**Featured Article**

**Imiquimod Treatment Induces Expression of Opioid Growth Factor Receptor: A Novel Tumor Antigen Induced by Interferon-α?**

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**ABSTRACT**

**Purpose:** Imiquimod represents a synthetic local immune response modifier that has demonstrated efficacy in clearing basal cell carcinoma. Via interaction with Toll-like receptor 7 on immune cells, imiquimod induces local production of cytokines, such as interferon (IFN)-α.

**Experimental Design:** To more closely define and elucidate mechanisms leading to basal cell carcinoma clearance *in vivo*, we examined gene expression profiles of skin basal cell carcinoma before and after treatment with 5% imiquimod cream (Aldara) by using high-density oligonucleotide arrays.

**Results:** We show that imiquimod predominantly induces genes involved in different aspects of immune response. In addition to effects on immunity, imiquimod treatment modulates the expression of genes involved in the control of apoptosis and oncogenesis. Array data indicated that imiquimod treatment induces expression of opioid growth factor receptor, a molecule recently reported to be a target for antitumor antibody responses. Immunohistochemistry revealed *in vivo* up-regulation of opioid growth factor receptor protein on tumor and on infiltrating cells after treatment. By using basal cell carcinoma cell lines treated with IFN-α or imiquimod, we show that opioid growth factor receptor up-regulation is IFN-α-mediated, rather than directly imiquimod-mediated. By using tissue microarray containing 52 basal cell carcinomas, we demonstrate opioid growth factor receptor expression in almost half of the cases. Expression of opioid growth factor receptor correlated with a longer recurrence-free period in basal cell carcinoma that occurred after radiotherapy (Kaplan-Meier analysis, *P* = 0.041).

**Conclusions:** In addition to its immunomodulatory and antiproliferative activity, opioid growth factor receptor seems to have a prognostic significance in basal cell carcinoma patients. Our data add to the growing list of basal cell carcinoma-associated tumor antigens.

**INTRODUCTION**

Basal cell carcinoma is the most common malignancy in the white human population worldwide, and its incidence has been increasing in the last decade (1). Basal cell carcinoma tends to grow slowly and rarely metastasizes. Basal cell carcinoma can be successfully treated by intralesional injections of interferons (IFNs) or by topical application of imiquimod (1). Imiquimod has been tested and found to be efficacious in clearing basal cell carcinoma [basal cell carcinoma (2–4)]. Imiquimod [1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine] belongs to the group of imidazoquinolones, synthetic local immune response modifiers that have demonstrated potent antiviral and antitumor activity (5). Imiquimod activates macrophages and other monocyte-derived immune cells via interaction with Toll-like receptor-7 (6) inducing the local production of cytokines such as IFN-α, tumor necrosis factor α, and interleukin (IL)-12, resulting in an enhanced innate immune response (5, 7). In addition, imiquimod induces migration and activation of skin Langerhans cells (8), all of which promote the biasing of naïve T cells to Th1 cells, which are potent producers of IFN-γ, thus leading to the enhancement of adaptive immunity. The stimulation of innate and adaptive immunity may finally lead to regression of viral-induced lesions and neoplasms (5, 7).

A functional genomic approach offers a unique possibility to define and analyze gene expression profiles in response to a specific drug treatment. The implementation of high-density oligonucleotide arrays containing more than 10,000 genes on a single array has enabled such a genome-wide assessment of changes in the overall expression profile (9). Using this approach, we have analyzed *in vivo* gene expression profiles of basal cell carcinomas treated with 5% imiquimod cream. In addition, we established basal cell carcinoma cell lines from skin lesions, which we treated with imiquimod or IFN-α, and their gene expression profiles were then compared with the ones from skin basal cell carcinoma after imiquimod therapy *in vivo*.

**MATERIALS AND METHODS**

**Patient Samples**

This study used material from a 3M Pharmaceuticals-supported study (10) investigating infiltrate alterations in skin...
basal cell carcinoma after treatment with 5% imiquimod cream (Aldara) approved by Institutional Ethics Committee. Before entering the study, all patients provided written informed consent for both studies. At the screening visit, a biopsy specimen was taken for histological confirmation of skin basal cell carcinoma. Patients applied the cream once daily 5 times/week for a maximum of 6 weeks. At the point when the tumor began to show signs of erosion, the tumor was surgically excised. The surplus material was available from five patients, one of whom had a second basal cell carcinoma lesion, which was treated on signed informed consent after completion of the study. An additional patient with M. Bowen was treated according to the same scheme, on signed informed consent.

Cell Lines

Establishment of Primary Basal Cell Carcinoma Cell Cultures. Four primary basal cell carcinoma cell cultures were established according to the previously described methodology (11). These basal cell carcinoma cell lines exhibited a low culture-doubling rate, ranging from 14 to 30 days. Basal cell carcinoma cell lines expressed keratin markers detected by two antibodies: Lu-5 (BMA Biomedicals AG, Augst, Switzerland) and NCL-L-AE1/AE3 (Medite AG, Nunningen, Switzerland). They were negative for expression of vimentin, neuron-specific enolase, and S-100. To remove contaminating keratinocytes, the primary basal cell carcinoma cultures were exposed to “calcium shift” (11). The increase in calcium concentration in growth medium led to their cornification in the next 96 h, whereas basal cell carcinoma cells flattened, elongated, and stratified but neither cornified nor died (11).

Immunomodulatory Treatment. The HCl salt form of imiquimod was provided by 3M Pharmaceuticals (St. Paul, MN). Primary basal cell carcinoma cell lines and human keratinocytes were stimulated with 15 μg/ml imiquimod or with 1000 units/ml IFN-α2a (Roferon-A; Roche) for 24 h. Primary human keratinocytes were obtained from foreskins as described elsewhere (12) and grown in defined keratinocyte serum-free medium (Gibco; Invitrogen AG, Basel, Switzerland) containing bovine pituitary extract and recombinant epidermal growth factor.

To evaluate the viability of the cells after the aforementioned treatments, the cells were stained with propidium iodine (Fluka Chemie GmbH, Buchs, Switzerland) and counted under an inverted epifluorescence microscope. Neither imiquimod nor IFN-α treatment induced a significant decrease in cell numbers after 24 h (37.25±8.18 cells/cm² before treatment versus 40.50±11.09 cells/cm² after imiquimod treatment or 38.75±14.5 cells/cm² after IFN-α treatment).

Gene Expression Profiling

Total RNA was isolated using TRizol reagent (Invitrogen AG). Double-stranded cDNA was generated using a Superscript cDNA synthesis kit (Invitrogen AG) using an oligo(dT)₁₅ primer containing a T7 RNA polymerase promoter at the 3’ end (Microsynth, Balgach, Switzerland). Labeled cRNA was prepared from double-stranded cDNA by in vitro transcription using a T7 polymerase [MEGAscript T7 kit; Ambion (Europe) Ltd.] in the presence of biotin-11-CTP and biotin-16-UTP (Enzo Diagnostics, Farmingdale, NY) and purified using RNase columns (Qiagen AG, Basel, Switzerland). Fifteen μg of biotinylated cRNA were fragmented and hybridized to HG-U95Av2 GeneChip arrays (Affymetrix, Santa Clara, CA) that contain probe sets representing ~12,000 genes. Chip hybridization, washing, and staining were performed according to the Affymetrix-recommended protocols.

After scanning of the probe arrays, digitalized image data were processed using Affymetrix Microarray Suite 5.0 software. In addition to determining the expression level for each gene (the so-called “signal”), this software assigns a detection call [absent (not expressed), present (expressed), or marginal (marginally expressed)] for each gene measured. The overall fluorescence intensity was scaled to a global intensity of 500 to enable the comparison between chips.

Data Analysis

The expression data for all genes were exported to Microsoft Excel. The following criteria were used to select differentially expressed genes on imiquimod (or IFN-α) treatment: (a) paired Student’s t test significance level of P < 0.05; (b) fold change cutoff of 1.5 in both directions; and (c) the present call(s) had to coincide with the regulation in question, e.g., if a candidate gene were to be called “up-regulated” after immunomodulatory treatment, at least one sample in the treated group had to be called present; vice versa, if a gene were to be called “down-regulated” after immunomodulatory treatment, at least one sample in the untreated group had to have a present call. The selected differentially expressed genes were normalized to a mean of 0 and a SD of 1, log₂-transformed, and subjected to average linkage hierarchical clustering using the uncentered Pearson similarity matrix (13). Clustering analysis was performed using the GENE CLUSTER program, and the figures were generated with the TREE VIEW program (13). The selected genes were annotated using the NetAffx analysis system offered by Affymetrix (14).

Real-Time Polymerase Chain Reaction

Approximately 1 μg of total RNA was reverse transcribed using oligo-(dT)$_{15}$ priming and avian myeloblastosis virus reverse transcriptase [1st Strand cDNA Synthesis Kit for Reverse Transcription-PCR (avian myeloblastosis virus); Roche Molecular Biochemicals, GmbH, Mannheim, Germany] at 42°C for 1 h. PCR amplifications were carried out using HotStart system (LightCycler-Faststart DNA Master SYBR Green I; Roche Molecular Biochemicals) in the LightCycler thermocycler, as described previously (15). The primer sets used and cycling conditions are shown in Supplementary Table 1. To generate external standards for subsequent quantification, PCR products were purified and cloned into the pCRII-TOPO vector using TOPO TA Cloning Kit (Invitrogen AG), according to the manufacturer’s recommendations. The incorporation of the insert into the vector was confirmed by sequencing and BLAST comparison.

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4 http://rana.lbl.gov.
Table 1 OGFR protein expression in basal cell carcinomas treated with topical imiquimod

<table>
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<tr>
<th>Patient</th>
<th>OGFR TU</th>
<th>OGFR INF</th>
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<td>–</td>
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NOTE: The duration of the treatment is presented in days (time point where the tumor showed signs of erosion and was excised).

Abbreviations: OGFR, opioid growth factor receptor; TU, tumor; INF, infiltrate; –, no expression; ±, single cell positivity; +, 5–35% of the cells positive; + +, 35–70% cells positive; + + +, >70% cells positive; Px, basal cell carcinoma sample not included in the microarray analysis due to unsatisfactory RNA quality; MB, M. Bowen sample.

Protein Isolation and Immunoblotting Analysis

After the RNA extraction with TRIzol, proteins were isolated from the intermediate fraction according to the manufacturer’s recommendation. For immunoblotting analysis, protein extracts were electrophoresed using Novex Tris-Glycine gels (Invitrogen AG) and the buffer system of Laemmli and subsequently transferred to nitrocellulose in 12.5 mM Tris base, 100 mM glycine in 20% methanol transfer buffer. The membranes were blocked with 10 mM Tris, 150 mM NaCl, 5% nonfat powdered milk, and 0.25% Tween 20 and incubated with primary antibody for 60 min. Primary antibodies were used at the following concentrations: opioid growth factor receptor, 0.25 µg/ml and actin, 0.2 µg/ml (clone I-19-R; rabbit polyclonal IgG; Santa Cruz Biotechnology Inc., Heidelberg, Germany). After incubation with the primary antibody, the membrane was washed extensively with 10 mM Tris-HCl, 150 mM NaCl, and 0.25% Tween 20 and incubated for 45 min with the secondary antibody coupled to horseradish peroxidase. Finally, the blots were developed with chemiluminescence (ECL; Amersham Biosciences, Freiburg, Germany). To reprobe the blots with another antibody, the membrane was stripped in 62.5 mM Tris-HCl, 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 50°C. To remove any remaining SDS, the membrane was washed three times with PBS and blocked again.

Tissue Microarray and Immunohistochemistry

A tissue microarray containing 52 basal cell carcinoma tissue samples was constructed as described previously (16). Paraffin-embedded tissue and tissue microarray sections, stained with H&E, were evaluated by an experienced dermatopathologist (R. D.) and subsequently stained with antihuman opioid growth factor receptor antibody raised against the tandem repeat region of opioid growth factor receptor, a kind gift from Dr. Joseph A. Mollick (Department of Medicine, Division of Oncology, Stanford University Hospital, Stanford, CA). Immunohistochemistry was performed with a 1:30 working antibody dilution using the alkaline phosphatase-anti-alkaline phosphatase technique, as described previously (10).

RESULTS

We generated gene expression profiles for six basal cell carcinomas and one M. Bowen sample before and after treatment with 5% imiquimod cream and for four basal cell carcinoma cell lines and two keratinocyte controls before and after treatment with IFN-α or imiquimod. Based on our filtering criteria, we found the expression of 1305 genes (~10% of the genes on the HG-U95Av2 chip) to be differentially regulated on treatment with imiquimod of the skin basal cell carcinoma. Of these genes, 372 (28.5%) genes were up-regulated, whereas 933 (71.5%) genes were down-regulated. Two-way clustering analysis, a mathematical approach that essentially organizes the data by grouping genes with a similar expression pattern, yielded two major array clusters (i.e., cell lines and skin samples) with this set of 1305 differentially expressed genes and, within each group, a subcluster relating to a particular type of the treatment (Fig. 1, A and B).

Topical Imiquimod Treatment Preferentially Modulates Genes Involved in Immune Response. The vast majority of up-regulated genes consisted of genes involved in different aspects of immune response. Given the previous reports showing that imiquimod is a potent IFN-α inducer (17–21), we first focused on this group of genes. Using hierarchical clustering, we compared the expression profiles of the skin samples before and after imiquimod treatment with basal cell carcinoma cell lines treated with IFN-α or imiquimod. The genes that group together according to this method are likely to be coregulated and functionally related (22). Fig. 1C shows the results of the clustering analysis, revealing that genes up-regulated in basal cell carcinoma cell lines and keratinocytes by IFN-α treatment solely represent the same group of genes up-regulated by imiquimod treatment of skin basal cell carcinoma. In vivo imiquimod induced the expression of different 2',5'-oligoadenylylate synthetase isoforms, IFN-induced proteins including MxA and MxB protein, as well as STAT1 and STAT2 transcription factors. By comparing the genes that were down-regulated in basal cell carcinoma cell lines treated with IFN-α (and cell line controls) and basal cell carcinoma skin lesions treated with imiquimod, we could define 79 genes expressed in both evaluated groups. Clustering analysis demonstrated that the majority of these genes grouped together, confirming that they belong to the same functional category (Fig. 1E).

Apart from IFN-α and IFN-γ, imiquimod induced a whole spectrum of cytokines (IL-1β), chemokines (RANTES, ecallentin, MIP-1α, and CXCL11), and cognate receptors [MIP-1α/RANTES receptor (CCR1), CCR7 receptor, IL-3 receptor α, IL-15 receptor α], some of which are presented in Fig. 2A.

In addition to the IFN-α cluster, we could define a IFN-γ cluster, namely, genes that are up-regulated by IFN-γ as a result of Th1 shift and the activation of T cells. Fig. 1D shows parts of the “infiltrate” cluster containing some of the IFN-γ-induced genes involved in antigen processing (TAP-1 and MECL-1) and
Fig. 1 Gene expression patterns determined by two-way hierarchical clustering analysis of 1305 genes. Relationships between experimental samples are summarized in the dendrogram, in which the pattern and length of the branches reflect the relatedness of the samples. Red indicates a high relative level of expression, whereas green represents a low relative level of expression. A, within the “cell lines” cluster, two major clusters are distinguishable:
the formation of immunoproteasome (PA28), function of activated T cells (granzyme B, LAG-3, and T-cell immune regulator 1), and autoregulatory apoptosis in T cells (tumor necrosis factor receptor 2).

This cytokine boom is likely responsible for the influx of immune cells observed in histology (Fig. 4, E–G), which is reflected in the up-regulation of genes representing receptors, e.g., killer cell inhibiting receptors and killer cell activating receptors. Toll-like receptors, and HLA molecules pertinent to these cells (summarized in Fig. 2B). Topical imiquimod treatment resulted in an enhanced expression of molecules related to dendritic cell development, such as CD1d, flt-3 ligand, and Toll-like receptor-3 (Fig. 2, B and C). Moreover, imiquimod seems to be a very potent inducer of costimulatory molecules such as CD86, CD40, ICAM-2 (CD102), or SLAM (CDw150; Fig. 2C). Infiltrating T cells seem to be activated and fully armed to kill, which is reflected in the up-regulation of cytotoxic (cytolytic) molecules such as granzyme B and granulysin (Fig. 2D). There was also an up-regulation of genes involved in respiratory burst and production of reactive oxygen species in phagocytes (neutrophils and macrophages), i.e., neutrophil cytosolic factors I and 4 (Fig. 2D).

Imiquimod Treatment Up-Regulates Opioid Growth Factor Receptor Expression: An Effect of Interferon-α? One molecule within the infiltrate cluster with no clear functional relation to this cluster drew our attention. We observed posttreatment induction of opioid growth factor receptor gene expression in skin basal cell carcinomas (2.5-fold increase; P = 0.005). Quantitative real-time PCR analysis confirmed microarray results, demonstrating up-regulation of opioid growth factor receptor expression in six of seven treated skin samples (one remained unchanged), as shown in Fig. 3A. Normalization of opioid growth factor receptor to PTCH gene expression (defined as basal cell carcinoma-specific) again revealed an increase in opioid growth factor receptor expression in five of six tested skin samples (Fig. 3B). Immunohistochemistry using the antibody raised against the immunogenic opioid growth factor receptor tandem repeat region revealed an impressive opioid growth factor receptor up-regulation in all skin basal cell carcinoma after imiquimod treatment (Table 1 and Fig. 4), including one M. Bowen sample. In addition to tumor cells, infiltrating cells also up-regulated opioid growth factor receptor expression (Fig. 4, D–G). Before treatment, basal cell carcinoma tumor cells demonstrated either weak or no opioid growth factor receptor immunoreactivity (Fig. 4, B and D). Normal epidermis demonstrated a nuclear pattern of opioid growth factor receptor expression in the cells from the basal to the granular cell layer (Fig. 4A). It is of note that the irregular nests of atypical, neoplastic keratinocytes in M. Bowen exhibited opioid growth factor receptor immunoreactivity before treatment (Fig. 4C).

To further differentiate whether imiquimod induces opioid growth factor receptor directly or via IFN-α, we analyzed opioid growth factor receptor expression in basal cell carcinoma cell lines (keratinocytes) treated with imiquimod and IFN-α for 24 h by real-time PCR. IFN-α up-regulated opioid growth factor receptor expression in two basal cell carcinoma cell lines (L1 and L2) and keratinocyte controls (t test, P < 0.05), whereas two cell lines demonstrated no significant change in opioid growth factor receptor expression (Fig. 3C). After treatment with imiquimod, there was no significant change in opioid growth factor receptor expression in any of the cell lines investigated. Immunoblotting analysis of basal cell carcinoma cell culture extracts demonstrated an increase in opioid growth factor receptor protein after IFN-α treatment (Fig. 3D). We observed no changes in opioid growth factor receptor protein expression after imiquimod treatment of basal cell carcinoma cell lines.

Opioid Growth Factor Receptor Expression in Basal Cell Carcinomas Treated with Radiotherapy Is Associated with Longer Recurrence-Free Time. Using a tissue microarray containing 27 basal cell carcinomas of aggressive, sclerosing type and 25 basal cell carcinomas of nonaggressive type (14 nodular and 11 superficial basal cell carcinomas), we demonstrate opioid growth factor receptor expression by tumor cells in 23 of 32 (44.2%) evaluated cases (Fig. 4I). Opioid growth factor receptor tissue microarray immunohistochemistry results are presented in Table 2A. Opioid growth factor receptor was predominantly expressed in aggressive, sclerosing basal cell carcinoma (16 of 23 opioid growth factor receptor-positive cases; Spearman’s ρ = −0.294; P = 0.035). Nonaggressive basal cell carcinomas displayed opioid growth factor receptor expression in seven cases (four nodular and three superficial basal cell carcinomas, respectively). In a group of 36 primary basal cell carcinomas, 13 of which have recurred after radiotherapy, opioid growth factor receptor expression was detected in 12 of 23 basal cell carcinomas that showed no recurrence (Table 2B). The absence of opioid growth factor receptor expression in the primary tumor correlated with the history of recurrence after radiotherapy (Spearman’s ρ = −0.457; P = 0.005). Moreover, primary basal cell carcinomas that did not recur during the follow-up period exhibited stronger opioid growth factor receptor expression than the ones that have recurred, irrespective of the histology type (Mann-Whitney U test, P = 0.020). In patients whose basal cell carcinomas recurred, opioid growth...
Fig. 2 Genes modulated by imiquimod treatment of the skin basal cell carcinoma. Bars represent the posttreatment fold change of the mRNA level of a particular gene when compared with the status before treatment. Black bars indicate up-regulation, whereas white bars represent gene down-regulation. Every gene is represented by gene name and GenBank accession number.
factor receptor expression correlated with a longer recurrence-free period (Kaplan-Meier analysis; log-rank test, \( P < 0.041 \)).

**Imiquimod Treatment Affects the Expression of Various Apoptosis Genes.** In the skin, imiquimod treatment induced a spectrum of genes implicated in apoptotic signaling and the execution of apoptosis (Fig. 2F). Clustering analysis revealed that the up-regulation of these genes was an infiltrate-specific phenomenon encountered exclusively on imiquimod treatment of the skin basal cell carcinoma. Namely, none of the cell lines investigated (treated with either IFN-\( \alpha \) or imiquimod) exhibited a similar pattern of expression.

Apart from induction, imiquimod-treated skin basal cell carcinomas showed down-regulation of additional apoptosis genes, among others, the decrease in bcl-2 homolog expression (Fig. 2F). Some of these genes organized together according to the two-way hierarchical clustering algorithm (Fig. 1G). Down-regulation of these genes was not observed in any of the treated cell lines.

**The Clearance of Skin Basal Cell Carcinoma Is Associated with the Decrease in Expression of Well-Defined and Potentially Novel Genes Implicated in Basal Cell Carcinoma Oncogenesis.** In the group of down-regulated genes, 63 genes were assigned to be involved in oncogenesis according to the known or predicted function (Fig. 2E). In the skin, imiquimod
treatment resulted in a decrease in expression of Patched (PTCH), GLI1, and GLI3 genes, which are implicated in basal cell carcinoma pathogenesis (23). Real-time PCR analysis showed down-regulation of PTCH in five of six tested samples, GLI1 down-regulation of four of six tested samples, and down-regulation of GLI3 in five of six tested samples (Fig. 5). In addition to these genes, we observed a down-regulation of genes reported to be expressed in other human epithelial cancers, such as frizzled (FZD)-7 (24), and c-erb-B2 (HER2). Because the expression of C-erb-B2 (HER2) has been reported previously in

![Fig. 4 Opioid growth factor receptor immunoreactivity in skin basal cell carcinoma. A, the pattern of opioid growth factor receptor expression in normal epidermis showing clear nuclear opioid growth factor receptor immunoreactivity in the basal, spinous, and part of the granular cell layer (original magnification, ×20). B, cytoplasmic opioid growth factor receptor protein expression in skin basal cell carcinoma (patient P5-2 before treatment). Tumor islets are indicated by arrows. Note the nuclear pattern of opioid growth factor receptor expression in neighboring epidermis, which is not present in tumor islets (original magnification, ×10). C, opioid growth factor receptor expression in Bowen’s disease (original magnification, ×40). D, absence of opioid growth factor receptor expression in skin basal cell carcinoma (patient 4 before treatment; original magnification, ×10). E–G, massive up-regulation of opioid growth factor receptor immunoreactivity in skin basal cell carcinoma after imiquimod treatment (patients Px, P1, and P2, respectively; original magnification, ×10). For better discrimination of the infiltrate, tumor borders are accentuated by a dotted black line. H, nuclear opioid growth factor receptor expression in basal cell carcinoma tumor cells after treatment (original magnification, ×100). I, tissue samples from the basal cell carcinoma tissue microarray stained with antibody against the immunogenic opioid growth factor receptor tandem repeat region (original magnification, ×10).]
basal cell carcinoma (25), we assessed the expression of FZD7 by real-time PCR. All basal cell carcinoma cases investigated demonstrated FZD7 expression. After imiquimod treatment, there was a down-regulation of FZD7 gene expression in five of six treated skin lesions (Fig. 5).

On the other hand, a group of genes (tyrosinase, Pmel-17/ gp100, Melan-A/MART-1, and p15/MAAT-1) involved in melanin biosynthesis and known as melanoma-associated antigens was singled out (Fig. 2E). The expression of these genes, although not detectable in any of the basal cell carcinoma cell lines or keratinocytes, was down-regulated in skin lesions on imiquimod treatment (Fig. 1H). Immunohistochemical staining showed that the melanocytes were the cells accounting for tyrosinase, gp100, and MART-1 expression in basal cell carcinoma tissue sections. After imiquimod treatment, the melanocyte density decreased, which is probably the reason for the decrease in expression of these markers (data not shown).

**DISCUSSION**

Imune response modifiers are substances that directly or indirectly (through the modification of one or more components of the immunoregulatory network) affect a specific immune function (26). Imidazoquinolone compounds, of which imiquimod is the best characterized to date, are such agents. Imiquimod is found to be efficacious in clearing superficial basal cell carcinoma (2–4). However, its exact mechanism of action has yet to be fully understood. To more closely define and elucidate the mechanism(s) leading to tumor clearance has yet to be fully understood. To more closely define and elucidate the mechanism(s) leading to tumor clearance, we examined gene expression profiles of basal cell carcinoma of the skin before and after treatment with 5% imiquimod cream by using Affymetrix oligonucleotide arrays. Of a total of 12,000 probe sets, we identified 1,305 that were differentially regulated in the skin basal cell carcinoma on imiquimod treatment. Of these genes, one third were up-regulated after imiquimod application, comprising mostly genes that were functionally involved in the immune response. We first focused on genes modulated by IFN-α, knowing that the regression of viral warts or high-grade intraepithelial neoplasia was associated with increased production of IFN-α, IFN-γ, and 2’5’-oligoadenylate synthetase (18, 19, 21). In addition to these, imiquimod treatment of the skin basal cell carcinoma induced many other IFN-α-regulated genes [various IFN-induced proteins, STAT1, STAT2, MxA, and MxB protein (27, 28)]. Topical imiquimod treatment resulted in an IFN-γ increase as well and in the induction of molecules preferentially regulated by this IFN type.

Apart from IFNs, imiquimod treatment induced the expression of various cytokines, of which IL-1β and MIP-1α were recently shown to be up-regulated in macrophages treated with S-28463, an imiquimod analog (29). In patients with genital warts, the erythema at the treatment site correlated with IFN-γ and RANTES mRNA expression (18), which were up-regulated in our samples as well.

Early infiltrate developing within 3–5 days after treatment initiation appears to be of lymphoid/myeloid origin, which is reflected in the up-regulation of genes encoding for different receptors and cell surface markers expressed in these cells. According to the expression of genes (e.g., IFN-γ, tumor necrosis factor receptor 2, CD97, ICAM-2, and granzyme B) previously described to be expressed in differentiated Th1 cells (30), imiquimod treatment effectively induced biasing of naïve T cells into Th1 cells. Imiquimod activates macrophages via interaction with Toll-like receptor-7 resulting in the secretion of IL-12 (6, 31), favoring the development of IFN-γ-producing T cells. Exposure to IL-12 results not only in the increased expression of Th1-specific genes but also in the induction of additional genes such as CCRI (MIP-1α/RANTES receptor),

<table>
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</table>

**NOTE.** The 52 samples in the TMA included 36 primary BCCs and 16 secondary BCCs.

Abbreviations: OGFR, opioid growth factor receptor; BCC, basal cell carcinoma; TMA, tissue microarray.

* Expression refers to tumor cells.

A. OGFR expression with respect to BCC histology in 52 samples in the TMA

B. OGFR expression in primary BCCs that recurred after radiotherapy
LAG-3, and SLAM (30), whose up-regulation we noted as well on imiquimod treatment. A particularly interesting finding was the up-regulation of CD1d, whose expression was recently shown on dermal dendritic cells and monocyte-derived dendritic cells (32).

Apart from monocytes/macrophages, plasmacytoid dendritic cells represent another important target for Toll-like receptor-7-mediated imiquimod activation (6). Activation of the Toll-like receptors on plasmacytoid dendritic cells results in the expression of costimulatory molecules (e.g., CD40 and CD86) necessary for the appropriate activation of antigen-specific T cells (5). Plasmacytoid cells are characterized by a particular set of cell surface molecules, some of which are quite specific for these cells (IL-3 receptor α) and have been up-regulated after imiquimod treatment in our samples (CD32, CD38, CD40, and CD43). IL-3 promotes survival but does not drive the plasmacytoid dendritic cells to proliferate (33). Flt3 ligand and granulocyte colony-stimulating factor, however, have the capability to increase the number of plasmacytoid dendritic cells in vivo (33, 34). Our data show the up-regulation of flt-3 ligand and granulocyte colony-stimulating factor receptor after imiquimod treatment. In view of a recent report demonstrating that imida-}

zooquinolones can directly induce plasmacytoid dendritic cell maturation (35), it is highly suggestive that in addition to activating these cells, imiquimod treatment induces a microenvironment promoting survival and maturation of plasmacytoid dendritic cells. The arrays that we used (HG-U95Av2) contain only Toll-like receptor-1, -2, -3, -5, and 6 genes. Nevertheless, we observed the increase in Toll-like receptor-2 (myelomonocytic cells, neutrophils, lymphocytes) and -3 [predominantly dendritic cells (36)] transcripts that correlated with the infiltrate characteristics observed by histology.

The posttreatment up-regulation of opioid growth factor receptor in the skin (on both the mRNA and protein level) represents a novel and intriguing finding. Opioid growth factor belongs to the group of endogenous opioid peptides and acts as negative regulator of cell proliferation, in addition to its role in pain modulation/transmission (37). Opioid growth factor action is targeted to DNA synthesis, with no effect on the apoptotic pathway (38). Opioid growth factor exerts its action through opioid growth factor receptor, which differs markedly from classical opioid receptors in terms of its function (growth), distribution (neural and nonneural), subcellular location (nucleus), and competitive inhibitory profile (37). Zagon et al. (39) demonstrated constitutive expression of opioid growth factor receptor in normal skin, suggesting its regulatory role in DNA synthesis in the basal cell layer. In addition, McLaughlin et al. (40) showed decreased opioid growth factor receptor protein expression in squamous cell carcinoma of the head and neck as compared with normal epithelium, unleashing tumor cells from growth control. Opioid growth factor receptor transcriptional levels, however, remained unaffected, which led the authors to conclude that the changes responsible for decreased opioid growth factor receptor expression are most likely due to translational/posttranslational defects (40). Using real-time PCR, we could demonstrate opioid growth factor receptor mRNA expression in skin basal cell carcinomas. Our findings confirmed the opioid growth factor receptor expression pattern described previously by Zagon et al. (39) in normal skin and, furthermore,
revealed weak or absent immunoreactivity in basal cell carcinoma tumor cells. After immunomodulatory treatment with imiquimod, we describe up-regulation of opioid growth factor receptor protein in tumor by using the antibody raised against the opioid growth factor receptor tandem repeat region. In addition, we show in vitro that opioid growth factor receptor up-regulation in keratinocytes and basal cell carcinoma cell lines is IFN-α-mediated. These findings correspond to in situ up-regulation of opioid growth factor receptor shown by immunohistochemistry. Two recent reports by Wu et al. (41) and Mollick et al. (42) imply that opioid growth factor receptor protein itself could be immunogenic by demonstrating anti-opioid growth factor receptor responses in cancer patients after immunotherapy. Opioid growth factor receptor protein structure is characterized by the presence of tandem repeats that share homology with the extracellular domain of MUC1 (42). It is conceivable that through up-regulation of opioid growth factor receptor variant, imiquimod might not only be suppressing tumor growth but also increasing tumor immunogenicity. Induction of opioid growth factor receptor in infiltrating cells might be a part of the local immune response control as well as an additional signal to produce specific cytokines (Th1/Th2 switch) or antibodies analogous to other opioid receptors (43). An additional positive side effect of opioid growth factor receptor up-regulation might be analgesia despite substantial tissue damage that develops locally. In a large series of more than 600 patients with anogenital warts treated with imiquimod, patients preferred imiquimod 5% cream over other therapies due to the lack of associated pain (44). Using a tissue microarray containing a panel of different basal cell carcinoma subtypes, we demonstrate opioid growth factor receptor expression in almost half of the evaluated tumors, primarily those with an aggressive phenotype. On the other hand, basal cell carcinomas that did not recur after radiotherapy show strong opioid growth factor receptor expression irrespective of their histology type. Even in the case of recurrence, opioid growth factor receptor expression had an impact on the length of the recurrence-free time. In addition to its putative immunomodulatory as well as antiproliferative activity, opioid growth factor receptor seems to have prognostic significance in basal cell carcinoma patients.

According to our array data, imiquimod treatment had an impact on the expression of various genes involved in apoptotic pathways. The expression pattern of some of these genes appeared to be part of the control of locally induced immune response. Noteworthy was the posttreatment down-regulation of a bcl-2 homolog in skin samples. Aberrant expression of bcl-2 in basal cell carcinoma was implicated in extended tumor cell survival without influencing cell proliferation (10, 45, 46). A recent study by Schön et al. (47) showed that imiquimod induces apoptosis through bcl-2-dependent translocation of cytochrome c, which is independent of membrane-bound death receptors (Fas, tumor necrosis factor-related apoptosis-inducing ligand, and tumor necrosis factor). Accordingly, we have shown that imiquimod induces down-regulation of bcl-2 protein in tumor cells in vivo, thereby not affecting tumor Fas expression (10).

Imiquimod treatment resulted in the reduction of the tumor volume, thus decreasing the expression of various genes implicated in oncocogenesis. We detected and confirmed the down-regulation of genes involved in the hedgehog signaling pathway (PTCH, GLI1, and GLI3) implicated in the pathogenesis of sporadic and hereditary basal cell carcinoma (23). Moreover, we demonstrated expression and down-regulation of another gene involved in Wnt signaling, FZD7, which is oncogenic in other epithelial cancer types (24, 48). Apart from alterations in the sonic hedgehog pathway and some members of the Wnt cascade (23), there is little available information on other genes involved in basal cell carcinoma tumor formation. Therefore, high-throughput microarray technology might be a good tool to define and investigate novel basal cell carcinoma tumor antigens.

In conclusion, our study using high-density oligonucleotide arrays provides for the first time a detailed insight into a complex set of in vivo changes after basal cell carcinoma treatment with imiquimod cream. We have summarized and confirmed the existing literature evidence on imiquimod mechanism of action in one go. Our approach revealed for the first time the up-regulation of opioid growth factor receptor by imiquimod, implying that this effect is likely IFN-α-mediated. Future studies will provide more evidence on the role of opioid growth factor receptor as a tumor antigen in basal cell carcinoma and other cancer types.

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REFERENCES


Imiquimod Treatment Induces Expression of Opioid Growth Factor Receptor: A Novel Tumor Antigen Induced by Interferon-α?

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