4 lymfeklieren die positief waren voor metastasen van het basaalcelcarcinoom. Omdat de prognose van patiënten met gemetastaseerd basaalcelcarcinoom ondanks behandeling met conventionele cytostatica slecht is, werd bij patiënte gezocht naar een behandelalternatief. In studieverband kon zij behandeld worden met een hedgehog-sigitaaltransductieremmer, vismodegib, waarvan op dat moment de eerste gunstige resultaten bij patiënten met gemetastaseerd basaalcelcarcinoom waren gepubliceerd.^{1,2} Tijdens de laatste poliklinische controle gebruikte patiënte het medicijn bijna

3 jaar in een dosering van 150 mg per dag, met als resultaat complete remissie van zowel het lokale tumorresidu ter plaatse van de dura als van de metastasen; al na 6 weken trad regressie op van de tientallen basaalcelcarcinomen elders op het lichaam (Figuur 4). De belangrijkste bijwerkingen bij patiënte zijn spierkrampen, smaakverlies, misselijkheid en haaruitval.

Figuur 1. Foto van patiënt A met op de vertex multiple, ulcererende tumoren die zich bevinden in de randen van een huidtransplantaat.

Figuur 2. Foto van patiënt A met verspreid over het lichaam multiple basaalcelcarcinomen in het kader van het basaalcelnaevussyndroom.

Figuur 3. CT-schedel met aantasting van schedelbot.

Figuur 4. Foto van patiënt A met regressie van de basaalcelcarcinomen na 3 jaar behandeling met vismodegib.

Beschouwing

Het basaalcelcarcinoom is de meest voorkomende vorm van huidkanker, een vorm die uiterst zelden (0,003-0,55%) metastaseert. In geval van metastasering gaat het meestal om grote (diameter > 10 cm), lang bestaande tumoren in het hoofdhalsgebied of in het genitale gebied. Metastasering vindt voornamelijk plaats naar lymfeklieren, longen en lever. De prognose is slecht, met een mediane overleving van 8-14 maanden.³ Naar de behandeling van gemetastaseerd basaalcelcarcinoom met radiotherapie en conventionele chemotherapie zijn geen prospectieve studies gedaan. Wel zijn er casussen beschreven van patiënten die succesvol behandeld zijn met cisplatine.³

Basaalcelnaevussyndroom

Basaalcelcarcinomen kunnen voorkomen in het kader van het basaalcelnaevussyndroom. Dit is een zeldzame, autosomaal dominant overervende aandoening met een incidentie van 1 per 57.000 personen. Aan dit syndroom ligt een kiembaanmutatie ten grondslag in het *patched-1* (*PTCH1*) tumorsuppressorgen, dat gelegen is op chromosoom 9q22. De belangrijkste klinische symptomen bij patiënten met dit syndroom zijn multipele basaalcelcarcinomen, odontogene keratocysten van de kaak, palmoplantaire huidputjes, calcificatie van de falx cerebri en ribafwijkingen. Patiënten kunnen tevens typische uiterlijke kenmerken hebben, zoals macrocefalie en 'frontal bossing'. Daarnaast hebben zij een verhoogd risico op het ontwikkelen van een medulloblastoom. Vaak al op jonge leeftijd ontstaan multipele basaalcelcarcinomen. De behandeling van deze talrijke tumoren kan zeer complex zijn. Radiotherapie is gecontra-indiceerd, aangezien het een snelle inductie van nieuwe tumoren kan veroorzaken.^{4,5}

Hedgehog-sigtaaltransductieremmers

Een nieuwe ontwikkeling voor de behandeling van patiënten met een gemetastaseerd of niet-resectabel basaalcelcarcinoom zijn remmers van de hedgehog-sigtaaltransductieroute, waartoe onder andere vismodegib en erismodegib behoren. Deze sigtaaltransductieroute is van belang in de embryogenese en is bij volwassenen voornamelijk actief in stamcellen. Extracellulaire hedgehog-eiwitten fungeren als ligand voor het PTCH1-transmembraaneiwit, een tumorsuppressoreiwit dat normaal gesproken het smoothed (SMO) eiwit remt. In vrijwel alle basaalcelcarcinomen worden inactiverende mutaties in het PTCH1-eiwit of activerende mutaties in het SMO-eiwit gevonden; beide resulteren in verhoogde activiteit van de hedgehog-sigtaaltransductieroute.⁶ Dit leidt tot een toename van celproliferatie en tot tumorvorming. Hedgehog-sigtaaltransductieremmers kunnen op verschillende manieren de sigtaaltransductieroute beïnvloeden; vismodegib en erismodegib activeren beide het SMO-eiwit. Vismodegib wordt oraal toegediend, van erismodegib is ook een topicale vorm beschreven.⁷

Vismodegib

De studie waaraan onze patiënte deelnam laat bij 30% van de patiënten met gemetastaseerd basaalcelcarcinoom en bij 43% van de patiënten met lokaal uitgebreide basaalcelcarcinomen een gedeeltelijke of complete respons zien door gebruik van vismodegib. De meest gerapporteerde bijwerkingen van het medicijn zijn spierkrampen, smaakstoornissen, gewichtsverlies, vermoeidheid, misselijkheid en haaruitval.⁸ Secundaire resistentie is eveneens beschreven, waarbij na een initiële respons de tumor alsnog recidiveert.⁹ Ook zijn er resultaten bekend van vismodegib bij patiënten met het basaalcelnaevussyndroom.¹⁰ Deze patiënten ontwikkelden significant minder nieuwe basaalcelcarcinomen en bij hen gingen bestaande basaalcelcarcinomen in regressie. Deze positieve resultaten kunnen waarschijnlijk verklaard worden door de aanwezigheid van een kiembaanmutatie in het *PTCH1*-tumorsuppressorgen.^{2,10} Vismodegib is in de Verenigde Staten geregistreerd voor gemetastaseerd en lokaal uitgebreid basaalcelcarcinoom waarbij chirurgische of radiotherapeutische behandeling niet mogelijk is. Naar verwachting zal de registratie van vismodegib in Europa nog dit jaar plaatsvinden. In verschillende academische centra in Nederland worden op dit moment studies uitgevoerd met hedgehog-sigtaaltransductieremmers

Conclusie

Onze casus is een voorbeeld van een patiënt met een zeldzame, agressieve presentatie van het veel voorkomende basaalcelcarcinoom. De casus is illustratief voor de effectiviteit van een nieuwe, veelbelovende groep van geneesmiddelen die zich richt op de remming van de hedgehog-sigtaaltransductieroute.

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Chapter 7

Vismodegib for basal cell carcinoma:
targeted therapy in case of locally
advanced or metastasised disease

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Abstract

The development of the hedgehog pathway inhibitor vismodegib provides a new treatment option for metastasised and locally advanced basal cell carcinoma in which surgical excision or radiotherapy is contraindicated. Only a fraction of patients with basal cell carcinoma are eligible for this therapy, but it is effective in the majority of those who do receive vismodegib. However, development of tumour resistance is quite common and adverse events frequently lead to discontinuation of therapy. Intermittent treatment or combination therapy could reduce the occurrence of tumour resistance and diminish toxicity. We present three patients who were successfully treated with vismodegib: a 73-year-old man with locally advanced basal cell carcinoma, an 82-year-old man with basal cell carcinoma that had metastasised to the lungs, and a 42-year-old man with Gorlin syndrome.

Inleiding

Het basaalcelcarcinoom is de meest voorkomende vorm van kanker in Nederland, met 37.500 nieuwe patiënten in 2014, en de incidentie stijgt nog steeds.¹ Het overgrote deel van de basaalcelcarcinomen is eenvoudig curatief te behandelen. De behandeling van voorkeur is excisie. Voor oppervlakkig groeiende basaalcelcarcinomen zijn daarnaast non-invasieve therapieën zoals 5-fluorouracilcrème, imiquimodcrème en fotodynamische therapie beschikbaar. In specifieke gevallen is radiotherapie geïndiceerd. Langer bestaande onbehandelde laesies kunnen leiden tot een zogenaamd lokaal uitgebreid basaalcelcarcinoom. Curatieve behandeling met chirurgie of radiotherapie is bij deze tumoren soms technisch niet meer mogelijk of heeft grote functionele gevolgen. Metastasering is uiterst zeldzaam (0,003-0,55%) en komt voornamelijk voor bij lokaal uitgebreide basaalcelcarcinomen in het hoofd-hals gebied of genitale gebied. De mediane overleving bij gemetastaseerde ziekte is 8 tot 14 maanden.² De meerwaarde van radiotherapie of conventionele chemotherapie bij gemetastaseerd basaalcelcarcinoom is tot nu toe niet aangetoond. De komst van 'targeted therapy' ofwel doelgerichte systemische therapie met 'hedgehog' (Hh)- signaaltransductieremmers heeft gezorgd voor nieuwe mogelijkheden in de behandeling van deze vergevorderde en gemetastaseerde basaalcelcarcinomen. In Nederland is nu vier jaar ervaring met de Hh-remmer vismodegib. In eerste instantie werd deze behandeling enkel gegeven in studieverband. Sinds 2014 is vismodegib in Nederland geregistreerd voor volwassenen met lokaal uitgebreid en/of gemetastaseerd basaalcelcarcinoom dat ongeschikt is voor operatief ingrijpen of radiotherapie en wordt het in de reguliere patiëntenzorg voorgeschreven in een aantal academische centra. Tumoren bevinden zich vaak in het hoofdhalsg gebied en indicatiestelling van systeemtherapie vindt dan ook bij voorkeur plaats in een multidisciplinair hoofd-halsteam, waarin onder andere een dermatoloog, hoofd-hals chirurg, radiotherapeut en internist-oncoloog participeren. Het aantal patiënten dat in ons land jaarlijks in aanmerking komt voor behandeling met Hh-remmers is klein. Centralisatie van zorg lijkt dan ook wenselijk. In 2015 werden in Nederland 25 patiënten behandeld, waarvan een groot deel slechts kortdurend of in studieverband. De kosten van behandeling met vismodegib bedragen ongeveer 60.000 euro per jaar. Vanuit de Nederlandse Vereniging van Dermatologie en Venereologie is een landelijke stuurgroep opgericht, met het doel een platform te creëren voor overleg en kwaliteitsbewaking bij alle patiënten die in Nederland worden behandeld.

Wij beschrijven drie patiënten die met vismodegib behandeld werden voor respectievelijk een lokaal uitgebreid basaalcelcarcinoom, een gemetastaseerd basaalcelcarcinoom en multipele basaalcelcarcinomen in het kader van het basaalcelnaevussyndroom. Aan de hand van deze casuïstiek bespreken wij de werking en bijwerkingen van vismodegib en de nieuwe ontwikkelingen rondom dit geneesmiddel.

Ziektegeschiedenissen

Patiënt A

Een 73-jarige man met een blanco voorgeschiedenis werd verwezen met een sinds vijftien jaar bestaand basaalcelcarcinoom op zijn voorhoofd, waarvoor hij nooit eerder medische hulp had gezocht (Figuur 1A). Een CT- en MRI-scan lieten destructie zien van de voorwand van de sinus frontalis en uitbreiding van de tumor in beide mediale ooghoeken. Curatieve chirurgie of radiotherapie zouden blindheid tot gevolg kunnen hebben. In verband met wondverzorgingsproblemen en dreigend visusverlies door tumorprogressie, werd gekozen voor behandeling met vismodegib 150 mg per dag. Na 5 maanden behandeling zagen we een complete klinische respons (Figuur 1B). Patiënt wordt inmiddels ruim een jaar continu behandeld met vismodegib met een aanhoudende complete respons. Zijn voornaamste bijwerkingen zijn spierkrampen, haaruitval en gewichtsverlies van ongeveer 5 kg.

Figuur 1A. Aangezicht van patiënt A met een lokaal uitgebreid basaalcelcarcinoom op zijn voorhoofd. Centraal is een defect van de voorwand van de sinus frontalis zichtbaar. **B.** Aanhoudende klinische respons na 1 jaar behandeling met vismodegib.

Patiënt B

Een 82-jarige man bezocht ons spreekuur met een sinds twaalf jaar bestaand ulcererend en makkelijk bloedend basaalcelcarcinoom op de scalp (Figuur 2A). Uit angst zocht patiënt niet eerder medische hulp. Een CT-hoofd-hals liet een zeer uitgebreid weke delen proces van de hoofdhuid zien, verlopend van de kruin rechts tot retro-auriculair met lokaal botaantasting tot en met de tabula interna. Daarnaast waren op een CT-thorax uitgebreide nodulaire afwijkingen in de longen zichtbaar, tot ruim 2 cm groot, suspect voor pulmonale metastasen (Figuur 2B). Anamnestic, bij lichamelijk onderzoek, laboratoriumonderzoek en op de eerdergenoemde beeldvorming waren geen aanwijzingen voor een andere primaire tumor. Gezien de lokale uitgebreidheid en de verdenking op pulmonale metastasen, werd gestart met vismodegib 150 mg per dag. Na 9 maanden behandeling zagen we duidelijke regressie van de tumor op de scalp (Figuur 2C) en volgens RECIST 1.1 stabiele ziekte (afname van 19% in de diameter) van de pulmonale metastasen. De voornaamste bijwerkingen zijn smaak- en eetlustverlies, partieel haarverlies en gewichtsverlies van 5 kg.

Figuur 2A. Patient B met een lokaal uitgebreid basaalcarcinoom van de hoofdhuid met doorgoei in het schedelbot.

Figuur 2B. Axiale CT-opname met 'maximum intensity projection' van de thorax met multiple nodulaire afwijkingen, passend bij pulmonaire metastasen.

Figuur 2C Regressie van de huidtumor na 9 maanden behandeling met vismodegib.

Patiënt C

Een 42-jarige man met het basaalcelnaevussyndroom kwam regelmatig voor oncologische controle op de polikliniek dermatologie. In het kader van dit syndroom was hij bekend met hydrocefalus, hypoplasie van het corpus callosum, mentale retardatie, skeletdeformaties aan handen en voeten, cheilognathopalatoschisis, odontogene keratocysten en multipale basaalcelcarcinomen. Patiënt woont in een instelling en wordt vertegenwoordigd door zijn zus. Patiënt heeft een honderdtal basaalcelcarcinomen gehad, welke meestal operatief verwijderd werden onder algehele anesthesie. Daarnaast werd herhaaldelijk non-invasieve topische behandeling toegepast met 5-fluourouracil- en imiquimodcrème. De behandelingen waren voor patiënt zeer belastend en na chirurgie ontstonden jeukende keloïden. Nadat er in 3 maanden tijd meer dan twintig nieuwe basaalcelcarcinomen optraden, werd gestart met vismodegib 150 mg per dag. Met het oog op de bijwerkingen werd gekozen voor intermitterende behandeling. Tijdens een behandelperiode van 3 maanden trad er een snelle en complete respons op van alle aanwezige tumoren. Behoudens gewichtsverlies van 5 kg had patiënt geen bijwerkingen. Inmiddels is vismodegib 6 maanden gestaakt en de tumoren komen langzaam terug.

Beschouwing

Werkinsmechanisme

De Hh-sigtaaltransductieroute is van belang in de embryogenese en is daarna betrokken bij stamcelonderhoud en weefselhomeostase. Extracellulaire Hh-eiwitten fungeren als ligand voor het patched-1 (PTCH1) transmembraaneiwit, een tumorsuppressoreiwit, dat normaal gesproken het oncogene smoothened (SMO) eiwit remt. Ongeveer 90% van de sporadische basaalcelcarcinomen heeft identificeerbare mutaties in *PTCH1* en 10% heeft activerende mutaties in *SMO*, beide leidend tot een verhoogde activiteit van de Hh-sigtaaltransductieroute. Dit heeft een toename van celproliferatie en tumorvorming tot gevolg. De werking van vismodegib berust op binding aan het SMO-eiwit, waardoor de Hh-sigtaaltransductieroute geremd wordt.³

Effectiviteit

Recent zijn de interim-resultaten van de STEVIE-studie gepubliceerd. Dit is het grootste onderzoek naar de veiligheid en effectiviteit van vismodegib, waarin 1277 patiënten met lokaal uitgebreid of gemetastaseerd basaalcelcarcinoom behandeld werden. Op het moment van analyse werden 499 patiënten een jaar gevolgd. Van de patiënten met lokaal uitgebreid basaalcelcarcinoom was de best gemeten respons bij 34% een complete respons bij 34% (n=153), bij 33% een partiële respons (n=149) en stabiele ziekte bij 27% (n=118). Progressieve ziekte was de best gemeten respons bij 2% van de patiënten. De mediane progressievrije ziekteduur was 24 maanden. Bij gemetastaseerd basaalcelcarcinoom was de effectiviteit lager. In de STEVIE-studie had 7% (n=2) van de patiënten met metastasen een complete respons, 31% (n=9) een partiële respons en 34% (n=10) stabiele ziekte. Progressieve ziekte vond plaats in 4% van de patiënten. De mediane progressievrije ziekteduur was 13 maanden.⁴

Bijwerkingen

Vrijwel alle patiënten die behandeld worden met vismodegib ervaren tenminste één bijwerking. De meest voorkomende bijwerkingen, bij ongeveer 50% van de patiënten, zijn spierspasmen, haar- en smaakverlies. Gewichtsverlies komt in 33% van de patiënten voor en zou gerelateerd kunnen zijn aan de smaakverandering, maar is waarschijnlijk ook te wijten aan een probleem in de vetopslag ten gevolge van de medicatie. Diarree, misselijkheid en vermoeidheid zijn andere veel voorkomende bijwerkingen. De ontwikkeling van cutane plaveiselcelcarcinomen, zowel in het tumorgebied als daarbuiten, is eveneens een gerapporteerde bijwerking.⁵ Derhalve is frequente dermatologische controle geïndiceerd en dient laagdrempelig histologisch onderzoek plaats te vinden in geval van verdachte huidafwijkingen. 'Serious adverse events', waaronder pneumonie, algehele gezondheidsachteruitgang en dehydratie, werden in de STEVIE-studie gerapporteerd bij 22% van de patiënten. Ongeveer 40% van de patiënten is vanwege bijwerkingen gestopt met

de behandeling. De gemiddelde behandelduur was 8,5 maanden.⁴ Goede begeleiding van de patiënt is dan ook essentieel en consultatie van een diëtiste ter voorkoming van gewichtsverlies kan nodig zijn.

Resistentie

Een deel van de basaalcelcarcinomen reageert niet of slechts gedeeltelijk op behandeling met vismodegib. Deze basaalcelcarcinomen hebben mogelijk een ander genetisch profiel dan de tumoren met een goede klinische respons. De tumoren die wel reageren hebben een gemiddelde responsduur van 24 maanden.⁴ Activerende *SMO* mutaties in de tumor, die voorkómen dat vismodegib kan binden, liggen ten grondslag aan deze zogenoemde secundaire resistentie.^{6,7} Deze mutaties zouden pre-existent aanwezig kunnen zijn; cellen met deze mutaties overleven behandeling en groeien uit tot (nieuwe) resistente tumoren. Een andere mogelijkheid is dat de mutaties ontstaan tijdens de behandeling. Als behandelstrategie ter voorkoming van resistentie biedt intermitterend gebruik of combinatiebehandeling met andere middelen mogelijk een uitkomst.

Neoadjuvante behandeling

Het is nog onduidelijk of neoadjuvante behandeling van basaalcelcarcinomen met vismodegib zinvol is. Terughoudendheid is gewenst, aangezien de behandeling er voor kan zorgen dat de tumor niet meer per continuïtatem groeit, waardoor snijrandonderzoek naar radicaliteit onbetrouwbaar wordt.⁸ Daarnaast zijn er sterke aanwijzingen dat tijdens behandeling met vismodegib indolente tumorcellen achterblijven, die na behandeling weer klinisch manifest worden.^{6,7} Dit betekent dat voor radicale resectie het gehele in eerste instantie aangedane gebied moet worden verwijderd. Een andere behandelingsmogelijkheid is de combinatie van radiotherapie en vismodegib. Dit lijkt effectief en goed verdraagbaar, maar de resultaten van een fase 2 studie volgen nog.

Basaalcelnaevussyndroom

Het basaalcelnaevussyndroom (BCNS), ook wel bekend als Gorlin syndroom, is een autosomaal dominant overervende aandoening met een incidentie van 1 per 57.000 personen. Een kiembaanmutatie in het *PTCH1*-gen ligt ten grondslag aan dit syndroom. De belangrijkste klinische verschijnselen van dit syndroom zijn multipele basaalcelcarcinomen op jonge leeftijd, palmoplantaire huidputjes, odontogene keratocysten van de kaak, calcificatie van de falx cerebri, ribafwijkingen, medulloblastoom op kinderleeftijd en typische uiterlijke kenmerken, zoals macrocefalie en 'frontal bossing'.⁹ Het aantal en de frequentie van het optreden van nieuwe basaalcelcarcinomen wisselt sterk per individu. De meeste tumoren zijn goed te behandelen met excisie of non-invasieve therapie. De behandeling kan echter complex zijn en de multipele, soms mutilerende, littekens kunnen voor een grote psychische belasting zorgen. Radiotherapie is bij deze aandoening gecontra-indiceerd, aangezien röntgenstraling inductie van

nieuwe tumoren veroorzaakt. Omdat de oorzaak van BCNS gelegen is in een *PTCH1* mutatie, reageren de basaalcelcarcinomen goed op behandeling met Hh-remmers en de ontwikkeling van nieuwe tumoren wordt geremd.¹⁰ Op dit moment is vismodegib niet geregistreerd voor onderhoudstherapie bij patiënten met BCNS. Voor een selecte groep van patiënten met BCNS is er zeker een plaats voor behandeling met Hh-remmers, deze mening wordt gedeeld door de landelijke stuurgroep. Momenteel worden patiënten met multipole basaalcelcarcinomen met en zonder BCNS in studieverband behandeld met vismodegib in verschillende doseringsschema's. Intermitterend doseren zou de toxiciteit van de behandeling verminderen, waardoor de behandeling beter verdragen wordt.

Conclusie

De komst van Hh-remmers, zoals vismodegib, heeft gezorgd voor nieuwe mogelijkheden in de behandeling van het lokaal uitgebreid en/of gemetastaseerd basaalcelcarcinoom dat ongeschikt is voor operatief ingrijpen of radiotherapie. Bij bepaalde patiënten met het basaalcelnaevussyndroom heeft onderhoudstherapie met Hh-remmers een plaats. Bijwerkingen komen relatief vaak voor en zijn frequent een reden om therapie te staken. Aandacht voor deze bijwerkingen en een goede begeleiding van de patiënt zijn belangrijk. Daarnaast is het optreden van resistentie een regelmatig voorkomend probleem. Intermitterende behandeling zou zowel de toxiciteit als de kans op resistentie kunnen verminderen. Behandeling met Hh-remmers vindt bij voorkeur plaats in een multidisciplinair hoofd-hals team en gezien het kleine aantal patiënten dat in aanmerking komt voor deze behandeling is centralisatie van zorg wenselijk.

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Chapter 8

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

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Letter to the editor

Sporadic basal cell carcinoma (BCC) is the most frequent malignancy in Caucasians. It mostly behaves indolently, however when neglected it can become destructive (locally advanced BCC).¹ Upregulation of Hedgehog (Hh) signalling is crucial in the development of almost all BCCs.² Vismodegib is a synthetic smoothened (SMO) antagonist that inhibits the Hh pathway, with response rates up to 47.6% in locally advanced BCC.³

We saw a 68-year-old woman with a 15-cm wide deeply ulcerating infiltrative BCC on her left shoulder, which initially started as a small asymptomatic red papule that was neglected for 20 years (Figure 1A). Magnetic resonance imaging (MRI) confirmed widespread tumour invasion of the shoulder girdle muscles but no evidence of lymphogenic or visceral metastasis. Because curative surgery and radiation therapy would impair shoulder function and enhance the risk of lymphedema of the arm, we treated our patient with vismodegib 150 mg once daily.

Treatment resulted in dramatic clinical tumour regression within 8 weeks with complete regression and scar tissue remaining after 16 weeks (Figure 1B). After 20 weeks of continuous treatment, multiple nodules became visible in the former tumour area (Figure 1C). MRI showed multifocal tumour recurrence in the skin and underlying muscles. Biopsies of two of the skin nodules revealed infiltrative BCC (Figure 2A-C). Because of this progressive disease, vismodegib was discontinued and complete surgical excision was performed (week 27).

Figure 1. Clinical pictures pre- and posttreatment. **A.** Basal cell carcinoma (BCC) before treatment with vismodegib. **B.** Complete clinical response 16 weeks after start of treatment. **C.** Newly developed tumour nodules, 27 weeks after start of treatment.

Figure 2. Histologic examination on pretreatment and posttreatment tissue. **A.** Tumour biopsy before treatment with vismodegib, showing infiltrative palisading basaloid cells reaching into the reticular dermis fitting the diagnosis of BCC. **B.** Biopsy from newly developed tumour nodule after 27 weeks of treatment with vismodegib, showing infiltrative basal cell carcinoma. **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. (A-C Hematoxylin-eosin stain; original magnification 32.5).

To unravel the mechanism of resistance, we performed mutation analysis on pretreatment tumour tissue, and on both responding tissue and recurrent tumour nodules (obtained at the time of surgical excision). With genomic DNA analysis of the *PTCH1* coding exons (GenBank RefSeq NM_000264.3) a presumably heterozygous c.1728_1728+1delinsAA (Figure 3a) mutation was found together with loss of heterozygosity in the primary BCC and in the recurrent tumour nodules, indicating that cells from the original tumour had survived. This mutation was not present in DNA isolated from buccal mucosa (saliva) or responding tissue.

Because vismodegib resistance in medulloblastoma is caused by acquired mutations in *SMO*,⁴ we reasoned that similar mutations might explain resistance in BCCs. We therefore sequenced *SMO* (GenBank RefSeq NM_005631.4) in two recurrent BCCs and detected two different, novel heterozygous missense *SMO* mutations (c.842G>T (p.Trp281Leu) in exon 4 and c.961G>A (p.Val321Met) in exon 5) (Figure 3B). These *SMO* mutations were not found in pretreatment tumour tissue, responding tissue, or buccal mucosa swab (not shown). The presence of 2 different mutations precludes a clonal origin of the resistant BCC nests, supporting tumour heterogeneity, even though they arose from the same clonal primary tumour. Possibly, even more *SMO* mutations might underlie tumour regrowth in this case, because not all new tumour nodules were biopsied. Our observation suggests that BCC can acquire resistance to vismodegib via different types of *SMO* mutations.

Tumour resistance is a challenge in targeted treatment of BCC. Possible approaches to overcome tumour resistance are combination therapy with different small molecule inhibitors, sequential or rotational therapy with nontreatment periods, or alternating different small molecule inhibitors. These approaches may lead to synergistic therapeutic effects, a lower application dosage, and even prevention of (acquired) tumour resistance. Experience with targeted therapy for melanoma has already shown that combination of different agents leads to a delay in development of tumour resistance.⁵ In vismodegib treated BCC, other *SMO* inhibitors, such as itraconazole and arsenic trioxide, are possible drug candidates for this strategy. In the future, mutational analysis on pretreatment and recurrent tumour tissue may contribute to anticipating the type of resistance, in order to proactively alter therapy and customize treatment of both patient and tumour characteristics.

Figure 3. Genomic analysis of *PTCH1* coding exons and sequence traces from *SMO* exon 4 and 5 of resistant tumour tissue. **A.** The top panel confirms the absence of a germline mutation in buccal swab. The primary tumour and resistant tumour, panel 2 and 4 respectively, both harbour the same splice site mutation (arrows) and skewed nucleotide (arrowhead) distribution (indicative of loss of heterozygosity). In the responding tissue, a *PTCH1* mutation could no longer be identified. **B.** Top panels represent resistant tumour 1 and lower panels resistant tumour 2. Arrows indicate the mutations in either exon 4 (left panels) for resistant tumour 2 or exon 5 (right panels) for resistant tumour 1. If the mutation is present in 1 tumour, it is not in the other and vice versa. Normal tissue, primary tumour, and responding tumour tissue display similar wild type sequence (not shown).

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Chapter 9

Epidermal cyst formation and hyperkeratosis in a patient treated with vismodegib for locally advanced basal cell carcinoma

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Introduction

Vismodegib, a hedgehog (Hh) pathway inhibitor, was approved in 2012 by the US Food and Drug Administration (FDA) 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 tumour 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 tumour 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 (Figure 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 (Figure 2A). 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 (Figure 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 (Figure 2B). Immunohistochemical analysis confirmed residual BCC cells by positive staining for Ber-Ep4, whereas epidermal cysts stained negative.

Figure 1A. 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 (*).

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 tumour 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. Tumour heterogeneity may underlie the emergence of SCC within the tumour 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 tumour, 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 tumour suppressor gene *patched-1* (*PTCH1*) prevent binding of Sonic Hh to the transmembrane protein PTCH1, resulting in activation of the Hh signalling pathway and eventually tumour formation. The Hh 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 Hh 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 10

Summary, discussion,
future perspectives
and valorisation

Part one

Basal cell naevus syndrome

Basal cell naevus syndrome (BCNS), also known as Gorlin syndrome, is an autosomal dominant disorder, characterized by multiple basal cell carcinomas (BCCs), odontogenic keratocysts, cerebral calcifications and palmoplantar pits.^{1,2} The syndrome is associated with germline mutations in the *Drosophila* homolog of *patched-1* (*PTCH1*) gene that encodes the PTCH1 protein. PTCH1 is part of the hedgehog (Hh) signalling pathway, which plays an important role in the regulation of cell growth and differentiation. BCNS belongs to the group of hereditary tumour syndromes, in which mutations in one or more genes predispose to the development of cancer. The development of sometimes hundreds of BCCs is a major problem in this group of patients.

Genetic basis of BCNS

In 1996 the *patched-1* (*PTCH1*) gene (MIM#601309) was first reported as a candidate gene for BCNS. Two different heterozygous mutations in the *PTCH1* gene were identified in two patients with BCNS.³ *PTCH1* is the human homolog of the *Drosophila patched-1* gene and is located on chromosome 9q22.3. With DNA sequencing analysis of the *PTCH1* gene, mutation detection frequency ranges from 40% to 85% in individuals with typical findings of BCNS.⁴⁻⁸ In **chapter 2** we describe a new database containing all previously published and newly identified *PTCH1* mutations. We established a database for *PTCH1* using the Leiden Open Variation Database (LOVD) version 3.0.⁹ The purpose of this database is to assemble molecular variants of the *PTCH1* gene in a standardised format. The database provides an open collection for both clinicians and researchers containing published and unpublished *PTCH1* mutations. To accomplish this, we listed the results from all blood samples sent to the two Diagnostic DNA Laboratories in the Netherlands for *PTCH1* mutation analysis between 1999 and 2015. In **chapter 3** we investigate whether there is a genotype-phenotype correlation in this population by collecting clinical symptoms from patients' medical records. In this research, we only looked at the patients who carried a *PTCH1* mutation in the blood. In the investigated Dutch cohort, we did not find a genotype-phenotype correlation based either on the location or on the type of *PTCH1* mutation. Our findings are consistent with the results of previous studies and are in line with the phenotype variability within families found in this study.^{4,10-13} The presence of a germline mutation in *SUFU* increases the risk of developing medulloblastoma in childhood.⁶ The presence of genetic variants (polymorphisms (SNPs), modifiers) that increase the risks of developing sporadic BCC, like MC1R and TERT-CLPTM1L, is associated with an earlier age of onset for BCCs in BCNS.¹⁴ The mutation detection percentage in our population is low (21%). Probably, relatively many patients with only minimal clinical symptoms of BCNS were tested. Another explanation can be that for many patients with clinical suspicion of BCNS, standard genetic tests are not yet suitable to detect the underlying genetic cause of the symptoms.

Postzygotic mosaicism in BCNS

In a substantial number of BCNS patients no *PTCH1* mutation is found in DNA extracted from blood. It is known that genomic rearrangements in *PTCH1* or deep intronic variants causing cryptic splicing cannot be detected with generally used genetic diagnostic tests.¹⁵ Also, mutations could be located in other genes involved in the Hh pathway (or yet unknown pathways), for example *SUFU* or *PTCH2*.^{6,16–18} Another explanation is the presence of genetic mosaicism. Low-grade mosaicism can be difficult or even impossible to detect in DNA extracted from blood. With standard techniques, e.g. Sanger sequencing, mutation loads under 5% are not detectable. Next generation sequencing (NGS) of target genes (i.e. *PTCH1*) in blood is more sensitive and has sufficient sequence read depth to not only reliably detect, but also quantify the degree of mosaicism¹⁹. Sometimes though, a mutation is only present in the skin and cannot be found in blood. When the presence of a mutation causes visible skin lesions, as in mosaic ichthyosis, the segmentally appearing skin disorder is an evident clue for the existence of segmental mosaicism caused by dominant mutations.²⁰ Mosaic skin diseases may present different patterns as described in **chapter 1**. Lesions can for example follow the lines of Blaschko, in broad or narrow bands, or appear in a checkerboard pattern.²¹ In BCNS, but also in other hereditary tumour syndromes, skin with only one affected allele has a normal appearance. Only a second hit of the other allele, through loss-of-heterozygosity (LOH) or somatic gene mutation, initiates the development of a BCC. As the presence of a BCC is a marker for affected skin, genetic mosaicism in the patient can be demonstrated when different BCCs share the same *PTCH1* mutation. In **chapter 4** and **5** we described three patients with BCNS caused by *PTCH1* mosaicism. Only in one patient there is a clear segmental distribution of skin lesions as they are limited to only one side of the body. This emphasizes the difficulty of recognizing a mosaic presentation of BCNS. To demonstrate a shared mutation in the different BCCs of these patients we used NGS in combination with a gene panel, consisting of a selection of genes participating in the Hh pathway including *PTCH1*, *PTCH2*, *SUFU* and *SMO*. Mosaic mutations in the latter has been described in Happle-Tinschert syndrome, that should be distinguished from mosaic BCNS^{22,23}

Mosaic manifestations of other hereditary tumour syndromes have been described as well, for example neurofibromatosis type 1.²⁴ In BCNS *PTCH1* postzygotic mosaicism could actually be more common than previously assumed. It is plausible that patients do not always fulfil the diagnostic criteria of BCNS and may even present only with multiple BCCs at a young age. Of note, low-grade mosaicism can affect the gonadal cells as well. If this is the case, patients have a chance, although probably very small, to give birth to a child with a germline mutation of the disease. Unfortunately, the degree of involvement of different organ systems is difficult to predict. Therefore, surveillance programs and treatment of BCCs should have the same approach as in patients with a germline mutation. So, in patients suspected of having BCNS and a negative mutation analysis on blood, even with NGS techniques, it is worthwhile to search for a shared mutation in their BCCs. In case of mosaic disease, genetic counselling can be adjusted.

Care pathways and patient customized health care

Currently, our department is developing an integrated care pathway for patients with BCNS. This project is a collaboration between different specialties: paediatrics, clinical genetics, maxillary surgery, (paediatric) neurology, ophthalmology, cardiology, gynaecology, psychology and medical oncology, with the dermatologist as the initiator of the project. The purpose of this plan is to standardize the health care for this group of patients based on best available evidence. The dermatologist has a central position in this care pathway, as the management of the multiple BCCs is a difficult and ongoing problem. Information about sun protective measures are very important in patients with BCNS, as individuals with a low tumour burden have significantly fewer UV signature somatic mutations.²⁵ Our dermatology department offers specialized consultations for patients with BCNS. Treatment of multiple BCCs can be very complex and has a high psychological impact on patients. Different treatment modalities are available, and choices are preferably made combining evidence-based medicine with shared decision-making. Maintenance therapy with Hh pathway inhibitors, such as vismodegib, can be considered in selected patients as I will discuss in more detail in part two of this chapter.

Future perspectives

Insight into the genetic cause of BCNS is not only helpful to understand the pathogenesis of this syndrome. For the individual patient, it is just as important, because it allows us to diagnose the disease at an early age and to offer patients correct screening programs and adequate genetic counselling.

Whole Exome Sequencing (WES) is increasingly used, especially in syndromes with no clearly defined candidate gene or in very heterogeneous diseases, because it enables the sequencing of all genes in one go. The method is however still costly and moreover it has a limited sequencing depth.¹⁹ In case of a high clinical suspicion of BCNS it is more efficient to look specifically at participants of the Hh pathway. We designed an NGS-based gene panel that includes *PTCH1*, *PTCH2*, *SMO* and *SUFU*. Its implementation in daily practice will provide a more effective, faster and less expensive diagnostic screening method for BCNS patients. Because of the higher sequencing depth, mosaic disease is more likely to be discovered. In patients with a high suspicion of BCNS in whom no *PTCH1* mutation can be detected in blood with regular Sanger sequencing, it is worthwhile to implement the gene panel, in order to analyze other genes involved in the Hh pathway, but also to reach a sequencing depth sufficient to detect low-grade mosaicism. To gain more information about the prevalence of mutations in other genes and the presence of mosaic disease in BCNS, it is of interest to look at the patients in our initial cohort that had no mutation in blood.

Part two

Treatment of locally advanced and metastasised basal cell carcinoma with vismodegib

The development of Hh pathway inhibitors provided a new treatment option for patients with metastasized or locally advanced BCC ineligible for surgery or radiotherapy. In **chapter 6** we described the first Dutch patient who was treated with vismodegib for a locally advanced BCC with locoregional metastasis of her BCC. An interesting detail is the presence of BCNS in this patient, already giving a preview of the effects of vismodegib on the multiple small BCCs in BCNS. Since the registration of the Hh pathway inhibitor vismodegib in the Netherlands in 2014, it is possible to treat patients outside the clinical trial setting. However, it is important to realise that only very few patients with BCC are candidates for this systemic therapy. In 2015 only 25 patients were treated for this indication in the Netherlands. Although treatment with Hh pathway inhibitors is initially effective, development of tumour resistance is common and adverse events frequently lead to discontinuation of therapy. Some patients with BCNS benefit from treatment with oral Hh pathway inhibitors, but especially in this group side effects are an important reason to discontinue therapy. An overview of treatment of BCC with Hh pathway inhibitors is given in **chapter 7** and some aspects of this treatment are discussed in more detail below. We published the topics mentioned above in a national journal, as we believe it is important to share experiences on new treatment options.

Efficacy and side effects

Vismodegib is a small molecule inhibitor targeting the Hh pathway. Binding of vismodegib to SMO leads to inactivation of this pathway.²⁶ Because most BCCs carry mutations in *PTCH1* (the inhibitor of SMO) resulting in upregulation of the Hh pathway,²⁵ vismodegib is initially effective in the vast majority of patients. The STEVIE trial assessed the safety and efficacy of vismodegib in patients with advanced BCC.^{27,28} In this trial 1215 patients (1119 laBCC, 96 mBCC) were treated with vismodegib with a mean follow-up of 18 months. The best measured response rate in patients with laBCC was a complete response in 33% and a partial response in 35%, stable disease in 25% and progressive disease in 2% of patients. The median duration of response was 23 months. In metastatic BCC the efficacy was lower. In only 5% of patients the best measured response was a complete response, in 32% a partial response, in 46% stable disease and progressive disease in 11%. The median duration of response was 12 months. At the clinical cut-off of the study, only 12% of patients were still receiving treatment with vismodegib. Main reasons to discontinue treatment were side effects and progressive disease. The presence of BCNS did not affect the efficacy of vismodegib.²⁸

In general, 30 to 40% of patients discontinued therapy because of side effects.²⁸ In patients treated because of BCNS-related multiple small BCCs even more than 50% could not continue therapy because of drug toxicity.^{29,30} Most frequently reported side effects, in about half of patients, are muscle cramps, dysgeusia and alopecia. Weight loss is reported in 33% of patients, possibly related with dysgeusia, but probably also due to changes in fat metabolism caused by the medication.²⁸ Diarrhoea, nausea and fatigue are other common side effects. The development of cutaneous squamous cell carcinoma (SCC) during vismodegib therapy has been reported as well.³¹ The actual clinical impact of this observation is not clear, as later studies did not find a higher incidence of SCC during treatment.³²

Proper patient support and consultation of a dietician during treatment are essential to prevent weight loss. Intermittent treatment is already implemented in clinical practice to reduce toxicity. Unfortunately, studies on intermittent dosing regimens only show reduction in toxicity to a certain level.³³

Drug resistance

A major problem in the treatment of BCC with vismodegib is the development of drug resistance. The majority of tumours initially show a complete or partial response to treatment with vismodegib. Only few BCCs do not respond at all, so called non-responders. The mean duration of response to therapy is 24 months.²⁷ It is thought that about 15-20% of patients develop resistance, the exact percentage is unknown.^{27,43} The number of resistant cases is probably underestimated because of the short follow-up and the slow growth of BCC. In **chapter 8** we describe two patients with locally advanced BCC, who are resistant to vismodegib. These cases illustrate how important it is to monitor the tumour area very carefully and perform sequential biopsies when needed.

Genetically, BCC is one of the most heterogenic forms of cancer.²⁵ However, for their growth BCCs are almost completely dependent on the Hh pathway. Genetic alterations that maintain Hh signalling in the presence of vismodegib are the primary mechanism of resistance. In **chapter 9** we describe a patient with acquired resistance to vismodegib due to *SMO* mutations. At the same time, it was found that drug resistance to vismodegib in the treatment of BCC is predominantly caused by *SMO* mutations that either directly impair drug binding or activate *SMO* to varying levels.^{35,36} The question is whether responsible mutations are acquired during treatment, or that they already existed before treatment. In the latter case, the minor clones may have benefited from a positive selection effect when other cells were killed by vismodegib. In the future, combination treatments should be developed to overcome or slow down the development of resistance. Drugs should inhibit *SMO* through different mechanisms or target the Hh pathway at points downstream of *SMO*. Several drug candidates are suggested in literature, for example itraconazol, bis-amides, arsenic oxide, protein-kinase inhibitors and mTOR inhibitors.³⁷⁻⁴⁰ There

are indications that imiquimod inhibits the Hh pathway as well.⁴¹ Furthermore, GLI-antagonists are very promising as they target the GLI transcription factors that are most downstream in the Hh pathway.⁴² Further research is needed to explore possible clinical applications of these drugs, especially in respect to pharmacokinetics and toxicity.

Hh inhibitors in basal cell naevus syndrome

Vismodegib has proven to be effective in the treatment of BCNS related BCCs. Existing BCCs disappear and development of new tumours is delayed.^{29,30} Unfortunately, discontinuation of treatment is common, mainly because of the side effects. Development of tumour resistance also occurs in patients with BCNS when it comes to laBCC or mBCC, but maybe to a more limited extent than in patients with sporadic BCC. Resistance is caused by the same underlying mechanism as in sporadic BCCs, as acquired *SMO* mutations are found in resistant tumour tissue.³⁰ However, the BCNS-related small BCCs seem to develop resistance less frequently.³⁰ This is probably because small BCCs are less heterogenic than the large laBCCs.²⁵ There is also evidence that syndromic BCCs have a lower mutational load and better genomic stability compared to sporadic BCCs, irrespective of the size of the tumour.⁴³ Studies on intermittent treatment dosing regimens in patients with multiple BCCs show that interruption of treatment does not compromise activity of vismodegib, but only leads to some reduction in toxicity.³³ Optimal treatment regimens should be personalized and aim to strike a balance between sufficient activity and acceptable side effects as these patients need long-term treatment. At this moment vismodegib is not registered for treatment of multiple BCNS-related small BCCs. However, surgical treatment of the high number of BCCs may have unacceptable high morbidity in some patients. Radiotherapy is contraindicated in BCNS because of the induction of new tumours. Therefore, in selected patients with BCNS there is an indication for maintenance therapy with Hh pathway inhibitors.

Neoadjuvant therapy

One should be very cautious in using vismodegib as a neoadjuvant therapy in clinical practice. Treatment with vismodegib can cause discontinuous growth of the tumour, leading to an unreliable histological margin assessment.⁴⁴ There is strong evidence that tumour cells remain present and become clinically manifest after treatment discontinuation.^{35,45} This means that to achieve a complete surgical resection the whole initially affected area should be removed anyway, so there is no benefit in reducing the tumour size before surgery. An interesting treatment option for non-syndromic locally advanced BCC is the combination of radiotherapy and vismodegib. This seems to be an effective and tolerable combination therapy, with a possible synergistic effect, but the results of a phase 2 trial are not yet available.⁴⁶⁻⁴⁸

Treatment settings and personalized medicine

A multidisciplinary oncological team in which a dermatologist, head and neck surgeon, oncological surgeon, radiotherapist, plastic surgeon and medical oncologist are represented, is the preferred setting for treatment of locally advanced BCC with Hh inhibitors.⁴⁹ Only then all treatment options can be considered and experiences shared. Not only tumour characteristics are important in deciding which treatment is best for the patient, also patient characteristics are of great significance. The majority of skin cancer occurs in the elderly. In the ageing population, frailty is an important issue. The term frailty refers to a state of vulnerability to poor resolution of homeostasis after a stressor event and is a consequence of cumulative decline in many physiological systems during a lifetime.⁵⁰ In frail elderly, a minor stressor can trigger disproportionate changes in health status. In frail elderly with advanced BCC, surgery may be technically achievable, but associated with a high morbidity when complicated by for example delirium or hospital acquired pneumonia. Treatment with surgery or radiotherapy may lead to an impaired quality of life, for example when the BCC involves the orbita and treatment requires an enucleation or exenteration of the eye or leads to vision loss by radiation-induced toxicity. In those patients, palliative treatment with vismodegib may be an option, but it is important to realise that side effects, like muscle cramps and dysgeusia, can also significantly influence quality of life. Involvement of a geriatrist in making treatment decisions can be helpful in these cases.⁵¹

Future perspectives on Hh pathway inhibitors for BCC

In the past decades, there has been a steep rise in the incidence of BCC. As BCC in general is a very slow growing tumour, it will take many years for it to become inoperable. As a logical consequence, the increase of neglected locally advanced tumours will lag many years behind the rise in incidence of BCC in general. As a direct result, in the nearby future an increasing number of patients with locally advanced or metastatic BCC that is ineligible for surgery or radiotherapy is to be expected. This emphasizes the need to optimize systemic treatment options for this group of patients.

Rapid advancement in our understanding of Hh signalling in BCC has provided a new systemic treatment option for this common form of skin cancer: Hh pathway inhibitors. We already have insight into the mechanism of the development of resistance to Hh inhibitors in the treatment of BCC. In a next stage, treatment strategies to overcome the development of resistance need to be investigated. Combination therapy probably provides the best perspective. Different drug candidates are proposed in literature. However, development of combination therapy still has a long way to go before it can be used in clinical practice.

For the small BCNS related BCCs topical Hh inhibitors are a promising treatment option. The topical SMO inhibitor patidegib has been developed for this indication.⁵² Results from a phase II trial in which patients with BCNS are treated with patidegib have not yet been published. A multicentre phase 3 trial will start this year, with the MUMC as participating centre in the Netherlands.

The past years have seen many significant achievements in the field of cancer genetics, driven by rapidly evolving technologies and decreasing costs of next-generation sequencing (NGS). NGS is already used in clinical practice in several forms of cancer to predict responsiveness to therapy. The term 'precision oncology' was introduced, referring to the molecular profiling of tumours to identify targetable alterations and to predict response to therapies. Precision oncology simply means to give the right drugs to the right patients.¹⁹ In BCC, an NGS targeted panel consisting of genes participating in the Hh pathway allows us to analyse tumour tissue relatively cheaply and fast. With this technique, it is also possible to analyse paraffin embedded tissue. In the future, NGS will probably contribute to making treatment decisions for locally advanced or metastatic BCC.

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Chapter 11

Samenvatting

Samenvatting

De hedgehog (Hh)-signaaltransductieroute speelt een belangrijke rol in de pathogenese van het basaalcelcarcinoom. In het overgrote deel van de basaalcelcarcinomen worden mutaties gevonden in het *PTCH1* gen. Dit tumorsuppressorgen codeert voor het PTCH1 eiwit, dat onderdeel uitmaakt van de Hh-signaaltransductieroute. Kiembaanmutaties in *PTCH1* liggen bovendien ten grondslag aan het basaalcelnaevussyndroom (BCNS). Dit syndroom wordt gekenmerkt door onder andere cerebrale calcificaties, palmoplantaire pits en multiële basaalcelcarcinomen op jonge leeftijd. Dit proefschrift gaat over de rol van de Hh-signaaltransductieroute in de diagnostiek van BCNS en over de Hh-route als aangrijpingspunt voor 'targeted' ofwel doelgerichte therapie met Hh-signaaltransductieremmers.

Hoofdstuk 1 geeft een algemene inleiding op het proefschrift. De functie van de Hh-signaaltransductieroute en het tumorsuppressorgen *PTCH1* en hun rol in de pathogenese van het basaalcelcarcinoom en BCNS worden uitgelegd. Daarnaast bevat het hoofdstuk achtergrondinformatie over genetisch mozaïcisme bij huidziekten en worden de doelstellingen van het proefschrift besproken.

In **hoofdstuk 2** beschrijven we de totstandkoming van een database voor het *PTCH1* gen, waarbij we gebruik maken van de Leiden Open Variation Database (LOVD). Aan deze database hebben we 331 reeds eerder gepubliceerde *PTCH1* mutaties en 117 nieuwe *PTCH1* mutaties toegevoegd. Deze mutaties werden gevonden bij 141 patiënten met een positieve *PTCH1* mutatieanalyse, uitgevoerd in het VU Medisch Centrum of het Maastricht Universitair Medisch Centrum. De database is vrij toegankelijk voor klinici en onderzoekers en kan online geraadpleegd worden op <http://www.LOVD.nl/PTCH1>.

Voor **hoofdstuk 3** verzamelden we de klinische kenmerken van 83 Nederlandse patiënten met een *PTCH1* mutatie in het bloed, om een eventuele genotype-fenotype correlatie te ontdekken bij patiënten met BCNS. We maakten verschillende heatmaps om deze correlatie te visualiseren. In de eerste heat-map werd de locatie van de mutatie in het gen uitgezet tegen de klinische kenmerken. In de tweede heatmap werd het type mutatie uitgezet tegen de klinische kenmerken. We vonden een grote variabiliteit van zowel genotype als fenotype, zonder aanwijzingen voor een genotype-fenotype correlatie

In **hoofdstuk 4** beschrijven we een patiënte met symptomen van BCNS, veroorzaakt door een postzygotische mutatie in het *PTCH1* gen. De graad van mozaïcisme werd bepaald middels Restriction Length Polymorphism Analysis en Droplet Digital Polymerase Chain Reaction. Het percentage cellen met een mutatie was bij benadering 16% in de verschillende weefsels van deze patiënte. Onze bevindingen tonen aan dat een laaggradig mozaïcisme, een klinisch beeld

kan geven dat voldoet aan de diagnostische criteria voor BCNS. Dit onderstreept het belang van genetisch onderzoek bij patiënten met een verdenking op BCNS, omdat de uitslag implicaties heeft voor de manier waarop de genetische counseling plaatsvindt. Standaard technieken zijn niet altijd geschikt om een laaggradig mozaïcisme op te pikken. Kwantitatieve genetische testen hebben de voorkeur, wanneer er een verdenking is op een genetisch mozaïcisme.

Hoofdstuk 5 borduurt voort op de inhoud van het vorige hoofdstuk. We beschrijven twee patiënten, bij wie we het vermoeden hadden dat er sprake was van BCNS, maar we geen *PTCH1* mutatie konden aantonen in het bloed. Bij beide patiënten verrichtten we een genetische analyse van hun basaalcelcarcinomen, waarmee er in de verschillende tumoren een identieke *PTCH1* mutatie kon worden aangetoond. Deze bevindingen tonen aan dat de symptomen van deze patiënten te verklaren zijn door een postzygotische *PTCH1* mutatie. Wanneer er bij patiënten met klinische kenmerken van BCNS sprake is van een negatieve *PTCH1* mutatieanalyse in het bloed, is het de moeite waard om een genetische analyse uit te voeren op verschillende basaalcelcarcinomen, aangezien hiermee genetisch mozaïcisme kan worden opgespoord.

Hoofdstuk 6 beschrijft de eerste Nederlandse patiënt die behandeld werd met de Hh-siginaaltransductieremmer vismodegib voor de indicatie gemetastaseerd basaalcelcarcinoom. Het basaalcelcarcinoom metastaseert slechts zelden, maar kent een slechte prognose. Er zijn geen prospectieve studies naar de behandeling van gemetastaseerd basaalcelcarcinoom met conventionele chemotherapie. Ten tijde van de casus waren Hh-remmers nog niet geregistreerd in Nederland voor de indicatie basaalcelcarcinoom. Patiënte kon in studieverband behandeld worden met de Hh-remmer vismodegib. Naast een aanhoudende complete remissie van de gemetastaseerde ziekte, verdwenen ook de multipole kleinere basaalcelcarcinomen, die zij had in het kader van BCNS.

Aan de hand van drie patiënten geven we in **hoofdstuk 7** een overzicht van de behandeling van gemetastaseerd en lokaal uitgebreid basaalcelcarcinoom met de Hh-siginaaltransductieremmer vismodegib. Ook bij bepaalde patiënten met BCNS heeft onderhoudstherapie met Hh-remmers een plaats. De effectiviteit van de behandeling voor de verschillende indicaties wordt besproken, evenals de bijwerkingen die kunnen optreden, zoals spierspasmen, haar-, en smaakverlies. De ontwikkeling van resistentie voor het medicijn is daarnaast een regelmatig voorkomend probleem. We geven aan dat de behandeling van het basaalcelcarcinoom met Hh-remmers bij voorkeur plaatsvindt in een multidisciplinair (hoofd-hals) team.

In **hoofdstuk 8 en 9** presenteren we twee patiëntencasuïstieken, waarbij er sprake is van resistentie voor vismodegib bij de behandeling van basaalcelcarcinoom. De eerste casus betreft een patiënte met een lokaal uitgebreid basaalcelcarcinoom op de linker schouder, waarbij er initieel een complete respons optrad, maar tijdens behandeling een recidief ontstond.

Genetische analyse van het recidief tumorweefsel, liet mutaties zien in het *smoothened* gen, die vóór de behandeling niet aanwezig waren in de tumor. Deze mutaties zijn verantwoordelijk voor de ontwikkeling van resistentie voor vismodegib. De tweede casus betreft een patiënt, met een lokaal uitgebreid basaalcelcarcinoom in het gelaat, waarbij de beoordeling van het behandelresultaat werd bemoeilijkt door het ontstaan van benigne huidafwijkingen in het tumorgebied tijdens behandeling met vismodegib. Beide patiënten werden uiteindelijk chirurgisch behandeld voor het basaalcelcarcinoom.

Hoofdstuk 10 besluit met een discussie en valorisatie, waarin we de resultaten van dit proefschrift in een breder perspectief plaatsen en we speculeren over toekomstige ontwikkelingen rondom het onderwerp van dit proefschrift.

Addendum

Curriculum vitae
List of publications and presentations
Dankwoord

Curriculum vitae

Marieke Reinders werd geboren op 18 oktober 1982 in Deurne. Ze groeide op in het Limburgse dorp Panningen, samen met haar zus en ouders. In 2000 behaalde zij haar gymnasiumdiploma aan het Bouwens van der Boijecollege in Panningen. In 2000 begon zij aan de studie geneeskunde aan de Universiteit van Maastricht en in 2006 studeerde zij cum laude af. Nadat zij een half jaar als poortarts op de spoedeisende hulp werkte in ziekenhuis Bernhoven in Veghel, kreeg zij een baan als arts niet in opleiding tot specialist op de afdeling dermatologie van het Catharina Ziekenhuis in Eindhoven. In 2007 werd zij aangenomen voor de opleiding tot dermatoloog in het Leids Universitair Medisch Centrum (opleider: R. Willemze). Tijdens deze opleiding kreeg zij de mogelijkheid om zich de Mohs' micrografische chirurgie eigen te maken in het Catharina Ziekenhuis in Eindhoven. Vanaf 2012 werkt zij als dermatoloog in het Maastricht Universitair Medisch Centrum. Marieke Reinders is getrouwd met Roel Hendrickx en zij wonen samen met hun twee zonen, Thomas en Maarten, in Roermond.

List of publications

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List of presentations

Genetic profiling of basal cell carcinomas detects postzygotic mosaicism in basal cell naevus syndrome.

Poster presentation, Annual Symposium of the Dutch Society of Human Genetics, 2018

Genetic mosaicism in basal cell naevus syndrome.

Grow School for Oncology and Developmental Biology MUMC, Pizza meeting, Maastricht, 2018

Genetic mosaicism in basal cell naevus syndrome.

Annual scientific meeting of the Dutch Association for Experimental Dermatology, Lunteren, 2018

Resistance for vismodegib in basal cell carcinoma treatment.

Annual meeting of the Dutch Association for Dermatology and Venereology, Maastricht, 2017

Multidisciplinaire behandeling van huidkanker.

Huidkanker, een gezamenlijke strijd voor een behandeling op maat, Oncologie symposium, Maastricht 2016

Huidplastieken in de praktijk.

Dermatologen in opleiding dagen, Amersfoort, 2015

Post-operatieve zorg en complicaties.

Workshop: eenvoudig sluiten van defecten in het gelaat.

COCOM Dermatochirurgie en flebologie, 2014 en 2017

Ulcus cruris, veneuze versus arteriële insufficiëntie.

Wondsymposium. LUMC, Leiden, 2012

Erfelijke tumor syndromen.

EADV review. WTC, Amsterdam, 2011

An exceptional large Mohs' case.

20th EADV Congress. Lisbon, Portugal, 2011

Gemetastaseerd basaalcelcarcinoom bij basaalcelnaevussyndroom.

Oncologie in de parel van de Ardennen. Dermato-oncologie congres. Spa, België, 2010

CT- versus MR-angiography for visualization of the Adamkiewicz artery.

Poster presentation, 10th Maastricht Medical Students Research Conference, Maastricht, 2006

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