Hedgehog pathway inhibitors promote adaptive immune responses in basal cell carcinoma

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Running Title: Hedgehog Pathway Inhibitors effect on Basal Cell Carcinoma

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Basal cell carcinoma (BCC) is the most common malignancy in humans. Despite the presence of cancer testis antigens, BCCs are tumors that escape from adaptive immune surveillance. Since 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.
Abstract

Purpose:
Basal cell carcinomas (BCC) 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 BCC patients before (23 patients) and after 4 weeks 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 were 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 morphological signs of squamous differentiation. In addition, the number of cilia-positive BCC cells was significantly decreased. An up-regulation 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 was 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.
Advanced basal cell carcinomas (BCCs) are a small subset of basal cell carcinomas 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 testis and other tumor antigens, BCCs escape from immune surveillance by the down-regulation 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) (7, 8). Primary cilia are involved in a number of signaling cascades including the hedgehog 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) (8, 13) or, less commonly, constitutively activate SMO (gain-of-function mutation) (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 up-regulated MHC class I expression on BCCs and attracts MHC class II+, CD4+, and CD8+ T cells into the tumor cell nests. The in situ cytokine and chemokine networks are altered to an immune-supportive network and cilia on BCCs were decreased during the treatment of Hh pathway inhibitors. This suggests that Hh pathway inhibitors promote adaptive immune reactions in BCCs.

**Materials and methods**

**Patients and treatments**
We investigated biopsies of 23 BCC patients 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 (150mg 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 sonedigib (200 or 800mg daily) in the BOLT study (a phase II study of efficacy and safety in patients with locally advanced or metastatic BCC) (15).

The overall response rate in those patients with measurable disease was according to Response Evaluation Criteria in Solid Tumors, Version 1.1 [RECIST, v1.1]. Evaluation of target lesions was performed as below. Complete Response: disappearance of all target lesions. Any pathological lymph nodes (whether target or non-target) 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 (3-6mm) 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, Carpinteria, CA), CD4, HLA-DR-class II (Novocastra, Newcastle, UK), MHC class I (RDI Research Diagnostics, Flanders, NJ), CD68 (DAKO), Foxp3 (Abcam, Cambridge, UK), and Sox9 (Millipore, MA). Staining was performed using kits supplied by Ventana (Ventana, AZ) or Dako REAL Detection System (kit 5005) (Carpinteria, CA). 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 haematoxylin and eosin (HE). The figures show representative paired pre- and post-treatment samples.

**Immunofluorescence**

Sections were deparaffinized with 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 with primary antibodies: acetylated Tubulin (T6793, Sigma) 1:1000 dilution and Sox9 (sc-20095, Santa Cruz) 1:50 dilution. Incubation with secondary antibody: Alexa-488 goat anti-mouse (Life Technologies) 1:1000 dilution and Alex-568 goat anti-rabbit (Life Technologies)
1:100 dilution. Incubation with DAPI (500ng/ml). The results showed representative paired pre- and post-treatment 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 RT2 HT First Strand Kit (330441, Qiagen, Germany) according to manufacturer’s instructions. Genes were evaluated with the Human cytokine & chemokine PCR array (PAHS-150ZA, Qiagen, Germany) with the Viia7 system from Applied Biosystems (Carlsbad, CA). 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, CA). Quantification of CD4, CD8, HLA-DR-class II, CD68, Foxp3+ cells in intratumoral and peritumoral regions was done by counting cells in high-power fields (HPF) of ×40 magnification. For each section, 4 ×40 HPF representative areas per sample were counted. Immunoreactivity of Ki67 (nuclear), Sox9 (nuclear), BerEP (cytoplasm), Bcl2 (cytoplasm), and MHC class I expression (membrane of tumor cells) on tumor cells was quantified by investigation of 4 ×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 high-power fields (HPF) of ×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 means ± standard deviation (SD). RT-PCR data include data of three independent experiments. P-values were calculated with a parametric Student’s t-test. * p < 0.05.

**Results:**

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

In all 23 pre-treatment biopsies, diagnosis of BCC was histologically confirmed in HE stained sections by a board certified dermatopathologist (RD). Twenty three patients (10 male, 13 female;
Further investigation was focused on 5 patients (Table 1) with paired biopsies taken before and after 4 weeks treatment with Hh pathway inhibitors, who consented to the biopsy for clinical research. All patients suffered from locally advanced BC without Gorlin's syndrome. Clinical presentation and histological 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 (Figure 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 (Figure 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 anti-apoptotic molecule Bcl-2 on the tumor cells after 4 weeks treatment with Hh pathway inhibitors (Figure 1A, 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 treatment compared to that before Hh pathway inhibitor treatment (Figure 2). We confirmed that there was no non-specific staining for cilia (Supplementary Figure S1A).

**Promotion of adaptive immune cell infiltration and up-regulation 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 pre-treatment biopsies, CD68+,CD4+,CD8+ and FoxP3+ cells were rare and solely located in the stroma. Immunohistological staining revealed an increase and invasion of CD8+ T cells into tumor cell nests (Figure 3A) with an upregulation of MHC Class I expression on tumor cell membranes (Figure 3B, Supplementary Figure S2). Interestingly, at week 4 an increase of peri- and intratumoral CD4+ T cells was detected in the tumor area (Figure 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 (Figure 3D).

Moreover, the number of CD68+ macrophages was significantly increased in the intratumoral and peritumoral areas after 4 weeks of treatment (Figure 4A). BCC has been shown to be associated with regulatory T cells (Tregs) that could contribute to an immunosuppressive activity
against tumor-specific T cell 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 (Figure 4B). Interestingly, the ratio CD8+/Foxp3+ was increased only in the intratumoral and but not in peritumoral areas (Fig. 4C). We confirmed that there was no non-specific staining (Supplementary Figure 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 (Figure 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 tumor necrosis factor 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 tumor necrosis factor receptor superfamily member 11B (TNFRSF11B, osteoprotegerin) (Figure 5). The expression level of interferon (IFN)-γ increased 1.63-fold but this was not significant.

**Discussion**

This study reports on the histological alterations on the tumor cell population and the inflammatory tumor microenvironment of patients with BCCs as a result of Hh inhibitor treatment. The 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 basal-cell carcinoma (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 up-regulation 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
signalling. 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 co-stimulatory 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. Hh signaling pathway reduced the
strength of the TCR signal in mature peripheral T cells (18, 19). Additionally, 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 chemo- and cytokines changed
during treatment (Figure 5). Although these are limited in number, our data suggest that these
changes in the immune environment may be crucial for tumor control and highlights 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 IL-10 (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, CXCL9 were up-regulated during treatment with Hh pathway inhibitors. These
chemokines are produced and secreted by innate immune cells such as macrophages and exert
their effect mainly on the adaptive immune system. It is known that epithelial cells can produce
CCL18, CCL21, and CXCL9 (22-24). These chemokines are thought to have a critical role in
tumor suppression. Interestingly, high CCL18 or CCL21 expression was reported as a favourable
prognostic factor in patients with colorectal cancer (25, 26). Additionally we found an
upregulation of VEGFA during treatment, which may support granulation reactions with
angiogenesis in regressive BCC lesions.
Although the expression level of IFN-γ increased 1.63-fold, and was not significant in this study, our current findings provide an indirect indication for an IFN-γ primed micro-environment favoring immune response. It is known that IFN-γ up-regulates MHC class I antigen presentation by inducing gene expression signatures that are related to MHC class I antigen processing and presentation including activation of JAK/STAT1 signal transduction pathway (27). Furthermore, changes in cytokine profiles with up-regulation of CXCL9 (also known as Monokine induced by gamma interferon (MIG)), which has chemotactic activity on T cells induced by IFN-γ, supports this hypothesis. A potential cross-talk between IFN-γ and the Hh pathway was recently described by Laner-Plamberger et. al (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 IFN-γ-STAT1 signaling, which can induce cell cycle arrest, apoptosis and anti-tumor 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 IFN-γ depend on when the samples are collected (29, 30). A previous report showed that IFN-γ mRNA expression in the skin infiltrated IFN-γ 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 IFN-γ 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 up-regulation 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 were 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.

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References


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,</td>
<td>Solid BCC</td>
<td>Regression of tumor with inflammatory infiltrate</td>
<td>120×80mm, 120×80mm</td>
<td></td>
</tr>
<tr>
<td>Location and Size</td>
<td>Histological Type</td>
<td>Histological Description</td>
<td>Remission</td>
<td>Measurements</td>
<td></td>
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<tr>
<td>2 Locally advanced, thoracic, ulcerated, 80x60 mm measuring BCC</td>
<td>Solid BCC</td>
<td>Regression of tumor with inflammatory infiltrate</td>
<td>PR after 12 weeks</td>
<td>80x60 mm, 50x40 mm</td>
<td></td>
</tr>
<tr>
<td>3 Locally advanced, facial, ulcerated 50x60 mm measuring BCC</td>
<td>BCC with basosquamous differentiation</td>
<td>Epithelial tumor rests infiltrated by inflammatory cells.</td>
<td>PR after 16 weeks</td>
<td>40x40 mm, 40x35 mm</td>
<td></td>
</tr>
<tr>
<td>4 Multiple locally advanced, recurrent BCC, 10x12 mm with lower leg</td>
<td>Superficial BCC</td>
<td>Superficial tumor rests and inflammatory infiltrate</td>
<td>PR after 12 weeks</td>
<td>10x12 mm, 10x10 mm</td>
<td></td>
</tr>
<tr>
<td>5 Multiple BCC, 45x15 mm with upper arm</td>
<td>Superficial BCC</td>
<td>Regression of BCC with focal basosquamous differentiation, inflammatory infiltrate and cystic formation.</td>
<td>MR after 12 weeks</td>
<td>45x15 mm, 45x10 mm</td>
<td></td>
</tr>
</tbody>
</table>

CR: complete remission according protocol
PR: partial remission according protocol
MR: minor remission (not qualifying for PR, but clinical improvement)

**Figure legends:**

**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 high power fields before and after 4 weeks treatment. Scale bar; 100 µm (B) Ki67, BerEP, Bcl2, and Sox9 expression on tumor cells was quantified by investigation of 4x40 HPF representative of different patients and different high power fields 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).
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 x 100 HPF representative areas per sample were counted in BCC biopsies from patients before and after 4 week treatment with Hh inhibitors (n=4). *, p < 0.05

Figure 3: CD4, CD8, HLA-DR-class II, and MHC class I expression.

MHC class I expression on tumor cells was quantified by investigation of 4 ×40 HPF of a representative biopsy pair and evaluated as grade 1 (< 10% of cells), grade 2 (10- 30% of cells) and grade 3 (>30% of cells). Quantification of CD4, CD8, HLA-DR-class II + cells in intratumoral and peritumoral regions was done by counting cells in high-power fields (HPF) of ×40 magnification. For each section, 4 ×40 HPF representative areas per sample were counted from patients before and after 4 week treatment with Hh inhibitors. *, p < 0.05

Figure 4: Immunohistochemical stainings of CD68, and Foxp3.

(A, B) For CD68 and Foxp3 staining, 4 x 40 HPF of a representative biopsy pair are shown and 5 pairs were counted in BCC biopsies from patients before and after 4 week treatment with Hh inhibitors (n=5). *, p < 0.05 (C) The ratio CD8+/Foxp3+ was increased only in the intratumoral and but not in peritumoral areas.

Figure 5: Changes in cytokine profile

PCR array of paired biopsies (n=3) before and after 4 weeks of treatment with Hh inhibitors showed an increase of the expression levels of different chemokines and cytokines: CCL18, CCL21, CXCL 9, VEGFA and TNFSF11 (RANKL), and consistent decrease during treatment of the expression levels of TNFSF11 (RANKL) receptor TNFRSF11B. *, p < 0.05
Figure 1

(A) Immunohistochemical staining of HE, Ki67, Sox9, BerEP, and Bcl2 in Pre and Post samples.

(B) Graphs showing changes in expression of Ki67+, Sox9+, BerEP+, and Bcl2+ before and after treatment.
Figure 2

A

Pre

Post

× 40

× 100

Acetylated tubulin
Sox9
DAPI

B

No. of cilia positive cells

Pre
Post

*
Figure 3

A. CD8

B. HLA-DR

C. CD4

D. HLA-DR

Graphs show the changes in CD8+ cells/HPF, MHC I expression, CD4+ cells/HPF, and HLA-DR+ cells/HPF before (Pre) and after (Post) treatment in intratumoral and peritumoral regions.
Figure 4

A

Pre  Post

CD68

Post  Pre  Pre  Post

Intratumoral  Peritumoral

CD68+ cells/HPF

*  *

B

Pre  Post

Foxp3

Post  Pre  Peritumoral

Foxp3+ cells/HPF

*  *

C

Pre  Post  Pre  Post

Intratumoral  Peritumoral

CD8+/Foxp3−
Figure 5

- **CCL18 (Fold-change)**
  - Patient 2: Before = 1, After = 7
  - Patient 3: Before = 1, After = 4
  - Patient 4: Before = 1, After = 3
  - *p = 0.035, SEM = 1.41

- **CCL21 (Fold-change)**
  - Patient 2: Before = 1, After = 5
  - Patient 3: Before = 1, After = 2
  - Patient 4: Before = 1, After = 1
  - *p = 0.046, SEM = 6.11

- **CXCL9 (Fold-change)**
  - Patient 2: Before = 1, After = 1
  - Patient 3: Before = 1, After = 1
  - Patient 4: Before = 1, After = 1
  - *p < 0.001, SEM = 0.06

- **VEGFA (Fold-change)**
  - Patient 2: Before = 1, After = 3.5
  - Patient 3: Before = 1, After = 2.5
  - Patient 4: Before = 1, After = 2
  - *p = 0.049, SEM = 0.58

- **TNFSF11 (Fold-change)**
  - Patient 2: Before = 1, After = 80
  - Patient 3: Before = 1, After = 50
  - Patient 4: Before = 1, After = 40
  - *p = 0.18, SEM = 23.2

- **TNFSF11B (Fold-change)**
  - Patient 2: Before = 1, After = 1.2
  - Patient 3: Before = 1, After = 1
  - Patient 4: Before = 1, After = 0.8
  - *p < 0.001, SEM = 0.06
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