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# Molecular aetiology and pathogenesis of basal cell carcinoma

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## Summary

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Recent insights into the cell biology of the epidermis and its appendages are transforming our understanding of the pathogenesis of basal cell carcinoma (BCC). The significant progress that has been made warrants a comprehensive review of the molecular and cellular pathology of BCC. The items addressed include environmental and genetic risk factors, the biology of the putative precursor cell(s), and the contribution of aberrations in processes such as apoptosis, cell proliferation, differentiation and signalling to carcinogenesis. Furthermore, established and novel treatment modalities are discussed with particular attention to future biological approaches.

Basal cell carcinoma (BCC), also called basalioma, basal cell epithelioma, rodent ulcer and Jacobs' ulcer, was first described in 1824<sup>1</sup> and is the most common cancer among Caucasians.<sup>2</sup> BCC accounts for approximately 75% of all skin cancers. Mortality rates are low,<sup>3</sup> but BCC may occasionally grow aggressively causing extensive tissue destruction.<sup>4</sup> Its frequency of metastasis is very low (<0.1%).<sup>5</sup> Metastasis to lymph nodes, lung, bone and liver has been described.<sup>6,7</sup>

BCCs are commonly subdivided according to their differences in histological appearance.<sup>8,9</sup> The major histological patterns are nodular, micronodular, superficial and morpheaform BCC. The nodular type is characterized by a rounded mass of neoplastic cells with well-defined peripheral contours and peripheral palisading. The superficial type is defined by one or more tumour foci that extend from the epidermis into the papillary dermis. Peripheral palisading occurs and the peripheral contours are smooth. The micronodular subtype grows as small nodules, hence the name. Peripheral palisading is usually present. The morpheaform subtype consists of tumour islands of varying size with an irregular outline and spiky configuration. Peripheral palisading is poorly developed. Mixed types of these histological patterns may occur, with the nodular-micronodular combination being the most common.<sup>10</sup> Furthermore, central nodular and peripheral morphea-like growth can occur. Finally, a so-called adenoid pattern is seen in 1–7% of the tumours and is mainly associated with the nodular growth type.<sup>11</sup> Nodular (~60%) and superficial (~25%) BCCs are often considered as non-aggressive subtypes, whereas morpheaform (~2%) and micronodular (~15%) BCCs are often referred to as aggressive subtypes, associated with a higher risk of local recurrence.<sup>12</sup>

## Risk factors

The risk for development of BCC is associated with environmental factors as well as several patient-dependent factors.

### Environmental risk factors

#### Sun exposure

BCCs generally occur on sun-exposed areas of the body<sup>13</sup> and high-risk patients are often fair-skinned with a history of burning, not tanning, when exposed to sunlight.<sup>14</sup> Male sex, older age and number of previous second-degree sunburns are also factors indicating a higher risk for development of BCC.<sup>15</sup> Corona et al.<sup>16</sup> showed in 2001 that there is a significant association between BCC development and recreational sun exposure during childhood and adolescence, as well as a strong relationship with family history of skin cancer. Patients with a BCC located on the trunk are at increased risk of developing multiple BCCs, and these tumours develop at a faster rate than BCCs located elsewhere on the body.<sup>17</sup> Grossman and Leffell<sup>18</sup> showed that there is a correlation between ultraviolet (UV)-B exposure and the development of skin cancer. However, the significant number of BCCs arising on non-sun-exposed areas of the body suggests that other risk factors may play a role in the development of BCC.<sup>19</sup>

#### Chemical carcinogens and radiation

Diepgen and Mahler<sup>20</sup> found that chemical carcinogens such as arsenic, coal tar products and psoralens as well as ionizing

radiation increase the risk of non-melanoma skin cancer, mainly squamous cell carcinoma (SCC). With respect to the relationship between smoking and skin cancer development, de Hertog *et al.*<sup>21</sup> showed an association with SCC but not BCC, while Boyd *et al.*<sup>22</sup> recently proved that an association exists between smoking and BCC in young women. Furthermore, exposure to fibreglass dust and dry-cleaning agents has also been reported to enhance the risk for BCC development.<sup>19</sup> However, this is one report and the findings in it have yet to be confirmed. We find it difficult to think of a pathogenic mechanism to explain the carcinogenic potential of fibreglass on skin.

Exposure to psoralens combined with UVA-treatment (PUVA) in psoriasis patients has been reported to result in an increased risk for BCC and SCC.<sup>23–25</sup> However, later studies failed to substantiate the increased risk of BCC and it is currently doubted whether BCC can really result from PUVA therapy.<sup>26,27</sup> Melanoma risk is also increased by PUVA.<sup>27</sup> Prior non-diagnostic X-ray treatment for skin conditions also enhanced the risk for BCC.<sup>19</sup> In general, it seems that DNA-damaging agents predispose more to SCC than they do to BCC. This observation is consistent with the spectrum of malignancies observed in congenital disorders of DNA repair (discussed below).

### Viral carcinogenesis

Several authors demonstrated an association between infection with the oncogenic types of human papillomavirus (HPV) and development of BCC,<sup>28–30</sup> while Harwood and Proby<sup>31</sup> showed that HPV could abrogate UV-induced apoptosis. Furthermore, HPV DNA was detected in BCC patients by Barr *et al.*<sup>28</sup> and Weinstock *et al.*<sup>30</sup> suggesting that HPV infection may play a role in developing BCC. To date, a causal connection has not been established and it seems doubtful that one will be found.

### Hereditary predisposition

#### Detoxifying proteins

Proteins that mediate detoxification processes, including individual responses to UV irradiation by protecting from oxidative stress, are likely to be involved in susceptibility for BCC.<sup>32</sup> For example, glutathione S-transferase (GST) enzymes are part of the cells' defence mechanism against harmful chemicals produced endogenously and in the environment.<sup>33</sup> UV irradiation causes oxidative stress in the skin, which leads to lipid peroxidation and DNA hydroperoxide formation.<sup>34</sup> GST is responsible for the disposal of these potential mutagens.<sup>35</sup> Cytosolic GST activity in mammalian tissues is due to the presence of multiple GST isozymes, which can be assigned to five classes, e.g.  $\alpha$ ,  $\theta$ ,  $\mu$ ,  $\pi$  and  $\sigma$ .<sup>36</sup> In human skin, GST activity is found predominantly in sebaceous glands and in the outer root sheath (ORS) of hair follicles, the  $\pi$ -class of GST being the predominant isozyme.<sup>37</sup> GST- $\pi$  has been suggested to be

an oncofetal protein that is re-expressed during carcinogenesis.<sup>38</sup> A significant increase in skin tumorigenesis is observed in mice lacking  $\pi$ -class GST.<sup>39</sup> This finding seems to contradict the oncogene hypothesis, but in humans, GST- $\pi$  is expressed in malignant melanomas,<sup>40</sup> whereas BCCs show only a weak expression of the protein.<sup>41</sup> Obviously, this apparent contradiction needs to be addressed if we are to understand the role of GST- $\pi$  in human tumorigenesis, if any. Conditional (skin-specific) GST- $\pi$  knockout mice could be used as a model system. Several polymorphisms in GST family members exist<sup>36,42</sup> and have been associated with impaired detoxification, thus influencing the risk for several cancers, including non-melanoma skin cancer.<sup>43,44</sup> A GSTT1 null genotype is associated with high UV sensitivity,<sup>45</sup> and a GSTM1 null genotype also predisposes for BCC, probably due to its role in defence against UV-induced oxidative stress.<sup>32,46</sup> Polymorphism of GSTM3 was also shown to increase risk for BCC.<sup>47</sup> Another genetic factor involved in detoxification of photosensitizing agents, and thus involved in BCC carcinogenesis, is polymorphism of CYP2D6 (the gene encoding cytochrome P450), which is correlated with an increased number of BCCs.<sup>46</sup> Furthermore, some allelic variants of CYP2D6 are associated with a multiple presentation phenotype of BCC<sup>48,49</sup> and these patients are also at higher *a priori* risk for developing BCC.<sup>50</sup>

#### DNA repair

In 1973, Milstone and Helwig<sup>51</sup> noted that patients with xeroderma pigmentosum (XP), a group of rare autosomal recessive disorders characterized by severe photosensitivity due to various defects in DNA repair, are prone to developing cutaneous cancers, mostly SCC but also BCC and malignant melanoma.<sup>52–54</sup> There are several variants of the disease, all caused by a different genetic defect in nucleotide excision repair, global genome repair, transcription-coupled repair or combinations thereof. Some of the genes involved are essential components of the TFIIH transcription complex; their absence is associated not only with UV sensitivity but also with sometimes severe neurological defects and growth retardation (as in de Sanctis–Cacchione syndrome).<sup>55</sup> Interestingly, at least two types of XP are caused by defects in DNA helicases that are involved in nucleotide excision repair and in transcription. Werner and Bloom syndromes are hereditary skin cancer disorders that are associated with helicase defects but, curiously, not with the development of BCCs.<sup>56,57</sup> Rothmund–Thomsen syndrome, which in some cases is caused by defects in a DNA helicase,<sup>58</sup> does seem to predispose to BCC.<sup>59</sup> The reason for this difference is poorly understood. The expression patterns of helicases may play a role but it is not clear why helicases should be tissue-specific. Chromosomal breakage disorders such as ataxia teleangiectasia and Nijmegen breakage syndrome do not predispose to BCC. Neither does Li–Fraumeni syndrome, which can be caused by germline mutations in the p53 gene,<sup>60</sup> nor dyskeratosis congenita, a disorder associated with failure to maintain telomeres.<sup>61,62</sup> Why these forms of genomic instability do not seem to be causally related to BCC

is uncertain. It may reflect a very basic biological difference between BCC and other malignancies. Whereas most tumours sooner or later show chromosomal instabilities,<sup>63</sup> BCC does not seem to do so.

#### Embryonic signalling pathways – Hedgehog, Wntless, Ectodysplasin and NF- $\kappa$ B

Patients with the nevoid BCC syndrome (NBCCS) or Gorlin syndrome,<sup>64</sup> show a rapid development of numerous BCCs at a young age. Whereas XP is an autosomal recessive disorder, NBCCS is an autosomal dominant disorder.<sup>65</sup> A human homologue to the *Drosophila* segment polarity gene *patched*, *PTCH1* (there is also a *PTCH2* of as yet unknown importance) is mutated in NBCCS patients, suggesting a contribution to the tumorigenesis.<sup>66,67</sup> As NBCCS patients normally inherit one mutated copy of the *PTCH* gene, tumours are likely to arise after inactivation of the remaining allele.<sup>68</sup> Haploinsufficiency of the *PTCH1* gene is probably responsible for the dysmorphisms. XP patients and sporadic BCCs may also show mutations in the *PTCH* gene.<sup>69–71</sup> The *PTCH1* gene product is part of a receptor for a protein called Sonic Hedgehog, which is involved in embryonic development.<sup>72</sup> Sonic hedgehog (SHH) is expressed in the Hensen node, the floorplate of the neural tube, the early gut endoderm, the posterior limb buds and throughout the notochord, and encodes a signal responsible for patterning the early embryo.<sup>73–75</sup> When SHH binds to *PTCH*, it releases smoothened (SMO), a transmembrane signalling protein, from inhibition by *PTCH* (Fig. 1).<sup>76</sup> It is now believed that *PTCH* modulates SMOH in an indirect manner,<sup>77</sup> although it is not known how. There is some evidence that *PTCH* may influence the localization or intra-membrane conformation of SMOH.<sup>78</sup> SMOH in turn signals to GSK3 $\beta$ , which phosphorylates GLI3 (a human ortholog of the *Drosophila* gene *cubitus interruptus*). It is assumed that the human orthologues of *Costal2*, *Fused* and *Suppressor of Fused* (*Su(Fu)*) then form a tetramer as they do in *Drosophila* but so far, only *Su(Fu)* has been demonstrated in humans.<sup>79</sup> The complex then translocates, possibly via the microtubule-binding activity of *Costal2*, which has kinesin activity, to the nucleus where it can regulate activity of target genes such as WNT genes, BMP and *PTCH1* itself (Fig. 1 and reviewed in<sup>80</sup>). WNT signals are transduced via the APC/Axin/DSH complex which inhibits GSK3 $\beta$  activity; as a result,  $\beta$ -catenin is not degraded and complexes with TCF/Lef1 into a transcription complex which is active in early hair follicle growth and in the initiation of anagen.<sup>81</sup>  $\beta$ -catenin, in turn, can influence the NF $\kappa$ B pathway (see below). Activating mutations of  $\beta$ -catenin give rise to pilomatricoma, another hair follicle tumour, clearly showing the importance of  $\beta$ -catenin-mediated signalling for hair follicle growth.<sup>82,83</sup> Such mutations have not yet been found in BCC, and it would be of interest to look for such alterations in sporadic BCCs. Thus, a staggeringly complex regulatory network emerges. It is of interest to note that it is essential for hair

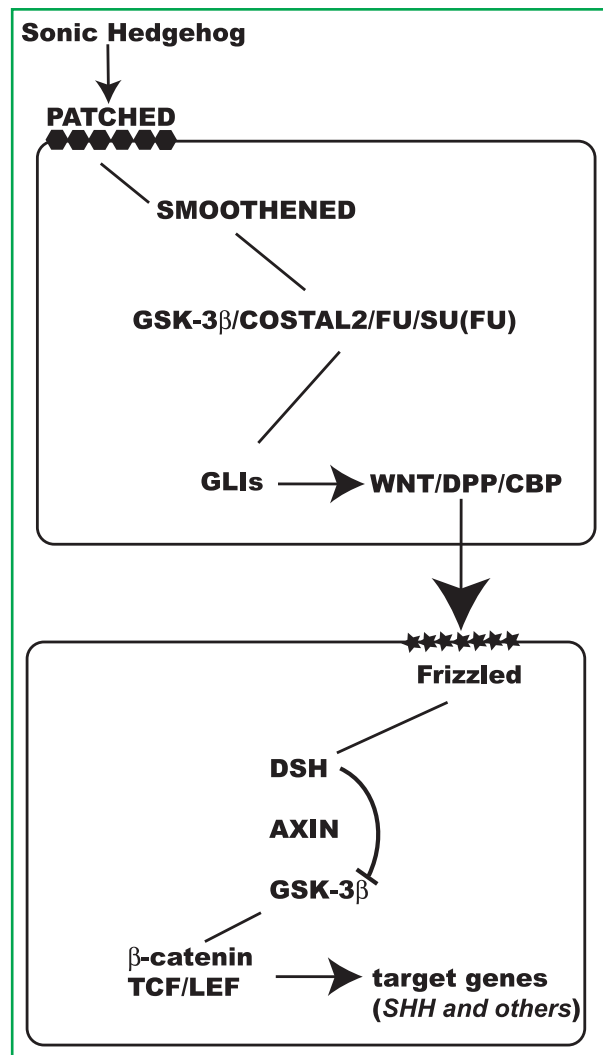


Fig 1. A simplified representation of the Sonic Hedgehog signalling network and its cross-talk with the Wnt signalling pathway. See the text for an explanation. GSK3 $\beta$ , glycogen synthetase kinase 3 $\beta$ ; FU, Fused; SU(FU), suppressor of fused; GLI, cubitus interruptus homologues; BMP, bone morphogenetic proteins; WNT, secreted Wingless signalling protein; CBP, CREB binding protein; TCF/LEF, transcription control factor/lymphoid enhancer factor; DSH, dishevelled family protein; APC, adenomatous polyposis coli protein.

follicle morphogenesis, suggesting that the development of BCC in the context of *PTCH1* mutations may represent uncontrolled hair follicle morphogenesis.

The high frequency of mutations in SMOH and *PTCH1* in BCCs, resulting in continuous activation of target genes, indicates that a disturbed HEDGEHOG pathway, resulting in excessive signalling, may be an important carcinogenic route.<sup>84</sup> UV irradiation enhances BCC development in *PTCH1* mutant mice.<sup>85</sup> Of the sporadic BCCs 20% show SMOOTHENED mutations<sup>86</sup> and 30–40% patched mutations.<sup>70</sup> In XP patients, the majority (~80%) of *PTCH1* mutations are UV-induced,<sup>87</sup> as expected more frequently than in sporadic BCCs,<sup>70</sup> where UV-signature mutations are seen in less than 50%.<sup>88</sup> *PTCH2*,

which is 57% identical to PTCH1, probably also serves as a receptor for hedgehog and related factors.<sup>89</sup> Mutations occur in sporadic BCC,<sup>90</sup> and it has been shown that when PTCH1 is mutated, PTCH2 mRNA is up-regulated.<sup>89</sup>

## NFκB

A good example of the importance of embryonic growth regulatory pathways in BCC carcinogenesis is the discovery that defects in one of the components of the NFκB signalling pathway can also cause BCC (own data, unpublished). The CYLD gene, which is mutated in familial cylindromatosis or Brooke–Spiegler syndrome, is a de-ubiquitinating enzyme that negatively regulates NFκB.<sup>91–93</sup> Its target is TNFR-associated factor 2 (TRAF2) and, to a lesser extent, TRAF6. Both TRAF2 and TRAF6 are implicated in the transduction of EDA/EDAR/EDARADD signals<sup>94–97</sup> and thus in the development of skin appendages, as mutations in EDA, EDAR, EDARADD and TRAF6 can all cause ectodermal dysplasias in humans and mice.<sup>96,98–100</sup> Recent data indicate that EDA can repress β-catenin-dependent transcription,<sup>101</sup> suggesting a regulatory connection between the EDA and PCTH pathways and hinting, again, at the central importance of the PTCH route for basal cell carcinogenesis. β-Catenin can control EDAR expression, showing that the EDA–NFκB pathway is subject to negative autotfeedback.

Normally, activation of NFκB is caused by phosphorylation of the NFκB inhibitor IκB through the IκB kinase complex.<sup>102</sup> The gene mutated in incontinentia pigmenti, NEMO (NFκB essential modulator), is part of the IκB kinase complex. Phosphorylation of IκB leads to its ubiquitination and subsequent disposal through the proteasome. CYLD was found to interact with NEMO.<sup>91–93</sup> This possibly transient interaction is required for its function, the de-ubiquitination of TRAF2 and TRAF6. The latter proteins are auto-ubiquitinating and need the ubiquitin tag for their normal function. Hence, CYLD down-regulates NFκB activation. Interestingly, CYLD mutations were recently found in familial trichoepithelioma. The latter tumour is also observed in familial cylindromatosis and resembles BCC to such a degree that a distinction is often difficult to make.<sup>103</sup> Indeed, we have recently observed the occurrence of a BCC in a patient suffering from familial cylindromatosis. These observations suggest that the NFκB pathway, which is involved in inflammation and in the embryogenesis of epithelial appendages, can also play a role in BCC. It should be of interest to examine sporadic BCCs for mutations in one of the components of the NFκB pathway. Likewise, patients suffering from multiple BCCs may harbour CYLD mutations.

## Unknowns

Finally, Rombo and Bazex syndromes are known to predispose to BCC. Both are characterized by the presence of numerous small cysts on the face and chest, as well as by hypotrichosis. The cysts contain vellus hairs.<sup>104,105</sup> Rombo syndrome is distinguished by striking degeneration of elastic fibrils in

sunlight-exposed areas causing dramatic skin alterations called atrophoderma vermiculatum.<sup>104,105</sup> In Bazex syndrome, so-called ice-pick scars are seen on the backs of the hands. This disorder is X-linked and has been mapped to Xq24–27.<sup>106</sup> Rombo syndrome is probably autosomal dominant, but otherwise very similar to Bazex. Identification of the causative genes should contribute significantly to our knowledge of hair follicle and BCC biology.

## Acquired genetic changes in basal cell carcinoma

### p53

The most common genetic aberrations in human skin cancers are found at the level of the p53 gene.<sup>107</sup> The p53 gene encodes a phosphoprotein that is involved in cell-cycle control and the maintenance of chromosomal stability.<sup>108,109</sup> In response to cellular stress, for example DNA damage, p53 is activated through phosphorylation.<sup>110,111</sup> MDM2 can associate with p53 and regulates its level and activity depending on the phosphorylation status of p53. When dephosphorylated, p53 will bind to MDM2 and is then degraded through the ubiquitin–proteasome pathway.<sup>112,113</sup> In response to DNA damage, p53 is phosphorylated by DNA damage-sensing proteins such as ATM and becomes detached from MDM2, resulting in stabilization and activation of target genes regulated by p53 (Fig. 2).<sup>114</sup> The response to DNA damage is either growth arrest, senescence or apoptosis.<sup>115</sup> The relative cellular content of p53 determines the response following DNA damage; when the content is low to moderate, cells will go into cell-cycle arrest to allow DNA repair, but when p53 levels are high, cells will progress to apoptosis.<sup>116</sup> p53 is capable of stimulating proapoptotic Bax expression<sup>117,118</sup> (see also below). In normal skin, wild type p53 is not detectable but appears within 2 h after UV irradiation, with peak levels at 24 h after irradiation and again undetectable levels at 36 h after irradiation.<sup>119</sup> Mutant p53 can accumulate in cells and p53 mutations have been detected in about half of all BCCs.<sup>120,121</sup> Furthermore, it was found that histologically proven aggressive BCCs are significantly associated with increased p53 expression, probably representing the mutated form although that assertion could not be established with certainty. However, it is striking that patients suffering from Li–Fraumeni syndrome do not show increased incidence of BCC. Consequently, it seems reasonable to assume that p53 mutations are secondary events in BCCs, occurring after tumour initiation. Considering the apparently limited contribution of DNA damage and chromosome instability to the BCC phenotype, the relevance of p53 mutations for BCC growth remains to be demonstrated. After all, in the absence of genetic damage, p53 activation will not occur. Moreover, one of the hallmarks of p53 dysfunction, aberrant mitosis, perhaps as a consequence of centrosome amplification,<sup>122</sup> has never been observed in BCC.<sup>123</sup>

In BCC patients, in a study comparing sunscreen users and non-users, it was shown that sunscreen users showed a significantly lower level of p53 mutations in their BCCs than

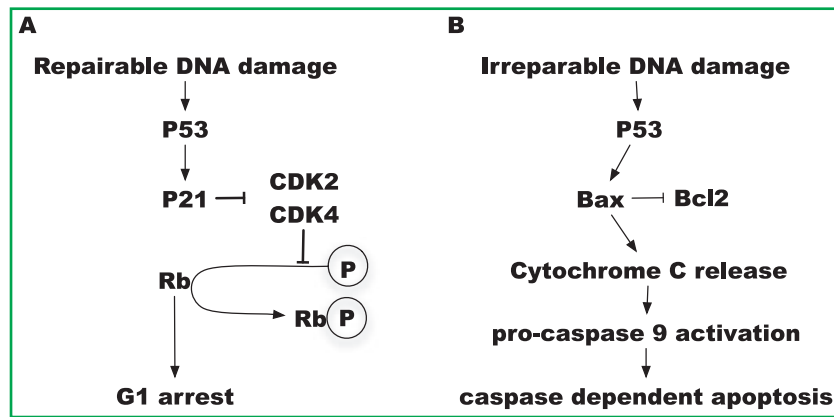


Fig 2. p53 signalling. DNA damage induces stabilization of p53. In the case of repairable damage (A), p53 triggers p21. This in turn inhibits cyclin-dependent kinases. As a consequence, Rb remains unphosphorylated and stalls the cell in the G1 phase of the cell cycle. In the case of irreparable DNA damage (B), p53 also induces Bax which then competes with Bcl2 in the mitochondrial membrane. As a result, cytochrome C is released from the mitochondria, triggering the caspase cascade that causes apoptosis.

non-users,<sup>124</sup> again suggesting that p53 mutations in BCCs are secondary events that may not contribute significantly to tumorigenesis. Mutational hotspots have been identified, with two-thirds of the mutations occurring at nine different sites.<sup>125</sup> Inactivation of p53 occurs predominantly by point mutation of one allele followed by loss of the remaining wild-type allele.<sup>126</sup> The p53 gene shows UV signature mutations, i.e. predominantly C(C) → T(T) conversions.<sup>125,127</sup> In 33% of BCCs found in Korean patients p53 mutations were detected<sup>69</sup> and up to 50% of the BCCs in Caucasian patients showed this mutation,<sup>120,121</sup> thus suggesting that different ethnic factors play a role in BCC carcinogenesis although differences in sun exposure (with the Westerners engaging more in recreational sunning) may just as well account for the differences observed.

## p63

The p63 gene, a p53 homologue, encodes multiple products and is restricted to cells with high proliferation potential and absent from cells undergoing terminal differentiation.<sup>128</sup> p63 has a nucleoplasmic distribution pattern in the basal compartment of stratified epithelia such as skin, tonsil, bladder and certain subpopulations of basal cells in prostate, breast, uterine cervix and bronchi.<sup>129–131</sup> p63-deficient mice have striking developmental defects such as absence or truncation of limbs, absence of hair follicles, teeth and mammary glands, and the skin lacks stratification and differentiation.<sup>132</sup> This indicates that p63 is essential for several aspects of differentiation during embryogenesis. Several isoforms of p63 can bind to p53 consensus sequences and activate p53 target genes. Isoform TAp63γ is capable of inducing cell-cycle arrest and apoptosis.<sup>133</sup> The ΔN isoforms, lacking the N-terminus, are unable to induce transcription, and have an antiapoptotic effect by rendering p53 and TA isoforms inactive. p63 is only rarely mutated in BCC.<sup>134</sup> It was shown that p63 functions not only as a stem-cell marker of keratinocytes<sup>135</sup> but may also maintain the stem-cell

phenotype.<sup>136</sup> In keeping with its basal localization in normal epidermis, BCC cells express p63.<sup>131,137</sup> It was shown that aberrant expression of p63 altered the UVB-induced apoptotic pathway suggesting that down-regulation of this protein in response to UV irradiation is important in epidermal apoptosis.<sup>138</sup>

## Immunological factors

### Immunosuppression

Organ transplant recipients are at greater risk for developing malignancies because of the prolonged, often life-long, immunosuppressive therapy.<sup>139–141</sup> SCC of the skin is the most common malignancy occurring in the setting of solid-organ transplantation and immunosuppression, and its incidence increases substantially with extended survival after transplantation.<sup>142</sup> SCC occurs more frequently in transplant patients,<sup>143</sup> whereas in the general population BCC is three to six times more frequent than SCC.<sup>144</sup> It was shown in Australian heart transplant recipients that the number of skin cancers is significantly correlated with both age at transplantation and duration of follow-up.<sup>145</sup> In Europe, 40% of renal transplant recipients develop skin cancer within 20 years after grafting.<sup>146</sup> Heart transplant recipients are at higher risk than kidney transplant recipients most probably due to the fact that they receive higher doses of immunosuppression agents,<sup>147</sup> but it cannot be overlooked that the different types of immunosuppressive agents have different effects in this respect. Increased incidence of BCC has not been described in organ recipients. From the available data, it seems clear that immunosuppression as practised after organ transplantation does not increase the risk of developing BCC. As stated above, the incidence of BCC seems not to be affected by PUVA treatment. A diminished response to skin application of dinitrochlorobenzene was found in people with SCC but not in patients with BCC, again supporting the notion that the incidence of BCC is not affected by immune status.<sup>148</sup>



### Human immunodeficiency virus

Seemingly in contradiction to the lack of an increase in the incidence of BCC in organ recipients, people suffering from acquired immune deficiency syndrome (AIDS) have shown an elevated risk for the development of BCC.<sup>149,150</sup> Human immunodeficiency virus (HIV) patients with BCC more frequently show blue eyes, blond hair, family history and extensive prior sun exposure.<sup>151</sup> The pigmentation phenotype is probably an independent risk factor that is added to the increased risk of BCC conferred by the immunosuppression. There have been some reports of BCCs metastasizing in people suffering from AIDS,<sup>152,153</sup> suggesting that immune surveillance is one of the factors determining the normally non-metastatic nature of the BCC. Why immunosuppression by HIV increases the risk of BCC, whereas pharmaceutical immunosuppression does not is not clear. The depletion of CD4 lymphocytes by HIV may lead to a more pervasive defect in adaptive antitumour immunity than does mere functional suppression by commonly used immunosuppressive compounds.

### Human leucocyte antigen (HLA) haplotypes

The major histocompatibility complex (MHC) genes code for membrane proteins that play important roles in controlling immune responses.<sup>154</sup> There are two classes of genes, class I (HLA-A, -B, -C) and class II (HLA-DR, -DQ, -DP), which play a role in host defence against the development and spread of tumours.<sup>155</sup> For example, loss of class I antigens is related to tumour progression in melanomas.<sup>156</sup> Furthermore, abnormalities in cell-mediated immunity have been reported in patients with multiple BCCs.<sup>157</sup> Whereas normal skin shows high levels of class I molecules, BCC shows either complete absence or heterogeneous expression.<sup>158</sup> All class I-negative tumours were histologically proven to be aggressive, whereas all non-aggressive BCCs were class I-positive. The low levels or absence of expression of class I antigens may result in escape from recognition by cytotoxic T cells, which then facilitates tumour growth.<sup>159</sup> Evidence for the involvement of HLA genes in the development of skin cancer was provided by Bouwes Bavinck *et al.*<sup>160</sup> These authors showed that the presence of HLA-DR7 and a decrease of HLA-DR4 are significantly associated with BCC. This corroborates the previous finding of Rompel *et al.*<sup>161</sup> that HLA-DR4 is decreased in BCC, especially in patients with multiple BCCs located on the trunk.<sup>162</sup> The authors suggested a protective role for HLA-DR4 against the development of BCC. HLA-DR1 is weakly associated with the development of multiple BCCs at an early age.<sup>163</sup> Furthermore, Bouwes Bavinck *et al.* presented two studies showing a correlation between HLA-A11 expression and skin cancer in immunosuppressed renal transplant recipients.<sup>164,165</sup> One of these studies showed that HLA-A11 was associated with resistance to skin cancer in renal transplant recipients,<sup>164</sup> while another study, in Australia, showed that renal transplant recipients with HLA-A11 had an increased

risk for developing skin cancer.<sup>165</sup> This apparent discrepancy may be the result of different genetic backgrounds and differential environmental factors.

### Human papillomavirus

Although HPV has been associated strongly with malignant progression of warts to SCC and with epidermodysplasia verruciformis,<sup>166</sup> different oncogenic subtypes of the virus were found in 60% of BCCs from immunosuppressed patients in contrast to 36% of BCCs from non-immunosuppressed patients, suggesting that these viruses may be involved in the development of BCC.<sup>167</sup> In renal transplant recipients with skin cancer HPV 5/8 DNA could be detected,<sup>28</sup> and Weinstock *et al.*<sup>30</sup> suggested immunosuppression to be a factor in BCC carcinogenesis by affecting HPV infection.

### Tumour stem cells

Several cell types have been suggested to be the precursor cells or stem cells for BCC: interfollicular basal keratinocytes, basal keratinocytes from hair follicles or sebaceous gland cells.<sup>168–173</sup> In general, stem cells have a relatively undifferentiated and slow-cycling phenotype, but can be stimulated to proliferate and give rise to transient amplifying cells which have a limited proliferative potential.<sup>174</sup> Stem cells may be the target of carcinogens and as such play an important role in tumorigenesis. One observation suggesting that uncommitted stem cells are the most likely cells of origin for human skin cancer is the fact that sunlight exposure in childhood may contribute to tumours many decades later.<sup>175</sup> As first suggested by Taylor *et al.*<sup>176</sup> the ultimate source of stem cells in the skin is the bulge region of the ORS.<sup>177–179</sup> As a result, hair follicles are likely to play an important role in skin homeostasis, wound healing and tumorigenesis.<sup>174</sup> Chemically induced BCCs in rats arise from hair follicles,<sup>180</sup> but it is not known whether this is also the case in humans.

Histologically, BCCs may resemble hair follicles,<sup>11</sup> and may show characteristics from both bulge region stem cells and transient amplifying cells.<sup>181</sup> In particular, BCC can histologically resemble trichoepithelioma, a benign hair follicle tumour.<sup>182</sup> The suprabulbar region of the ORS of the hair follicle has an immunohistochemical profile that is almost indistinguishable from that of a BCC.<sup>169,183,184</sup> The hair follicle hypothesis is further supported by the fact that when a carcinogen is added in the anagen phase, in which the hair follicle bulge region cells undergo transient amplification, BCCs are generated more frequently.<sup>185</sup> Furthermore, BCCs seldomly occur on non-hairy skin.<sup>181</sup> Support for the hair follicle hypothesis can be found in the expression of the basal cell adhesion molecule (B-CAM) in normal and diseased skin.<sup>186</sup> The fact that this cell-surface protein is preferentially expressed in suprabasal cell layers and the ORS of the hair follicle, and also shows high levels of expression in BCCs, suggests that BCCs originate from hair follicles rather than from basal keratinocytes, which are negative for B-CAM in

normal skin. However, the lack of cytokeratin 15 expression in the tumour cells suggests that BCCs do not differentiate towards a hair bulge cell fate.<sup>187</sup>

Howell and Mehregan<sup>188</sup> reported that the tiny pits in the epidermis of palms and soles characteristic of Gorlin syndrome<sup>64</sup> (or NBCCS, see above) occasionally show basaloid budding into the dermis, and have therefore suggested that they resemble tiny BCCs. This observation was taken to support the idea that BCCs can originate from interfollicular epidermis. There is no further proof to support this notion. Finally, our current understanding of the molecular genetics of BCC as outlined above also supports the notion that the hair follicle stem cell is the progenitor cell of the BCC. In all, it seems as if the BCC cell is a hair follicle stem cell in which the normal differentiation and anagen-initiation programme, of which the SHH network forms the backbone, has gone awry.

### Carcinogenesis

Tumour formation results from a disruption of the normal balance between cell proliferation and cell death.<sup>189</sup> Three large categories of genes affect cellular proliferation and survival, i.e. growth-promoting oncogenes, tumour suppressor genes and mutator or caretaker genes.<sup>190</sup> The normal counterparts of oncogenes, i.e. the proto-oncogenes, are crucial in regulating normal cell cycling and division, differentiation and apoptosis.<sup>191–193</sup> When these become mutated or amplified they can overcome the normal restraints of cell growth.<sup>194,195</sup> So-called 'tumour suppressor' genes can be involved in differentiation pathways that are coupled to cell growth, PTCH being a good example in point, and mutations or deletions of such genes have been reported in various types of cancer.<sup>196–198</sup> However, mismatch repair genes are also classified as tumour suppressors and the functional distinction between oncogenes, proto-oncogenes and so-called tumour suppressors is blurring. A more useful distinction is that in general (proto-)oncogenes need to be activated, and tumour 'suppressor' genes inactivated for malignant transformation to occur.<sup>199</sup> Defects in the latter appear to be more common than defects in oncogenes.<sup>200</sup> 'Mutator' or caretaker genes (involved in DNA repair) maintain the genome integrity and when their function is altered, mutations can accumulate more frequently.<sup>190</sup>

### Proliferation vs. differentiation in basal cell carcinoma

Proliferation indices vary greatly for the different subtypes of BCC, but in general relatively high percentages of proliferating tumour cells are found.<sup>201–204</sup> Based on immunohistochemical detection of the proliferation marker Ki-67, an average of 20% of the cells in BCC are found to be proliferating.<sup>202,203,205,206</sup> The proliferating cell nuclear antigen (PCNA) is present in <10% of non-recurrent BCCs, while recurrent BCCs show PCNA expression in >30% of the tumour cells.<sup>207,208</sup> In nodular and superficial BCC the proliferative activity is mainly restricted to the periphery of the tumour nests, whereas morphea-like tumours show a more

scattered pattern of proliferating cells.<sup>202,206</sup> An explanation for the zonal distribution of proliferation-potent cells may be that tumour cells migrating towards the centre of the tumour nests become more differentiated, or have less access to nutrients, resulting in lower proliferation potential.<sup>209</sup> Alternatively, adhesive properties may determine both behaviours.

Markers for the arrest of cell proliferation include the A-type lamins, also sometimes known as statins.<sup>210</sup> Nuclear lamins are intermediate filament proteins that form a network at the nucleoplasmic site of the nuclear membrane and can be divided in two subtypes, i.e. A-type lamins (lamin A, lamin A $\Delta$ 10 and lamin C) and B-type lamins (lamin B1 and lamin B2).<sup>211,212</sup> Aberrant expression patterns of lamins have been described in cancer and it is thought that the nuclear matrix plays a role in carcinogenesis.<sup>213</sup> In general, A-type lamin expression is correlated with a non-proliferating, differentiated state of cells and tissues,<sup>214</sup> and therefore altered expression of A-type lamins can be expected in cancer. In BCC, it was recently reported by Venables *et al.*<sup>215</sup> that the absence of lamin A correlated with rapid growth, while the absence of lamin C correlated with slow growth. Furthermore, it was reported that the expression of A- and B-type lamins varies with differentiation in normal epidermis<sup>216</sup> and skin tumours.<sup>216,217</sup> These authors support the idea that expression of A-type lamins, but not B-type lamins is associated with the differentiation phenotype of the tumours. Recently, Tilli *et al.*<sup>218</sup> reported four stages in BCC development based on different patterns of A-type lamin expression. Stage 1 comprises lamin A-negative, Ki-67-positive BCCs, representing the origin of BCC, while stage 2 comprises lamin A-positive, Ki-67-positive BCCs. As tumour growth slows down, lamin C is first relocated to the nucleolus in stage 3 and in stage 4 lamin C expression is largely diminished.

The fact that BCC shows relatively high percentages of proliferating tumour cells is not in line with the clinical finding that BCC is usually a slow-growing tumour.<sup>219</sup> Therefore, cell loss must be considered as an important factor in the net growth of BCC. Already in 1972, Kerr *et al.*<sup>220</sup> reported that a high apoptotic rate in BCC might account for the seemingly paradoxically slow growth rate. Furthermore, Mooney *et al.*<sup>221</sup> showed that BCCs do indeed exhibit a high apoptotic rate as based on *in situ* end-labelling of nicked DNA ends [using TUNEL staining, terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labelling].

### Apoptosis in epidermis and basal cell carcinoma

Apoptosis, a form of programmed cell death, is characterized by cell shrinkage and fragmentation.<sup>222</sup> Apoptosis is one process among others that is necessary for the correct development of an embryo,<sup>223</sup> and to eliminate autoreactive lymphocytes.<sup>224</sup> Abnormal, unwanted or damaged cells are removed by apoptosis without the involvement of the immune system, but through rapid phagocytosis of apoptotic cells before lysis, which prevents inflammation.<sup>225</sup> In this



respect the process of programmed cell death can be clearly distinguished from accidental cell death, i.e. necrosis.

During the different phases of apoptosis various sets of molecules act in an orchestrated fashion. These include:

**1 Death ligands and death receptors.** The family of death receptors is characterized by two to five copies of cysteine-rich extracellular repeats and a death domain within the intracellular carboxy-terminus of the receptor (the death domain).<sup>226</sup> When these death receptors are bound by ligands, apoptosis can be induced. Fas is an example of a type I transmembrane receptor which mediates apoptosis upon binding of the oligomerizing Fas ligand (FasL).<sup>227</sup> Fas is expressed on several different cell types, while expression of FasL is restricted to immune cells, including T and B lymphocytes, macrophages and natural killer cells.<sup>228,229</sup> Ligation of FasL to Fas causes rapid death-inducing signalling complex formation, which recruits and activates pro-caspase-8, thus triggering the apoptotic caspase cascade (see below).

**2 Bcl-2 protein family.** Many studies concentrate on the Bcl-2 family of apoptosis-regulating proteins.<sup>230,231</sup> Bcl-2 was first discovered in B-cell lymphomas showing a t(14:18) translocation,<sup>232</sup> resulting in a Bcl-2-immunoglobulin-heavy chain fusion gene.<sup>233</sup> This leads to overexpression of the antiapoptotic Bcl-2 protein. The protein has been shown to suppress apoptosis induced by various stimuli, such as depletion of interleukin (IL)-3 and IL-4<sup>234,235</sup> p53-induced apoptosis,<sup>236</sup> glucocorticoid treatment,<sup>237</sup> and c-myc induced apoptosis.<sup>238</sup> Bcl-2 expression has been localized to long-lived (stem) cells in self-renewing human tissues.<sup>191</sup> The protein is associated with the membranes of mitochondria, endoplasmic reticulum and nucleus,<sup>239</sup> and bears seven phosphorylation sites of which ser70 is critical for the apoptosis-suppressing function of Bcl-2.<sup>240</sup> A large number of Bcl-2-related proteins<sup>230,231,241,242</sup> have been isolated, which can act either as apoptosis-inducing (e.g. Bax, Bcl-xs) or apoptosis-suppressing agents (Bcl-xl). Heterodimerization between these family members determines whether a cell will die or not.<sup>243,244</sup>

**3 Caspases.** The aspartate-specific cysteine protease (caspase) cascade appears to be the main pathway for clearance of cellular constituents during the execution phase of apoptosis.<sup>245</sup> Several human caspases have been identified that share similarities in amino acid sequence, structure and substrate specificity.<sup>246,247</sup> Caspases show a high specificity for the conserved QACXG sequence, resulting in the cleavage after aspartic acid (Asp) residues.<sup>248</sup> The caspase family comprises apoptotic initiators (e.g. caspase-2, -8, -9 and -10) and apoptotic executioners (e.g. caspase-3, -6 and -7).<sup>246</sup> Caspase-3 seems to be responsible for the majority of apoptotic effects, and is supported by caspase-6 and -7. These three executioner caspases are important in the cleavage and degradation of several substrates, target proteins that are involved in RNA splicing, DNA repair, and scaffolding of the cytosol and the nucleus. Upon induction of apoptosis, caspase-3 cleaves the inhibitor of the caspase-activated DNase, resulting in degradation of DNA into oligonucleosomal fragments.<sup>249,250</sup> Lamin A is cleaved by

caspase-6<sup>251,252</sup> while in addition, cytoskeletal filaments such as cytokeratins are cleaved by caspase-6.<sup>253</sup> The externalization of phosphatidyl-serine at the cell membrane during apoptosis is also caspase-dependent.<sup>254</sup> The nuclear matrix protein poly(ADP-ribose) polymerase is also proteolysed by caspases during apoptosis.<sup>255</sup>

Inhibitors of apoptosis proteins, which are constitutively present in cells,<sup>256,257</sup> for example, bind to and inhibit caspase-3 and -7 as well as pro-caspase-9, but not caspase-1, -6, -8 or -10.

When apoptosis occurs inappropriately it may cause degeneration of normal tissue architecture or function. On the other hand, when apoptosis fails to occur this can give rise to dysregulation of tissue homeostasis, as a result of which neoplasms can arise.

Apoptosis in the epidermis is a common phenomenon. In the morphogenesis of human fetal skin and maintenance of adult epidermis apoptosis plays a pivotal role.<sup>258</sup> For example, the apoptosis machinery is activated during the normal terminal differentiation process in keratinocytes.<sup>259</sup> In fetal skin, cells undergoing apoptosis are present in several epidermal cell layers, whereas in neonatal epidermis these are found in the terminally differentiating granular cell layer, and in adult skin the spinous cells also show occasional apoptosis.<sup>258</sup> Furthermore, apoptosis occurs upon excessive UV light exposure, resulting in irreparable DNA damage (see also Fig. 2).<sup>260</sup> A significant negative correlation between the expression of either p53 or bcl-2 with the development of BCC has been described previously.<sup>261</sup> Mutation of p53 or overexpression of bcl-2 is sufficient to enhance the formation of BCC by suppressing apoptosis.<sup>262,263</sup>

Altered expression of Bcl-2 family member proteins in non-melanoma skin cancer has been reported extensively before,<sup>264</sup> suggesting that dysregulation of expression of these proteins may be a possible explanation of the indolent growth behaviour of BCC.<sup>206,265</sup> Bcl-2 is in general homogeneously expressed in BCC,<sup>206,266–268</sup> while the apoptosis-inducing Bax protein is also expressed at high levels.<sup>206,264</sup> These data clearly show that a considerable proportion of cells in BCC are in principle capable of undergoing apoptosis, corroborating the earlier findings of Mooney *et al.*<sup>221</sup>

Another apoptosis-inhibitor protein called survivin is expressed in 81% of BCCs, whereas it is not detected in normal skin, suggesting a contribution to the progression of BCC.<sup>269</sup>

Also, Fas-mediated apoptosis may be important for skin homeostasis. Hill *et al.*<sup>270</sup> suggested that dysregulation of Fas–FasL interactions may be central to the development of skin cancer. In normal skin, Fas is expressed in cytoplasmic membranes of the basal cell layer, while after sun exposure the expression of Fas is up-regulated in the entire epidermis. After further UV exposure, Fas expression is again down-regulated, resulting in negative staining in BCC.<sup>271</sup> BCCs express FasL, however, strongly and diffusely, providing evidence for an escape from local immunosurveillance by the induction of apoptosis in the peripheral T lymphocytes.<sup>272</sup>

## Current and future therapeutic modalities – from surgical to biological

Various surgical and non-surgical therapies are available for the treatment of BCC.<sup>273</sup> Medical history of the patient, age, tumour localization and size, physical condition, histological outcomes and cosmetic aspects will eventually determine the choice of therapy. Furthermore, Telfer *et al.*<sup>274</sup> published guidelines for the management of BCC, presenting evidence-based guidance for treatment. In spite of the fact that surgical excision is still the most prominent therapy used, non-invasive therapies such as photodynamic therapy (PDT)<sup>275</sup> or topical application of 5-fluorouracil (5-FU)<sup>276</sup> are currently becoming more and more interesting in selective cases, especially because of the improved cosmetic outcome.

### Surgery

Throughout this review we have used the term 'basal cell carcinoma' instead of 'basalioma', in keeping with current practice in the Netherlands. 'Carcinoma' suggests malignancy as well as full metastatic potential. Dermatological surgeons treat BCC as such and justify mutilating procedures by referring to the presumed malignant nature of the lesion, dreading recurrence as if it were melanoma. While definitely capable of causing local tissue destruction if left untreated, BCCs rarely, if ever, metastasize. Hence one may wonder whether it is really necessary to eradicate every last trace of tumour surgically. A recurrence often can be quite easily treated and will follow only about 1.6–5% of conventional excisions aimed at free margins (a procedure that can leave undetected residual cells, particularly in the morpheaform growth type).<sup>277,278</sup> As discussed below, biological therapies offer great promise and may be used as adjuvants to conventional excision, allowing for more conservative surgery in the future.

### Induction of apoptosis

Many currently used antineoplastic agents exert their therapeutic effects through the induction of apoptosis. Different cell types vary profoundly in their susceptibility, suggesting the existence of distinct cellular thresholds for apoptosis induction.<sup>279</sup> For example, BCC cells overexpressing IL-6 are resistant to UV irradiation and PDT-induced apoptosis.<sup>280</sup> Furthermore, it was shown that *de novo* p53 synthesis or stabilization of p53 is essential to induce apoptosis in BCC.<sup>281</sup> Overexpression of the antiapoptotic Bcl-2 has also been linked to resistance of cancers to various chemotherapeutic drugs.<sup>282</sup> In BCC, interferon (IFN)- $\alpha$  induces apoptosis and is thus effective in the treatment.<sup>283</sup> Untreated BCC cells express FasL but not the receptor, but in IFN- $\alpha$ -treated BCC patients the tumour cells express both FasL and receptor, whereas the peritumoral infiltrate mainly consists of Fas-receptor-positive cells.<sup>284</sup> Therefore, with IFN- $\alpha$  treatment, BCC most likely regresses through apoptosis.

Topical treatment of BCC with 5-FU has also been proven to be very successful. Up to 90% of treated BCCs show regression when 5-FU is applied in a phosphatidyl choline-based cream<sup>285</sup> or when it is locally injected in an epinephrine-containing gel.<sup>276</sup> The regression of tumours treated with 5-FU is probably caused by enhancing apoptosis in the tumour cells.<sup>286</sup> Recently, Nakaseko *et al.*<sup>287</sup> reported that apoptosis is involved in regression of the lesion after PDT in actinic keratosis. This therapy is also used for treatment of BCC,<sup>288</sup> where tumour cells may also undergo apoptosis.

Phytochemicals known to induce apoptosis are also being applied in cancer prevention and therapy.<sup>289</sup> Recently, Levin and Maibach<sup>290</sup> published an overview of plant-derived drugs and treatments in dermatology. It was shown that application of green tea polyphenolic fractions reduced UV-induced erythema, gave rise to a decrease in sunburns and could also reduce the number of UV-induced mutations in DNA.<sup>291</sup> Oral and topical application of black tea extracts also decreased photochemical damage to the skin.<sup>292</sup> Furthermore, in mice bearing skin tumours, tumour growth was inhibited by 70% after treatment with black tea, which was established by inhibition of proliferation and enhanced apoptosis.<sup>293</sup> Ajoene, an organosulphur compound of garlic,<sup>294</sup> has been shown to induce apoptosis in human promyeloleukaemic cells.<sup>295</sup> Recently, it was shown that ajoene can induce apoptosis in the human keratinocyte cell line HaCat and has a diminishing effect on BCC *in vivo* by down-regulating the expression of the apoptosis-suppressing protein Bcl-2.<sup>296</sup> Apart from the induction of apoptosis by directly targeting its mediators, the obvious role of the SHH pathway in BCC growth suggests that interference with this pathway may also be used to treat BCC. Indeed, it was recently shown that a SHH antagonist, the *Veratrum* alkaloid cyclopamine (11-deoxojervine) can be used to treat BCC.<sup>297</sup> Cyclopamine binds directly to Smoothened, which explains its activity in tumours characterized by activated SHH pathways.<sup>298</sup> Interestingly, its application to the surface of the tumour resulted not only in the rapid induction of apoptosis but also influenced the differentiation status in seven of seven tumours.<sup>299</sup>

### Modulation of differentiation

Retinoids (vitamin A metabolites and analogues) have also been shown to have suppressive effects on tumour promotion when administered in high doses, and the mechanism appears to be associated with modulation of growth, differentiation and apoptosis.<sup>300</sup> However, clinical experience suggests that the antitumour activity of retinoids when administered in tolerable doses is limited as a result of adaptation of the tumour's retinoid metabolism.<sup>301</sup> Retinoic acid metabolism blocking agents (RAMBAs) such as liarozole, possibly combined with retinoids in a relatively low dose, may offer a more tolerable and effective means of slowing tumour progression.<sup>301,302</sup>

## Immunomodulation

Because BCCs often elicit a strong inflammatory response, recent studies have sought to evaluate the effect of immunomodulatory compounds. One of the most promising is imiquimod, a Toll-like receptor 7/8 agonist that enhances the endogenous cytokine response (among others, INF- $\alpha$ , IL-10), stimulating the T-helper 1-mediated inflammatory responses. Several recent studies suggest that imiquimod can be used as a monotherapy, with excellent complete response rates (80% and more).<sup>303–306</sup> The tumours are infiltrated by macrophages and show an extensive apoptotic response.

## Conclusion

In order to develop better pharmacological treatments for BCC, we need to understand its biological nature. As long as it is considered a malignancy, radical surgery seems to be justified, in particular for tumours with a morpheaform growth pattern. Hence the crucial question is whether BCC is truly cancer. If we are defining cancer as a clonal expansion of cells that are no longer under host control, and are not subject to replicative senescence and apoptosis, BCC is true cancer. On the other hand, the lack of defects in the control of genomic integrity and the apparent inability to metastasize seem to suggest that there are fundamental differences between BCC and other malignancies. For example, there seems to be an important difference between frank malignancies and BCC with regard to the timing of the activation of the SHH pathway in the process of tumorigenesis. In many tumour types, such as those of the lung or the pancreatic duct, the SHH route has to be reactivated since it is not normally active in those tissues during adult life but only during embryogenesis. In the normal adult hair follicle on the other hand, the SHH pathway is subject to cyclic activation.<sup>307</sup> Hence, aberrations in this constitutional activity of the SHH pathway in the hair follicle may be initiating events in BCC tumorigenesis.<sup>308</sup> In other tumours, on the other hand, SHH activation seems to contribute to tumour progression rather than to initiation. In view of the foregoing, it is our opinion that the designation 'basalioma' is most appropriate, because it emphasizes the proliferative nature of the disorder, and at the same time indicates the limited malignant potential.

The availability of proapoptotic and immunomodulatory compounds for the treatment of BCC may change therapy in the near future. For the majority of patients, surgery may no longer be the first treatment option. Tumours that are currently resected with a wide margin, a procedure that can result in mutilating defects, might be treated by limited primary excision followed by adjuvant therapy using an immunomodulator, a proapoptotic agent and/or a cell-signalling modulator.

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