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ORIGINAL PAPER

Induction of the members of Notch pathway in superficial basal cell carcinomas treated with imiquimod

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Abstract Basal cell carcinoma of the skin (BCC) is the most common skin tumor in Caucasians worldwide. Different therapeutic options are available to treat BCC, including topical immunotherapy. Imiquimod is topical Toll-like receptor 7 agonist that activates anti-tumor immune response and has been recently approved for the treatment of superficial BCC (sBCC). We sought to investigate the influence of imiquimod treatment on the members of the Notch signaling pathway, whose activity is known to be decreased in BCCs. Six patients with sBCC were evaluated for Notch1, Jagged1 and Delta1 expression before (pre-treatment) and after the beginning of the topical treatment (post-treatment) with imiquimod using real-time PCR and immunohistochemistry. We show selective transcriptional up-regulation of Notch pathway members (Notch1, Jagged1 and Delta1) in tumor cells of the sBCC post-treatment. Furthermore, we demonstrate minor increase of Notch1 protein expression on infiltrating cells as well as strong increase in Jagged1 protein expression in regressing sBCC tumors post-treatment. In this way, imiquimod may act as a stimulator of the Notch pathway in sBCC tumor cells by up-regulating protein expression of the Notch ligand, Jagged1. Via induction of Notch signaling imiquimod may exert tumor suppressor function, which together with its proinflammatory properties results in tumor regression.

Keywords Basal cell carcinoma · Imiquimod · Notch1 · Jagged1 · Delta1

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Introduction

Basal cell carcinoma (BCC) is the most frequent malignant skin tumor in the white human population worldwide and it accounts for about 75% of all skin cancers [23]. BCC belongs to non-melanoma skin cancers, like squamous cell carcinoma of the skin [21, 23]. Its incidence has increased in the last decade, due to the sun exposure and increased life expectancy. To date, the pathogenesis of BCC is not fully elucidated. Even though the role of activated Hedgehog pathway in BCC development has been established in numerous studies, other pathways seem to be of equal importance, such as Notch-signaling pathway [6]. There is circumstantial evidence that BCCs originate from the basal layer of the outer root sheath of the hair follicle [9], which closely resembles the interfollicular basal layer of the epidermis with respect to protein expression patterns, including Notch [14]. Notch cell-to-cell signaling promotes differentiation of epidermal stem cells [4, 11, 12]. Notch and its ligands, Delta and Jagged, play an important role in development of normal epithelial tissue and maintaining homeostasis [12]. Notch members are involved in the control of cell fate determination and differentiation of epithelial stem cells. Notch signaling is not only involved in the development of normal epithelial tissues, but it also seems to play a role in tumorigenesis [20]. Dysregulation of Notch signaling pathway has been observed in different cancers, most which originate from epithelial structures [1]. In contrast to other organs, Notch1 appears to act as a tumor suppressor in the skin [11, 15]. Ablation of Notch1 from epidermal cells in mice leads to an uncontrolled proliferation of the basal epidermal layer and results in BCC-like tumors [15].

The newly registered drug for the external treatment of superficial BCCs (sBCC) is a topical immune response modifier named imiquimod (marketed as 5% cream,

Aldara®) [24, 25]. Imiquimod is a small synthetic compound that activates Toll-like-receptor 7 (TLR-7), which is expressed on plasmacytoid dendritic cells, macrophages and monocytes. This results in an increased production of inflammatory cytokines, such as interferon-α and potent stimulation of anti-tumor immunity that eventually leads to tumor destruction [24, 25].

In this study, we sought to investigate changes in Notch signaling pathway in patients, whose sBCC were treated with imiquimod. We show transcriptional up-regulation of Notch pathway members (Notch1, Jagged1 and Delta1). In addition, we demonstrate minor increase in Notch1 as well as strong increase in Jagged1 protein expression in regressing sBCCs treated with imiquimod.

Materials and methods

Patients

The analyses performed herein utilized the material from six patients with sBCC participating in two clinical open-label studies that investigated the changes in the inflammatory infiltrate in sBCC treated with a 5% imiquimod cream (Aldara, 3M Pharmaceuticals, St Paul, MN). Both studies were approved by the Institutional Ethics Committee. Before entering either study, patients provided written informed consent. At the screening visit that took place 2 weeks before treatment initiation, a biopsy specimen was taken for histologic confirmation of sBCC (pre-treatment samples). Patients applied imiquimod to the target sBCC lesion once daily until the treated tumor showed signs of erosion (generally after 3–5 days), which was then followed by complete excision of the tumor (post-treatment samples). Every pre- and post-treatment tissue sample was divided in two, one half was immediately snap frozen and stored at −80°C, whereas the other half was fixed in formalin and subsequently embedded in paraffin.

Real-time quantitative PCR

Total RNA was extracted from frozen tissue samples using TRIzol reagent (Invitrogen AG, Basel, Switzerland) according to the manufacturer's recommendations. Approximately, 1 μg of total RNA was reverse transcribed using first Strand cDNA Synthesis Kit for RT-PCR (Roche Diagnostics, Rotkreuz, Switzerland) at 42°C for 90 min. PCR amplifications were carried out with the HotStart system (LightCycler-Faststart DNA Master SYBR Green I, Roche Diagnostics) in the LightCycler thermocycler (Roche Diagnostics). Primer sets for PTCH, GAPDH, Notch1, DELTA1 and JAGGED1 were purchased from Search LC (GmbH, Dossenheim, Germany).

Immunohistochemical analysis

Paraffin-embedded tissue sections were stained with the following primary antibodies: anti-Notch1 (clone A6, mouse IgG2b, Acris Antibodies GmbH, Hiddenhausen, Germany) and anti-Jagged1 (clone c-20, polyclonal goat IgG, Santa Cruz Biotechnology Inc., Santa Cruz, CA). After antigen retrieval, immunohistochemistry was performed using alkaline phosphatase–anti-alkaline phosphatase technique, as previously described [25].

Results

Real-time PCR reveals selective up-regulation of Notch pathway members upon imiquimod treatment

To assess early changes in sBCC responding to topical imiquimod treatment, we analyzed gene expression levels of Notch1, DELTA1 and JAGGED1 gene in six patients by real-time quantitative PCR. Knowing that GAPDH gene is expressed by all cell types present in analyzed biopsies, we first analyzed the expression of above-mentioned genes normalized to GAPDH expression. Notch1 gene levels were up-regulated in one patient (patient 4; Fig. 1a). JAGGED1 gene expression levels remained unchanged during imiquimod treatment. DELTA1 mRNA expression was slightly increased in three patients (patients 2, 4 and 5).

Next, we focused on the transcriptional changes in tumor cells and used normalization to patched (PTCH) gene instead of GAPDH. All patients demonstrated a decrease in overall PTCH expression 3–5 days after imiquimod treatment (Fig. 1b) reflecting the ongoing tumor regression, i.e. reduction of the viable tumor tissue. After normalization to PTCH, we could observe increase of Notch1 expression post-treatment in all patients (Fig. 1c). Two patients showed a strong up-regulation of Notch1 by 118-fold (patient 1) and 310-fold (patient 4) in post-treatment biopsies as compared to pre-treatment samples. JAGGED1 mRNA levels were similarly increased in all patients after topical application of imiquimod. Patient 1 showed again a strong increase of 150-fold in JAGGED1 mRNA levels. DELTA1 gene expression was increased in patients 1–5 after treatment with imiquimod. Patient 1 presented once again with high up-regulation of Delta1 as compared to other patients.

To evaluate changes in expression levels of Notch1, JAGGED1 and DELTA1 in the infiltrate, we also used normalization to CD4 gene. An increase in CD4 cell population has been described as an early event in the first week of the imiquimod treatment [3, 26]. Three out of six patients (patients 2, 3 and 6) in our current study showed increased relative CD4 mRNA levels post-treatment (Fig. 1b). With

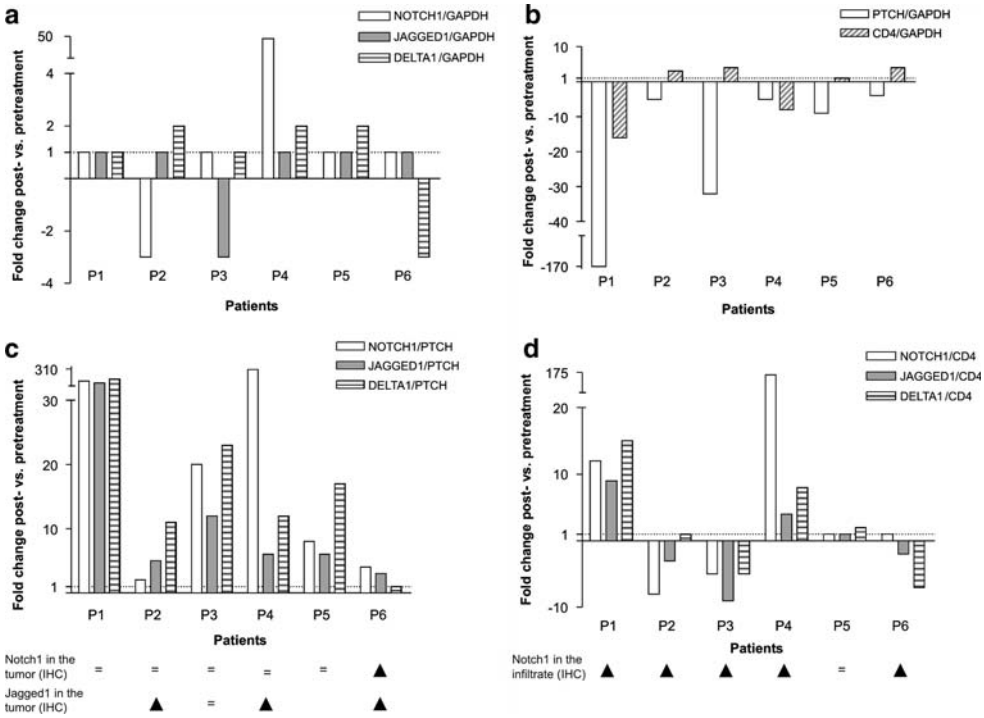

Fig. 1 Gene expression of Notch1, Jagged1 and Delta1 in sBCC before and after topical therapy with imiquimod (**a**, **c** and **d**). For each sample, mRNA expression of the target gene was normalized to mRNA expression of the GAPDH (**a**), PTCH (**c**) and CD4 (**d**) gene and shown as a ratio (e.g. Notch1 copy number per µl/Ptch copy number per µl). *Bars* represent fold change increase in post-treatment versus pre-treatment samples. **c** and **d** contain additional information of protein levels of the respective gene product assessed by immunohistochemistry (IHC) (*triangle* stands for up-regulation; *equal to* stands for no change). **b** shows relative expression of PTCH and CD4 genes normalized to GAPDH

respect to CD4, Notch1, JAGGED1 and DELTA1 gene up-regulation occurred in patients 1 and 4, whereas patient 5 also showed a slight increase in DELTA1 gene expression (Fig. 1d).

Our results show that imiquimod treatment induces selective and heterogeneous transcriptional up-regulation of Notch pathway members in treated lesions.

Jagged1 protein and not Notch1 is primarily up-regulated in imiquimod treated tumors

To further study the effect of imiquimod on Notch pathway in BCCs, we carried out immunohistochemical stainings on the paraffin-embedded tumor tissue samples using antibodies against Noch1 and Jagged1 (Table 1). As expected, Notch1 protein was expressed to the granular layer of the tumor-surrounding epidermis in all evaluated patients. Tumor cells were generally negative for Notch1 protein expression pre- and post-treatment, which contrasted the real-time PCR results (Figs. 1a, c, d, 2a). Only one patient (patient 6) demonstrated increased immunoreactivity of the tumor cells to Notch1 after therapy with imiquimod with partially matching PCR data (Fig. 2a). While looking for other cell types expressing Notch1 receptor, we found an increase in Notch1 protein expression on infiltrating immune cells in five patients (patients 1–4 and 6) post-treatment, which was corresponding to PCR data only in patients 1, 4 and 5 (Figs. 1d , 2a).

Low levels of Jagged1 expression were detected in the surrounding epidermis of the four patients (patients 2–5),

Table 1 Immunohistochemical analysis of Notch1 and Jagged1 expression in sBCCs treated with imiquimod

Patients	Notch1			Jagged1		
	Epidermis	Tumor	Infiltrate	Epidermis	Tumor	Infiltrate
P1 0	Always positive to granular layer	–	–	–	++	Always negative
P1 IM		–	±	No tumor		
P2 0		–	±	–	–	
P2 IM		–	+	±	++	
P3 0		–	–	±	+	
P3 IM		–	+	±	+	
P4 0		–	–	±	–	
P4 IM		–	±	±	++	
P5 0		–	±	±	+	
P5 IM		–	±	No tumor		
P6 0		–	–	+	+	
P6 IM		+	+	+	++	

Expression is graded as: (–) no expression, (±) single cell expression, (+) expression in less than 40% of infiltrate, (++) expression in more than 80% of infiltrate. 0, pre-treatment; IM, post-treatment; no tumor, no evidence of tumor on the post-treatment slide

with patient 6 expressing somewhat more Jagged1 intraepidermally. Jagged1 expression was confined to basal cell layer and did not seem to change upon imiquimod treatment. Serial cuts of paraffin-embedded tumors resulted in the lack of tumor tissue on the slides used to assess Jagged1 expression in patients 1 and 5. Only four patients were, therefore, evaluable for changes in the tumor post-treatment. At pre-treatment, tumor cells displayed different levels of Jagged1

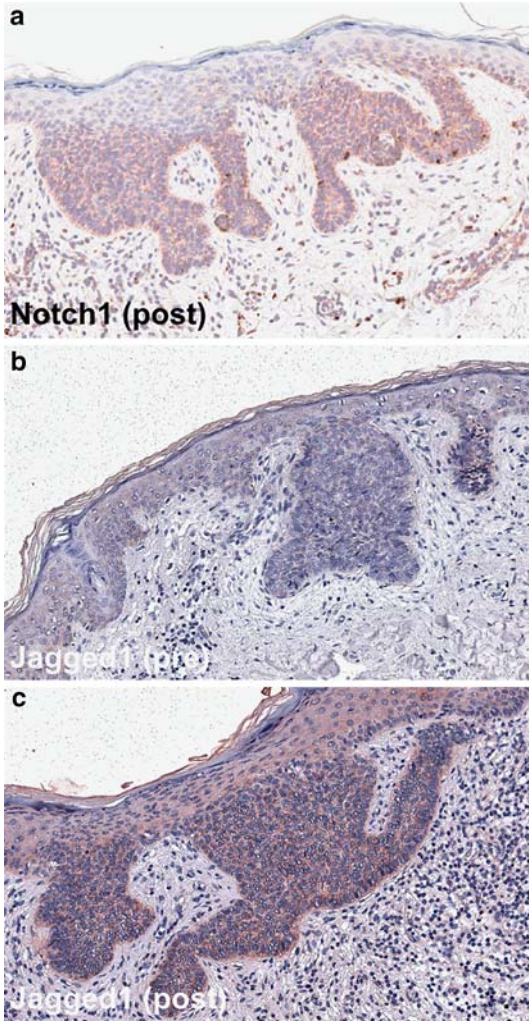

Fig. 2 Immunohistochemistry of Notch1 and Jagged1 protein in sBCC before (**b**) and after topical therapy with imiquimod (**a**) and (**c**), respectively. Original magnification was ×10

expression ranging from lack of (Fig. 2b) to true protein expression. In contrast to Notch1 protein expression, Jagged1 protein was strongly induced in tumors of three patients (patients 2, 4 and 6) upon imiquimod treatment (Fig. 2c). Infiltrating immune cells stayed negative for Jagged1 protein expression irrespective of the treatment. Jagged1 protein expression correlated with PCR data only partially (Fig. 1a, c, d). Taken together, imiquimod seems to preferentially induce protein expression of the Notch ligand, Jagged1 rather than the expression of Notch1 receptor itself in treated tumors.

Discussion

The Notch system was first described as being responsible for neurogenesis and ectodermal specification in *Drosophila* [2]. In mammals, there are four evolutionary conserved Notch receptors (Notch1–4) that are activated by five canonical Notch ligands Jagged1 and 2, Delta-like 1, 3 and 4. Notch signaling controls the development of most tissues by influencing cell death, proliferation and fate specification through cell–cell communication [2, 10]. Notch acts a control of the developmental processes though binary decision, lateral inhibition and boundary formation [12, 25]. The essential characteristic of Notch-mediated cell communication depends on differential expression of ligand and receptor on the cell surface [2, 4, 8, 10]. The cells stimulate each other to produce high levels of ligands and this again results in high receptor activation. High levels of ligand expression with subsequent Notch activation causes cell differentiation (and arrests the cell growth) and hence controls the cluster size. Notch plays an important role in the development and organization of different healthy tissues, but it also plays a role in tumorgenesis [1, 20]. Notch signaling is decreased in uncontrolled proliferating conditions, such as psoriasis [22]. Impaired Notch is also described to promote the development of squamous cell carcinoma of the skin and melanoma [13, 18]. Conversely, Delta/Notch signaling is increased in cells that undergo normal differentiation program, as in cell layers of the normal adult human epidermis [22]. Manipulation of Notch signaling could be therefore advantageous for the treatment of cancer. However, the function and the regulation of the Notch family members in the control of human skin tumors are not yet fully understood. In the skin, Notch seems to function as a tumor suppressor, as shown by Nicolas et al. [4, 15]. Loss of Notch1 in young mice induces hyperproliferation of the basal epidermal layer and deregulates expression of multiple differentiation markers, p21 is decreased and Gli2 is overexpressed [15]. Tumors that spontaneously develop in mice lacking Notch1 display BCC-like phenotype.

Expression of Notch1 and its ligands varies in the different layers of the epidermis [22]. In the healthy skin Notch1, Delta1 and Jagged1 are detectable in the whole epidermis, with pronounced expression of the latter two in the basal layer. Thélu and coworkers examined BCCs and found that the protein expression of Notch receptor and the ligands, Delta1 and Jagged1 was severely lowered in tumor regions [22]. They also observed expression of neither of the proteins in the regions with pallisading cells penetrating the dermis. In absence of Notch1, Delta1 and Jagged1 missing or decreased Notch signaling leads to disorder in epidermal differentiation and proliferation [22]. Our data demonstrate that Jagged1 protein is preferentially up-regulated following treatment with imiquimod. Jagged1 plays an important role in the differentiation of keratinocytes, as the activation of Notch pathway triggers terminal keratinocytes differentiation [4, 11, 12, 14]. As mentioned above, in BCCs the expression of Notch receptor and ligand is decreased in tumor regions [22]. When keratinocytes enter a pathological

status, they neither transcribe ligands nor receptor and thus the differentiating signal is absent [4, 22]. Our study shows that immunomodulatory treatment with imiquimod selectively up-regulates gene expression of Notch receptors (Notch1) and ligands (Jagged1, Delta1) in treated tumors. The results of the normalization to PTCH gene should be, however, interpreted with caution. Reduction of the viable tumor tissue may result in an artificially increased gene/PTCH ratio and would correspond, e.g. to the observed lack of Notch1 protein expression on tumor cells. Nevertheless, post-transcriptional modifications leading to discrepancy between RNA and protein levels cannot be excluded, as there are no currently available functional data on Notch1 regulation following TLR triggering. This effect of imiquimod on the skin seems to be rather drug and not disease-specific, since Jacobs et al. reported expression of NOTCH2 and 4 in the skin and common viral warts, but failed to detect their up-regulation after imiquimod treatment [7]. On the other hand, up-regulation of the ligand protein (Jagged1) would provide the missing signal for inducing differentiation and could theoretically influence the tumor phenotype [19]. So it seems that treatment with imiquimod has the potential to overcome BCC's biology and to stimulate the Notch pathway through the up-regulation of the Nocht-ligand Jagged1.

Imiquimod is a potent immune response modifier that activates TLR7, which results in an activation of NF-κB [5] and increased production of proinflammatory cytokines and a potent stimulation of anti-tumor Th1 immunity [24, 25]. Treatment with imiquimod induces massive peritumoral and intratumoral infiltration. Notch and its ligands play an important and critical role in cell fate determination of many cell types, including the lymphoid and dendritic cells [16, 27]. We observed only rare expression of the ligand Jagged1 on mononuclear cells in the infiltrate. Apart from T-cell development, signaling through Notch receptors appears to regulate the proteins that are crucial for peripheral T-cell activation and differentiation, such as NF-κB and IFN-γ [17]. Jagged1 is implicated to be a downstream target of NF-κB activation providing the loop between these two pathways [17]. Given the putative intersection of Notch and NF-κB signaling, it is conceivable that by activating TLR-7 imiquimod activates other downstream pathways in immune cells that enable mounting of an efficient immune response and tumor elimination.

In conclusion, our present study describes that imiquimod stimulates expression of Jagged1 protein in sBCC tumor cells. Via induction of Notch signaling imiquimod may exert tumor suppressor function, which together with its proinflammatory properties results in tumor regression.

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References

1. Allenspach EJ, Maillard I, Aster JC, Pear WS (2002) Notch signaling in cancer. Cancer Biol Ther 1:466–476
2. Artavanis-Tsakonas S, Rand MD, Lake RJ (1999) Notch signaling: cell fate control and signal integration in development. Science 284:770–776
3. Barnetson RS, Satchell A, Zhuang L, Slade HB, Halliday GM (2004) Imiquimod induced regression of clinically diagnosed superficial basal cell carcinoma is associated with early infiltration by CD4 T cells and dendritic cells. Clin Exp Dermatol 29:639–643
4. Blanpain C, Horsley V, Fuchs E (2007) Epithelial stem cells: turning over new leaves. Cell 128:445–458
5. Carmody RJ, Chen YH (2007) Nuclear factor-kappaB: activation and regulation during toll-like receptor signaling. Cell Mol Immunol 4:31–41
6. Holikova Z, Massi D, Lotti T, Hercogova J (2004) Insight into the pathogenesis of sporadic basal cell carcinoma. Int J Dermatol 43:865–869
7. Jacobs S, Grussendorf-Conen EI, Rosener I, Rubben A (2004) Molecular analysis of the effect of topical imiquimod treatment of HPV 2/27/57-induced common warts. Skin Pharmacol Physiol 17:258–266
8. Kolev V, Kacer D, Trifonova R, Small D, Duarte M, Soldi R, Graziani I, Sideleva O, Larman B, Maciag T, Prudovsky I (2005) The intracellular domain of Notch ligand Delta1 induces cell growth arrest. FEBS Lett 579:5798–5802
9. Kruger K, Blume-Peytavi U, Orfanos CE (1999) Basal cell carcinoma possibly originates from the outer root sheath and/or the bulge region of the vellus hair follicle. Arch Dermatol Res 291:253–259
10. Lai EC (2004) Notch signaling: control of cell communication and cell fate. Development 131:965–973
11. Lefort K, Dotto GP (2004) Notch signaling in the integrated control of keratinocyte growth/differentiation and tumor suppression. Semin Cancer Biol 14:374–386
12. Lowell S, Jones P, Le Roux I, Dunne J, Watt FM (2000) Stimulation of human epidermal differentiation by delta-notch signalling at the boundaries of stem-cell clusters. Curr Biol 10:491–500
13. Nickoloff BJ, Hendrix MJ, Pollock PM, Trent JM, Miele L, Qin JZ (2005) Notch and NOXA-related pathways in melanoma cells. J Investig Dermatol Symp Proc 10:95–104
14. Nickoloff BJ, Qin JZ, Chaturvedi V, Denning MF, Bonish B, Miele L (2002) Jagged-1 mediated activation of notch signaling induces complete maturation of human keratinocytes through NF-kappaB and PPARgamma. Cell Death Differ 9:842–855
15. Nicolas M, Wolfer A, Raj K, Kummer JA, Mill P, van Noort M, Hui CC, Clevers H, Dotto GP, Radtke F (2003) Notch1 functions as a tumor suppressor in mouse skin. Nat Genet 33:416–421
16. Olivier A, Lauret E, Gonin P, Galy A (2006) The Notch ligand delta-1 is a hematopoietic development cofactor for plasmacytoid dendritic cells. Blood 107:2694–2701
17. Osborne BA, Minter LM (2007) Notch signalling during peripheral T-cell activation and differentiation. Nat Rev Immunol 7:64–75
18. Proweller A, Tu L, Lepore JJ, Cheng L, Lu MM, Seykora J, Millar SE, Pear WS, Parmacek MS (2006) Impaired notch signaling promotes de novo squamous cell carcinoma formation. Cancer Res 66:7438–7444
19. Savill NJ, Sherratt JA (2003) Control of epidermal stem cell clusters by Notch-mediated lateral induction. Dev Biol 258:141–153
20. Shou J, Ross S, Koeppen H, de Sauvage FJ, Gao WQ (2001) Dynamics of notch expression during murine prostate development and tumorigenesis. Cancer Res 61:7291–7297
21. Stulberg DL, Crandell B, Fawcett RS (2004) Diagnosis and treatment of basal cell and squamous cell carcinomas. Am Fam Physician 70:1481–1488

22. Thelu J, Rossio P, Favier B (2002) Notch signalling is linked to epidermal cell differentiation level in basal cell carcinoma, psoriasis and wound healing. BMC Dermatol 2:7
23. Urosevic M, Dummer R (2002) Immunotherapy for nonmelanoma skin cancer: does it have a future? Cancer 94:477–485
24. Urosevic M, Dummer R (2004) Role of imiquimod in skin cancer treatment. Am J Clin Dermatol 5:453–458
25. Urosevic M, Dummer R, Conrad C, Beyeler M, Laine E, Burg G, Gilliet M (2005) Disease-independent skin recruitment and activation of plasmacytoid predendritic cells following imiquimod treatment. J Natl Cancer Inst 97:1143–1153
26. Urosevic M, Maier T, Benninghoff B, Slade H, Burg G, Dummer R (2003) Mechanisms underlying imiquimod-induced regression of basal cell carcinoma in vivo. Arch Dermatol 139:1325–1332
27. Wicking C, McGlinn E (2001) The role of hedgehog signalling in tumorigenesis. Cancer Lett 173:1–7