Hedgehog Pathway Inhibitors Promote Adaptive Immune Responses in Basal Cell Carcinoma

Atsushi Otsuka1, Jil Dreier1, Phil F. Cheng1, Mirjam Nægeli1, Holger Lehmann2, Lea Felderer3, Ian J. Frew2, Shigeto Matsushita1,3, Mitchell P. Levesque1, and Reinhard Dummer1

Abstract

Purpose: Basal cell carcinomas (BCCs) are tumors ignored by immune surveillance. Activated Hedgehog (Hh) signaling within primary cilia is a key driver in the pathogenesis of BCCs. We examined immune alterations during treatment with systemic Hh inhibitors.

Experimental Design: We investigated biopsies from patients with BCC before (23 patients) and after 4 weeks of treatment (5 patients) with Hh signaling inhibitor. Ber-Ep4, BCL-2, Ki-67, CD4, CD8, MHC class I, HLA-DR-class II, and SOX9 were analyzed by immunohistochemistry. Primary cilia were analyzed by double immunofluorescence of acetylated tubulin and SOX9. Differential gene expression for 84 cytokines and chemokines was analyzed in 3 patients.

Results: After 4 weeks of treatment, we found reduction of Ki-67, SOX9, Ber-Ep4, and BCL-2 expression in tumors associated with morphologic signs of squamous differentiation. In addition, the number of cilia-positive BCC cells was significantly decreased. An upregulation of MHC I expression on the cell membranes of residual tumor cells and an influx of CD4+, HLA-DR-class II+, and CD8+ cells with invasion into the tumor cell nests were found. Finally, qPCR arrays showed the differential expression of genes involved in modulating immune responses.

Conclusions: We show that Hh pathway inhibitor-induced tumor regression is accompanied by a dynamic change of the microenvironment with a disruption of immune privilege involving an influx of cytotoxic T cells, activation of the adaptive immune functions, and a profound alteration of the local chemokine/ cytokine network.

Introduction

Advanced basal cell carcinomas (BCCs) are a small subset of BCCs that cause significant morbidity and remain a therapeutic challenge due to their local invasiveness and proximity to vital structures (1). Despite the presence of cancer tests and other tumor antigens, BCCs escape from immune surveillance by the downregulation of HLA class I expression (2). Recently, targeted therapy based on knowledge of BCC pathogenesis has become available either commercially or in the context of human clinical trials. These orally available drugs, such as vismodegib and sonidegib, inhibit the Hedgehog (Hh) signaling pathway, and have improved the therapeutic repertoire (3–6).

The Hh pathway plays a crucial role in patterning and organogenesis during early development, and is largely inactive in the adult, except for its function in tissue repair and maintenance (7). The central components of the Hh pathway consist of three secreted ligands (Sonic Hh, Indian Hh, and Desert Hh), a negative regulatory receptor [Patched (PTCH)], a positive regulatory receptor [Smoothened (SMO), and glioma-associated oncogene (GLI) transcription factors (GLI1, GLI2, and GLI3; refs. 7, 8)]. Primary cilia are involved in a number of signaling cascades, including the Hh pathway (9). The primary cilium is a microtubule-based organelle that protrudes from the plasma membrane and acts as a sensor for extracellular signals. Hh signaling requires primary cilia. PTCH is located at the primary cilium in the absence of Hh; upon Hh binding, PTCH moves out of the cilium and SMO moves in and activates the GLI transcription factors (10, 11). In addition, a recent study showed that ciliary ablation strongly inhibited activated SMO-induced BCC-like tumors (12).

Vismodegib and sonidegib inhibit SMO and achieve significant tumor regressions including complete response in locally advanced and metastatic BCCs (3–6). Hh pathway activation was linked to BCC after the initial discovery of germline loss-of-function mutations in PTCH in patients with nevoid BCC syndromes (13). Most BCCs have mutations in the Hh signaling pathway that inactivate PTCH1 (loss-of-function mutation; refs. 8, 13) or, less commonly, constitutively activate SMO (gain-of-function mutation; ref. 14). These mutations cause active Hh pathway signaling, which in BCCs support proliferation of the neoplasia (3).

In this study, we demonstrate that inhibition of the Hh pathway results in upregulated MHC class I expression on BCCs and attracts MHC class II+, CD4+, and CD8+ T cells into the tumor

1Department of Dermatology, University Hospital Zurich, Zurich, Switzerland. 2Institute of Physiology, University of Zurich, Zurich, Switzerland. 3Department of Dermato-Oncology/Dermatology, National Hospital Organization Kagoshima Medical Center, Kagoshima, Japan.

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A. Otsuka, J. Dreier, and P.F. Cheng contributed equally to this article.

Corresponding Authors: Reinhard Dummer, University Hospital of Zurich, Gloriastrasse 31, CH-8091, Zurich, Switzerland. Phone: 41-44-255-2507; Fax: 41-44-255-8988; E-mail: reinhard.dummer@usz.ch; or Mitchell P. Levesque, mitchellpaul.levesque@usz.ch

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Translational Relevance
Basal cell carcinoma (BCC) is the most common malignancy in humans. Despite the presence of cancer testsis antigens, BCCs are tumors that escape from adaptive immune surveillance. Because Hedgehog (Hh) pathway inhibitors are used in the clinic to treat patients with advanced or metastatic BCC, we investigated immune alterations during this systemic therapy. In this report, we show that Hh pathway inhibitor treatment reduces tumor burden and is accompanied by a recruitment of cytotoxic T cells into the tumor and reduction in the frequency of ciliated cells, which appear to be required for Hh inhibitor efficacy. We propose that these immune responses are crucial for long-term tumor control and hypothesize that a combination of Hh inhibitors with immune modifiers might be therapeutically beneficial.

Materials and Methods

Patients and treatments
We investigated biopsies of 23 patients with BCC treated with vismodegib (n = 22) or sonidegib (n = 1). Biopsies were collected after informed consent (EK number 647) was given. Patients were treated with vismodegib (150 mg daily) in the STEVIE study (a single-arm, open-label, phase II, multicenter study to assess the safety of vismodegib in patients with locally advanced or metastatic BCC) or with sonidegib (200 or 800 mg daily) in the BOLT study (a phase II study of efficacy and safety in patients with locally advanced or metastatic BCC; ref. 15).

The overall response rate in those patients with measurable disease was according to RECIST Version 1.1 (RECIST, v1.1). Evaluation of target lesions was performed as below. Complete response was disappearance of all target lesions. Any pathologic lymph nodes (whether target or nontarget) must have reduction in short axis to <10 mm. Partial response: at least a 30% decrease in the sum of diameters of target lesions, taking as reference the baseline sum diameters.

Punch biopsies (3–6 mm) were taken before (23 samples) treatment initiation and after 4 weeks (5 samples, detailed information on the patients: Table 1) of treatment. Punch biopsies were outside the initial biopsy areas.

Immunohistochemistry
All tissues used for immunohistochemistry were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were deparaffinized in xylene and rehydrated. Epitope retrieval was performed in antibody-specific buffers. The following antibodies were used: Ber-Ep4, Bcl-2, Ki-67, CD8 (DAKO), CD4, HLA-DR-class II (Novocastra), MHC class I (BDI Research Diagnostics), CD68 (DAKO), Foxp3 (Abcam), and Sox9 (Millipore). Staining was performed using kits supplied by Ventana or Dako REAL Detection System (kit 5005). Antigen-specific antibodies were applied and visualized with either the iVIEW DAB detection Kit (Ventana) or the ChemMate detection Kit (Dako). Slides were counterstained with hematoxylin and eosin (H&E). The figures show representative paired pre- and posttreatment samples.

Immunofluorescence
Sections were deparaffinized in xylene and rehydrated with 70% EtOH. Antigen retrieval was performed at 110°C in a pressure cooker for 5 minutes in citrate buffer. Blocking was performed for 30 minutes at room temperature in blocking solution (10% goat serum, 0.3% Tween). Incubation was carried out with the following primary antibodies: acetylated Tubulin (T6793; Sigma; 1:1,000 dilution) and Sox9 (sc-20095; Santa Cruz Biotechnology; 1:50 dilution). Incubation was carried out with the following secondary antibodies: Alexa-488 goat anti-mouse (Life Technologies; 1:1,000 dilution) and Sox9 (Millipore). Staining was performed using kits supplied by Ventana or Dako REAL Detection System (kit 5005). Staining was performed using kits supplied by Ventana or Dako REAL Detection System (kit 5005). Antigen-specific antibodies were applied and visualized with either the iVIEW DAB detection Kit (Ventana) or the ChemMate detection Kit (Dako). Slides were counterstained with hematoxylin and eosin (H&E). The figures show representative paired pre- and posttreatment samples.

RT-PCR
Total RNA was extracted from a punch of frozen tumor material using TRIzol (Invitrogen) according to manufacturer's instructions. cDNA was made from the RT2 HT First Strand Kit (330441; Qiagen) according to manufacturer's instructions. Genes were evaluated with the Human cytokine & chemokine PCR array.

Table 1. Clinical presentation and histology of the 5 patients with paired biopsies

<table>
<thead>
<tr>
<th>Patient</th>
<th>Clinical presentation</th>
<th>Histology before treatment</th>
<th>Histology after 4 weeks</th>
<th>Tumor size (after 4 weeks and 8 weeks)</th>
<th>Best response after weeks of vismodegib treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Locally advanced, facial, ulcerated, 150 × 80 mm measuring BCC</td>
<td>Solid BCC</td>
<td>Regression of tumor with inflammatory infiltrate</td>
<td>120 × 80 mm, 120 × 80 mm</td>
<td>CR after 16 weeks</td>
</tr>
<tr>
<td>2</td>
<td>Locally advanced, thoracic, ulcerated, 80 × 60 mm measuring BCC</td>
<td>Solid BCC</td>
<td>Regression of tumor with inflammatory infiltrate</td>
<td>80 × 60 mm, 50 × 40 mm</td>
<td>PR after 12 weeks</td>
</tr>
<tr>
<td>3</td>
<td>Locally advanced, facial, ulcerated, 50 × 60 mm measuring BCC</td>
<td>BCC with basosquamous differentiation</td>
<td>Epithelial tumor rests infiltrated by inflammatory cells</td>
<td>40 × 40 mm, 40 × 35 mm</td>
<td>PR after 16 weeks</td>
</tr>
<tr>
<td>4</td>
<td>Multiple locally advanced, recurrent BCC, 10 × 12 mm with lower leg</td>
<td>Superficial BCC</td>
<td>Superficial tumor rests and inflammatory infiltrate, differentiation, inflammatory infiltrate, and cystic formation</td>
<td>10 × 12 mm, 10 × 10 mm</td>
<td>PR after 12 weeks</td>
</tr>
<tr>
<td>5</td>
<td>Multiple BCC, 45 × 15 mm with upper arm</td>
<td>Superficial BCC</td>
<td>Regression of BCC with focal basosquamous differentiation, inflammatory infiltrate, and cystic formation</td>
<td>45 × 15 mm, 45 × 10 mm</td>
<td>MR after 12 weeks</td>
</tr>
</tbody>
</table>

Abbreviations: CR, complete remission according protocol; MR, minor remission (not qualifying for PR, but clinical improvement); PR, partial remission according to protocol.
Figure 1. Histology and immunohistochemical stainings of Ki67, Sox9, BerEP, and bcl2. A, HE staining and Ki67, Sox9, BerEP, and bcl2 by immunohistochemical staining of a representative pair of biopsies with different HPFs before and after 4 weeks of treatment. Scale bar, 100 µm. B, Ki67, BerEP, Bcl2, and Sox9 expression on tumor cells was quantified by investigation of 4 × 40 HPF representative of different patients and different HPFs per sample and evaluated as grade 1 (<10% of cells or none staining for Sox9), grade 2 (10%–30% of cells or weak staining for Sox9), and grade 3 (>30% of cells or strong staining for Sox9).
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(PAHS-150ZA; Qiagen) with the Viaa7 system from Applied Biosystems. Fold change and \( P \) values were calculated by RT2 Profiler PCR Array Analysis (Qiagen). Genes were normalized to the three housekeeping genes on the array.

**Image analysis and quantification**

Images of the stained paraffin sections were acquired with Scanscope Image (Image Scope; Aperio Technologies). Quantification of CD4, CD8, HLA-DR-class II, CD68, and Foxp3\(^+\) cells in intratumoral and peritumoral regions was done by counting cells in high-power fields (HPF) of \( \times 40 \) magnification. For each section, \( 4 \times 40 \) HPF representative areas per sample were counted. Immunoreactivity of Ki67 (nuclear), Sox9 (nuclear), Ber-EP4 (cytoplasm), Bcl2 (cytoplasm), and MHC class I expression (membrane of tumor cells) on tumor cells was quantified by investigation of \( 4 \times 40 \) HPF representative areas per sample and evaluated as grade 1 (<10% of cells), grade 2 (10%–30% of cells), and grade 3 (>30% of cells). The number of cilia-positive cells in each section was determined by counting cells in HPFs of \( \times 100 \) magnification. The nucleated cells are counted in all experiments. A point on the immunological figures represents each single counted field.

**Statistical analysis**

Data of immunological studies and RT-PCR are presented as the mean ± SD. RT-PCR data include data of three independent experiments. \( P \) values were calculated with a parametric Student \( t \) test. *, \( P < 0.05 \).

**Results**

**Reduction of Ki67, Sox9, Ber-EP4, Bcl-2, and cillum expressions on BCC after the treatment with Hh pathway inhibitors**

In all 23 pretreatment biopsies, diagnosis of BCC was histologically confirmed in HE-stained sections by a board-certified dermatopathologist (R. Dummer). Twenty-three patients (10 male, 13 female; 46–90 years; mean, 70 years) were all partial responders of Hh pathway inhibitor treatment. Further investigation was focused on 5 patients (Table 1), with paired biopsies taken before and after 4 weeks of treatment with Hh pathway inhibitors, who consented to the biopsy for clinical research. All patients suffered from locally advanced BCC without Gorlin syndrome. Clinical presentation and histologic diagnosis of these patients are summarized in Table 1. In all cases, comparison of HE-stained sections from before and after 4 weeks of Hh pathway inhibitor treatment showed a tumor regression with a reduction of the tumor nest (Fig. 1A). The morphology was altered with a more eosinophilic staining pattern and signs of cornification suggesting a transdifferentiation into a squamous phenotype. In one case, we observed the formation of cystic structures filled with keratin (Fig. 1A).

We examined the expression of tumor- or BCC-associated proteins by immunohistochemical staining. In all cases, we found dramatic reduction of the expression of the proliferation marker Ki-67, the stemness marker Sox9, the hair follicle–specific antigen Ber-EP4, and the antiapoptotic molecule Bcl-2 on the tumor cells after 4 weeks of treatment with Hh pathway inhibitors (Fig. 1A and B). This was in one case associated with cyst formation.

The primary cilium has emerged as an important organelle that is required for Hh signaling (9). However, it is still unknown how cilia change on human BCCs during Hh pathway inhibitor treatment. Therefore, we next examined the expression of cilia that are identified by acetylated tubulin. In all cases investigated, we found ciliated BCC cells by immunofluorescence. Intriguingly, the number of cilia-positive cells on BCC is significantly decreased after 4 weeks of treatment compared with that before Hh pathway inhibitor treatment (Fig. 2). We confirmed that there was no nonspecific staining for cilia (Supplementary Fig. S1A).

**Promotion of adaptive immune cell infiltration and upregulation of MHC class I in BCCs following Hh inhibitor treatment**

We next analyzed the effect of Hh pathway inhibitor treatment on immune reactions in BCCs. In pretreatment biopsies, CD68\(^+\), CD4\(^+\), CD8\(^+\), and Foxp3\(^+\) cells were rare and solely located in the stroma. Immunohistochemical staining revealed an increase and invasion of CD8\(^+\) T cells into tumor cell nests (Fig. 3A) with an upregulation of MHC class I expression on tumor cell membranes (Fig. 3B; Supplementary Fig. S2). Interestingly, at week 4, an increase of peri- and intratumoral CD4\(^+\) T cells was detected in the tumor area (Fig. 3C). In addition, the number of HLA-DR-class II\(^+\) mononuclear cells was significantly increased in the intratumoral and peritumoral areas after 4 weeks of treatment (Fig. 3D).

Moreover, the number of CD68\(^+\) macrophages was significantly increased in the intratumoral and peritumoral areas after 4 weeks of treatment (Fig. 4A). BCC has been shown to be associated with regulatory T cells (Treg) that could contribute to an immunosuppressive activity against tumor-specific T-cell

**Figure 2.** Expression of cilia and SOX9. A, cilia and Sox9 by immunohistochemical staining of a representative biopsy pair before and after 4 weeks treatment. B, 3 \( \times \) 100 HPF representative areas per sample were counted in BCC biopsies from patients before and after 4 weeks of treatment with Hh inhibitors (\( n = 4 \)). * \( P < 0.05 \).
responses (16). Therefore, we next analyzed the number of Foxp3+ Tregs. The number of Tregs was significantly increased in the intratumoral and peritumoral areas after 4 weeks of treatment (Fig. 4B). Interestingly, the ratio CD8+/Foxp3+ was increased only in the intratumoral but not in peritumoral areas (Fig. 4C). We confirmed that there was no nonspecific staining (Supplementary Fig. S1B).

Change of the cytokine and chemokine milieu after Hh pathway treatment

It is likely that there is an immunological change after the treatment of Hh pathway inhibitors. To address this issue, we performed qRT-PCR using an array that included 84 cytokines and chemokines. The results showed 6 genes to be differentially expressed after Hh pathway inhibitor treatment. Of these 6 genes, 5 were upregulated by at least 2-fold after the treatment and 1 was downregulated at least 2-fold after treatment (Fig. 5). There was a significant increase of the expression levels of the chemokines and cytokines: chemokine (C-C motif) ligand (CCL) 18, CCL21, chemokine (C-X-C motif) ligand (CXCL) 9, vascular endothelial growth factor A (VEGFA), and TNF ligand superfamily member 11 (TNFSF11, receptor activator of nuclear factor kappa-B ligand, RANKL). Furthermore, the qPCR results showed a consistent decrease during treatment of the expression levels of TNF receptor superfamily member 11B (TNFRSF11B, osteoprotegerin; Fig. 5). The expression level of IFNγ increased 1.63-fold, but this was not significant.

Discussion

This study reports on the histologic alterations on the tumor cell population and the inflammatory tumor microenvironment.
of patients with BCCs as a result of Hh inhibitor treatment. These data provide evidence that the Hh pathway–induced tumor regression leads to a dramatic change in the microenvironment that is characterized with a disruption of immune privilege and the activation of the adaptive immune effector functions.

A previous study demonstrated that vismodegib showed a 30% and 60% response rate for metastatic and locally advanced BCC (5). However, most responses were partial responses. In addition, significant adverse events with negative impact on the quality of life, including muscle spasms, alopecia, dysgeusia, weight loss, and fatigue, were occurred in more than 30% of patients. Therefore, improvement of the therapy using Hh pathway inhibitors is required. In this study, we demonstrated that Hh pathway inhibitors promoted adaptive immunity via upregulation of MHC class I and infiltrating immune cells into the tumor sites. A combination therapy with immune modifiers has a possibility to improve the efficiency by shortening the treatment-related drug exposure and achieving a complete remission rate.

We demonstrated substantial alterations in the immune microenvironment with an intra- and peritumoral increase of CD4+ and cytotoxic CD8+ T cells and an upregulation of MHC class I during tumor regression under treatment with Hh pathway inhibitors. In addition, reduction of primary cilia was observed after Hh pathway inhibitor treatment. Primary cilia have been implicated in Hh pathway signaling, and ablation of cilia in SMO-activated cells inhibits tumor growth (17). Before treatment, all BCC cells are ciliated, suggesting that they are responsive to Hh signaling. By inhibiting Hh signaling, the BCC cells lose their cilia and subsequently stop proliferating, as seen in the reduction of Ki-67 staining.

T-cell activation requires both T-cell receptor (TCR) and costimulatory molecule ligation by professional antigen-presenting cells (APC), and the outcome of the stimulatory signal is influenced by the microenvironment of the T cell and the APC. The Hh signaling pathway reduced the strength of the TCR signal in mature peripheral T cells (18, 19). In addition, the repression of the Hh signaling pathway in T cells increased T-cell activation (20). This suggests that the Hh pathway inhibitor has direct effects on peripheral T cell and activates adaptive immune responses. The precise mechanism of the Hh inhibitor on immune modulation requires further investigation.

RT-PCR results showed that the expression levels of different chemokines and cytokines changed during treatment (Fig. 5).
Although these are limited in number, our data suggest that these changes in the immune environment may be crucial for tumor control and highlight the poorly understood interface between targeted therapy and immune responses. BCCs frequently express multiple cancer testis antigens, but are also associated with relative absence of MHC class I molecules from tumor cells, and it has been hypothesized that BCCs produce immunosuppressive factors such as IL10 (2, 21) and therefore are resistant to adaptive immune response. This might explain the absence of infiltrating CD8+ cells in BCC. We found that the chemokines CCL18, CCL21, and CXCL9 were upregulated with active Hh/GLI signaling (28). mRNA levels including SOCS1 promoter, and STAT1 phosphorylation was reduced in cells induced by IFNγ, which has chemotactic activity on T cells. A potential crosstalk between IFNγ and the Hh pathway was recently described by Laner-Plamberger and colleagues (28). They demonstrated that suppressor of cytokine signaling 1 (SOCS1) is a direct target of Hh/GLI signaling in human keratinocytes and medulloblastoma cells and a potent inhibitor of IFNγ–STAT1 signaling, which can induce cell cycle arrest, apoptosis, and antitumor immunity. It was shown that the transcription factors GLI1 and GLI2 activated the SOCS1 promoter, and STAT1 phosphorylation was reduced in cells with active Hh/GLI signaling (28). mRNA levels including IFNγ depend on when the samples are collected (29, 30). A previous report showed that IFNγ mRNA expression in the skin-infiltrated IFNγ-producing T cells increased at the peak of 12 hours and diminished after 24 hours in skin allergic inflammation (30). In addition to the limited number of samples in this work, the biopsy time point may be too late to directly document the IFNγ peak.

In vitro studies on bone tissue showed that Hh signaling indirectly induced osteoclast differentiation by upregulating osteoblast expression of parathyroid hormone–related protein (PTHrP), which promoted RANKL expression (31). Mouse models on human bone tissue emphasized these results. RANKL was induced by activation of the Hh pathway, and the expression of RANKL was inversely associated with that of HLA-G5, especially with Hh stimulation (32). Our analyses of cytokine profiles showed an upregulation of RANKL during treatment with Hh inhibitors. RANKL is also expressed by Th cells and is thought to be involved in dendritic cell maturation as well as the regulation of T-cell–dependent immune responses.
In conclusion, we demonstrated that Hh pathway inhibitor treatment–induced tumor regression was accompanied by a recruitment of cytotoxic T cells into the tumor and reduction in the frequency of ciliated cells, which appeared to be required for Hh inhibitor efficacy. Therefore, we propose that these immune responses are crucial for long-term tumor control and hypothesize that a combination of Hh inhibitors with immune modifiers might be therapeutically beneficial.

Disclosure of Potential Conflicts of Interest

R. Dummer is a consultant/advisory board member for Roche and Novartis. No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: A. Otsuka, P.F. Cheng, M. Nágeli, J.I. Frew, M.P. Levesque, R. Dummer

Development of methodology: M. Nágeli, M.P. Levesque, R. Dummer

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Otsuka, P.F. Cheng, M. Nágeli, H. Lehmann, I. Felderer, R. Dummer

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Otsuka, J. Dreier, P.F. Cheng, M. Nágeli, J.I. Frew, M.P. Levesque, R. Dummer

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