Tazarotene-Induced Gene 3 Is Suppressed in Basal Cell Carcinomas and Reversed *In Vivo* by Tazarotene Application

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Basal cell carcinomas are the most common form of skin cancer. Tazarotene is a retinoic acid receptor selective retinoid that upregulates a tumor suppressor, tazarotene-induced gene 3 (TIG-3), in keratinocytes and psoriasis. Expression of TIG-3 in basal cell carcinomas was studied in an opened-label pilot biomarker study of 22 patients with basal cell carcinomas who applied tazarotene 0.1% gel for up to 12 wk prior to excision. Nineteen paired baseline and treated specimens were compared using immunohistochemistry and *in situ* hybridization. Compared to overlying normal epidermis, TIG-3 protein and mRNA were decreased in 14 and 18 of 19 basal cell carcinomas (74% and 95%), respectively ($p < 0.001$). Tazarotene treatment was associated with increased TIG-3 protein and mRNA expression in basal cell carcinomas compared to baseline levels ($p \leqslant 0.001$ and $p = 0.028$, respectively). Sixty percent of basal cell carcinomas treated with tazarotene decreased in size by at least 25%. Ten of 19 lesions improved histologically, including three complete responses. There was a correlation between the increased expression of TIG-3 protein and histologic improvement ($p = 0.020$), suggesting that suppression of TIG-3 may underlie the development of basal cell carcinomas. This association suggests that reversal of TIG-3 expression may help to explain the mechanism of retinoid action in epidermal differentiation and chemoprevention. ***Key words: chemoprevention/retinoids/skin cancer/translational research/tumor suppressor. J Invest Dermatol 121:902–909, 2003***

Basal and squamous cell carcinomas arise from epidermal keratinocytes, and comprise the majority of skin cancers, the most common malignancy in human (Kanjali and Duvic, 1998). A complex genetic program underlying normal epidermal differentiation leads to terminal differentiation, i.e., keratinocyte apoptosis and barrier formation (Eckert *et al*, 1997). Epidermal carcinogenesis is associated with ultraviolet-light-induced genetic mutations in the tumor suppressor, p53 (Brash *et al*, 1996; Ananthaswamy *et al*, 1997), and sonic hedgehog signaling pathway proteins Lacour, 2002; Tojo *et al*, 2002). Psoriasis is a nonmalignant, epidermal hyperproliferation induced in genetically susceptible individuals by reversible cellular inflammation and cytokine expression (Veal *et al*, 2002). The cytokine inducible nuclear transcription factor, NF-κB, is implicated in the control of epidermal proliferation and may be common to both psoriasis and skin cancer (Seitz *et al*, 1998).

Vitamin A analogs or retinoids have been used clinically to treat carcinomas arising from oral mucosa and skin (Kraemer *et al*, 1988) as well as for chemoprevention of second primary cancers in head and neck and lung cancer (Lippman *et al*, 1996). A clear understanding of their specific mechanism(s) of action in cancer, however, has lagged behind clinical application. Retinoids bind to tissue-specific retinoid receptors: the retinoic acid receptor (RAR-α, RAR-β, and RAR-γ) and retinoid X receptor (RXR-α, RXR-β, and RXR-γ). RAR and RXR are ligand-dependent transcription factors that form homodimers or heterodimers, bind to DNA sequences or response elements, and also interact with other transcription factors to modulate the expression of genes (Chambon, 1995). Retinoids and their receptors influence expression of keratin genes in epidermal cells (Aneskievich and Fuchs, 1992).

To better understand retinoid action, cells in cell culture systems have been treated with receptor-selective retinoids, and a panel of genes that are induced or reduced during treatment with selective retinoids have been isolated (Balmer and Blomhoff, 2002). Tazarotene is a synthetic RAR-β/γ selective retinoid approved for the topical treatment of acne and psoriasis (Elder *et al*, 1992; Weinstein *et al*, 1997; Balmer and Blomhoff, 2002; Webster *et al*, 2002). Differential display PCR was employed to identify mRNAs that were induced by tazarotene treatment of human keratinocytes, and over 30 genes, called RARRES for retinoic acid receptor responders or TIGs for tazarotene-induced genes, have been isolated (Nagpal *et al*, 1996a; 1996b; 1997; Thacher *et al*, 1996; DiSepio *et al*, 1998; Duvic *et al*, 1998; Jing *et al*, 2002). Only few of these genes have been further characterized. TIG-1, a membrane bound protein recently implicated as a tumor suppressor, is diminished in prostate carcinoma cell lines and tumors (Nagpal *et al*, 1996a; Jing *et al*, 2002).

Our laboratory has been interested in the tazarotene-induced gene 3 (TIG-3) because it is homologous to H-rev, a known class

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Abbreviations: CCR, complete clinical response; IHC, immunohistochemistry; ISH, *in situ* hybridization; MR, minor response; PD, progressive disease; PR, partial response; RAR, retinoic acid receptor; RARRES, retinoic acid receptor responders; RXR, retinoid X receptor; SD, stable disease; TIG-3, tazarotene-induced gene 3.

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II tumor suppressor that inhibits growth of cancer cell lines *in vitro* (Hajnal *et al*, 1994; DiSepio *et al*, 1998). TIG-3 gene was localized to chromosome 11q23, a site of loss of heterozygosity in several cancer lines (Murakami *et al*, 1998; Di Iasio *et al*, 1999; Duvic *et al*, 2000). Expression studies of TIG-3 have shown that the carboxy-terminus is required for cell location and growth inhibition (Deucher *et al*, 2000). In breast cancer cell lines, growth inhibition by retinoid treatment correlates with TIG-3 inducibility suggesting a causal relationship (DiSepio *et al*, 1998). With regard to epidermal hyperproliferation and development of skin cancers, we previously found that TIG-3 expression is diminished in psoriasis lesions and basal cell carcinomas (BCC), and is significantly lower in advanced squamous cell carcinomas (DiSepio *et al*, 1998; Duvic *et al*, 2000).

BCC are common, indolent skin tumors arising from keratinocytes in the interfollicular basal epidermis, hair follicles, and sebaceous glands, and they can become locally invasive and cause significant mortality (Kruger *et al*, 1999). Of interest, topical tazarotene gel used for 8 mo was previously reported to induce clinical remission in 16 of 30 BCC (53%), but the mechanism of action and histology were not addressed (Peris *et al*, 1999). To determine whether a relationship exists between application of topical tazarotene, clinical improvement in BCC, and induction of TIG-3, we performed a prospective clinical molecular biomarker trial assessing TIG-3 expression in patients' skin. All growth patterns of BCC demonstrated decreased or loss of expression of TIG-3 protein and mRNA that was reversed *in vivo* with treatment of BCC with tazarotene for up to 12 wk.

MATERIALS AND METHODS

Patients and study design The experiments conducted in this study were done in accordance with Helsinki principles and had institutional IRB approval. Twenty-two adults (shown in **Table I**) with 35 histologically proven BCC signed approved informed consent to participate in a prospective, opened label pilot study conducted at a single institution. Two weeks after a 3 mm diagnostic punch biopsy of the BCC was performed, patients were instructed to apply tazarotene 0.1% gel (kindly provided by Allergan, Irvine, CA) to BCC tumors once daily for 6–12 wk. The lesions were clinically evaluated every 4 wk for tumor response using bidimensional measurements and photography. Oncologic responses were graded as CCR (complete clinical disappearance of tumor), PR (>50% partial reduction in size), MR (minor response of 25%–50% reduction in size), SD (stable disease, 0%–25% decrease) or progression (PD, increase in tumor size). All BCC were removed by Mohs micrographic surgery (Rapini, 1999) and the tissue was examined histologically. The degree of local irritation was assessed as absent or present (mild, moderate, or severe) (**Table II**).

Table I. Patient demographics and response to tazarotene treatment

Characteristics	Patients with BCC
Age – y	$n=22$
Median	67
Range	50–85
Gender – no. of patients (%)	$n=22$
Female	3 (14)
Male	19 (86)
Histologic patterns – no. of lesions (%)	$n=35$
Superficial	7 (20)
Nodular	15 (43)
Infiltrative	2 (6)
Mixed	11 (31)
Clinical response – no. of lesions (%)	$n=35$
Complete clinical response	1 (3)
Partial response (>50%)	9 (26)
Minor response (>25%<50%)	11 (31)
Stable disease (>0%<25%)	12 (34)
Progressive disease	1 (3)
Lost follow-up	1 (3)
Duration of treatment – no. of patients (no. of lesions)	22 (35)
≤6 wk	10 (11)
>6–12 wk	12 (24)

Histopathology Skin specimens were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained as previously described (Stoler *et al*, 1988; Duvic *et al*, 2000). BCC were graded as having one of four growth patterns: superficial (including multicentric), nodular (including micronodular), infiltrative (including morpheaform), or mixed (including a combination of any two or all of the above types or adenoid differentiation) (Rippey, 1998). Histopathology was evaluated in all 35 lesions (**Table I**). Nineteen paired pre- and post-treatment skin biopsy specimens from 14 patients were available and of sufficient quantity and quality to evaluate TIG-3 protein and mRNA expression (**Table III**). Normal adjacent skin from tumors numbered 4, 5, and 7 at baseline as well as normal control skin from six adult mastectomy patients were used as the positive controls. All BCC were excised with Mohs micrographic surgery at the end of the study and normal healing was observed.

Immunohistochemistry (IHC) IHC was performed as previously described (Duvic *et al*, 2000). TIG-3 rabbit polyclonal antibody raised to recombinant TIG-his tag protein$_{1-164}$ was a gift from Dr Richard Eckert and Dr Anne Deucher, Department of of Biochemistry, Case Western Reserve, Cleveland, OH (Deucher *et al*, 2000). The primary antibody was at a dilution of 1:500 and was detected with horse antirabbit secondary antibody at 1:100 using a Vector Stain ABC kit (Vector Laboratories, Burlingame, CA). Primary antibody was omitted as a negative control.

***In situ* hybridization (ISH)** ISH was conducted using plasmid (pAGN-TIG-3) with the 600 bp 3′ TIG-3 cDNA in reverse orientation as previously described (DiSepio *et al*, 1998; Duvic *et al*, 2000). One microgram of cDNA template was linearized with Not I or Hind III and transcribed using either Sp6 or T7 polymerase to yield antisense and sense (negative control) riboprobes, respectively. Riboprobes were transcribed in the presence of UTP-digoxigenin using a Genius 4 kit, according to the manufacturer's instructions (Roche, Indianapolis, IN). The probe concentrations were estimated using a dot-blot method and serial dilutions with a Dig DNA labeling and detection kit (Roche). ISH was performed as described with the modification of using active DEPC water and using ethanol to reduce background staining (Duvic *et al*, 2000; Xu *et al*, 2001). Baseline and treated tissue sections with normal skin controls were analyzed simultaneously for expression of TIG-3 mRNA to ensure reliable comparison.

Semiquantitative analysis of staining intensity The staining intensity was evaluated by either IHC or ISH, graded by two separate blinded observers, and recorded for each specimen of normal control skin, tumor, and adjacent skin. For IHC, staining intensity was graded as 0 (no staining), 1 (faint staining, light brown), 2 (light staining, yellow brown), 3 (moderate brown), 4 (dark brown), or 5 (dark black staining obscuring the architecture). For ISH, staining intensity was graded as 0 (no staining), 1 (light blue, faint staining), 2 (blue staining), 3 (moderate staining, purple color), 4 (strong, very deep purple), and 5 (dark black staining obscuring the architecture). The scales are shown in **Fig 1**.

Statistical analysis Means, standard deviation, frequency, and summary data are given whenever appropriate. Fisher's exact test, the χ^2 test, and the general linear model (GML) univariate procedure (SSPS, version 11.5 for Windows) were used to assess the association between two binary variables, such as the association between the clinical responses and the modulation of TIG-3. A two-tailed paired sample *t* test was used to compare TIG-3 expression between BCC lesions and the overlying epidermis or from BCC lesions before and after treatment. Two-sided p-values were determined in all analyses.

RESULTS

Clinical response and safety As shown in **Table I**, 11 lesions from 10 patients were treated with topical tazarotene 0.1% gel for less than 6 wk and 24 lesions from 12 patients were treated for 6–12 wk. The tumors ranged in size from 0.3×0.3 to 5.5×2.0 cm with a median dimension of 1.0×1.5 cm. Twenty-

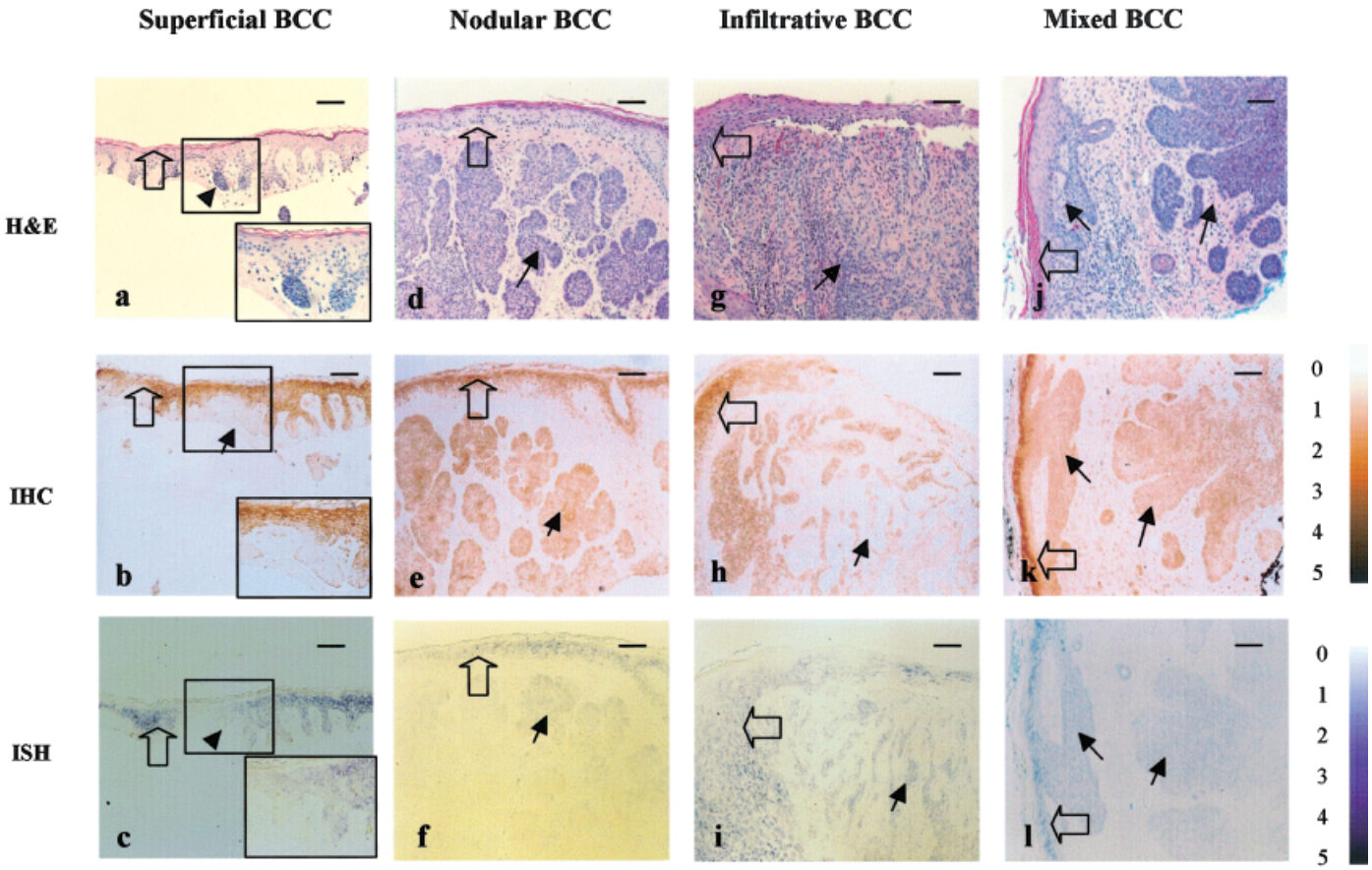

Figure 1. TIG-3 protein and mRNA expression are decreased in all types of BCC compared to the overlying epidermis. Four BCC of representative histology (superficial, nodular, infiltrative, and mixed) were studied using hematoxylin and eosin staining for morphology (*top row*: *a*, *d*, *g*, *j*), IHC for TIG-3 protein (*middle row*: *b*, *e*, *h*, *k*), and ISH for mRNA expression (*bottom row*: *c*, *f*, *i*, *l*) as described in *Materials and Methods*. *Black arrows* indicate tumor areas and *empty arrows* indicate the overlying epidermis. The *boxed inserts* in (*a*), (*b*), and (*c*) indicate where superficial BCC is budding from the epidermis. *Scale bar*: 50 μm.

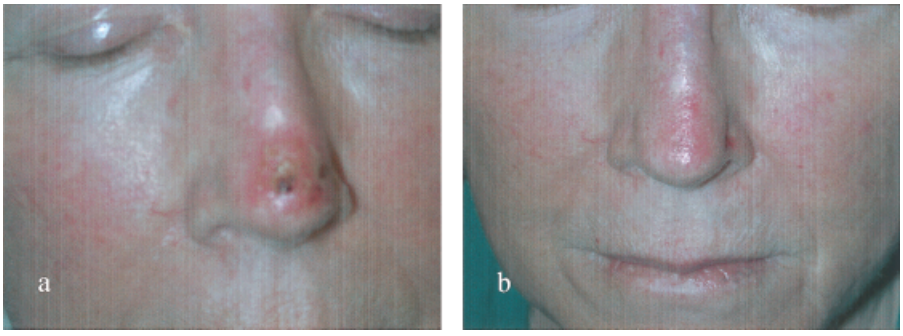

Figure 2. One female patient with a nodular BCC on the tip of the nose. (*a*) Baseline photograph taken 1 wk after biopsy on 4-03-01. (*b*) After 1 mo of topical tazarotene gel 0.1% application on 5-02-01 prior to surgical removal.

one of the 35 BCC treated (60%) showed a reduction of 25% or more in tumor size (CCR + PR + MR) at the time of surgical excision. Nine of 35 lesions (26%) had partial regression (PR). An example of a responding patient is shown in **Fig 2**. Eleven lesions (31%) exhibited minor responses (MR), 12 BCC (34%) were judged as stable (SD), and only one (3%) progressed slightly in size during therapy. One patient was lost to follow-up. In the group of BCC treated for less than 6 wk, one was judged CCR, one partially regressed, and five had minor responses. The overall response rate was eight PR and six MR for 24 BCC treated for 6–12 wk.

Table II. Symptoms or signs reported with topical tazarotene gel application

Symptom or sign	Frequency among patients ($n = 22$)
Burning or itching	1 (5%)
Telangectasias	6 (27%)
Erythema or inflammation	10 (45%)
Flaking, excoriations	3 (14%)
Erosions or ulcerations	4 (18%)
Crusting, scabbing	14 (63%)

Table III. Expression of TIG-3 protein and mRNA in 19 BCC and overlying epidermis before and after topical tazarotene treatment

				TIG-3 staining intensity (IHC)[b]				TIG-3 staining intensity (ISH)[c]			
		Growth pattern[a]		Baseline		Treated		Baseline		Treated	
ID	Sites	Baseline	Treated	BCC	Epidermis	BCC	Epidermis	BCC	Epidermis	BCC	Epidermis
1	Shoulder	M (N + I)	M (N + S)	2	4	2	3	2	3	2	3
2	Nose	N	M (N + A)	2	3	2	2	1	2	3	3
3	Clavicle	M (N + S)	S	2	4	4	3	1	2	2	3
4	Abdomen	S	S	0	4	2	4	0	4	0	1
5	Nasal ala	I	I	1	3	2	1	1	3	2	1
6	Temple	M (N + S)	M (N + S + I)	2	4	2	3	1	3	1	1
7	Helix	N	M (N + I)	1	4	1	2	0	0	0	0
8	Auricular	M (N + I)	S	2	2	3	2	0	1	0	1
9	Ear	N	M (N + S)	1	3	3	2	0	3	1	1
10	Upper back	S	S	1	5	2	5	2	4	3	4
11	Mid-back	S	S	1	1	1	2	3	3	2	4
12	Nose	M (N + A)	S	2	5	3	3	1	2	2	4
13	Nose	S	n/a[d]	2	3	n/a	3	1	3	n/a	2
14	Frontal scalp	N	N	2	2	3	5	1	3	3	4
15	Forehead	N	n/a	3	5	n/a	5	1	3	n/a	4
16	Chest	M (N + S)	n/a	2	5	n/a	4	2	5	n/a	2
17	Temple	N	M (N + S)	1	4	3	4	1	4	2	4
18	Neck	N	N	1	1	3	2	1	3	3	4
19	Ala	N	S	1	1	3	3	1	2	3	4
Mean ± SD				1.53 ± 0.69	3.32 ± 1.38	2.44 ± 0.81	3.05 ± 1.18	1.05 ± 0.78	2.79 ± 1.13	1.81 ± 1.11	2.63 ± 1.42
Paired Student *t* test				[e]p < 0.001		[f]p = 0.186		[g]p < 0.001		[h]p = 0.001	
				[i]p < 0.001		[k]p = 0.399		[j]p = 0.028		[l]p = 0.718	

[a]Growth pattern morphology: N, nodular; S, superficial; I, infiltrative or morpheaform; M, mixed − a combination of any two or all of the above patterns.
[b]Staining intensity of IHC: 0, no staining; 1, light yellow, faint staining; 2, light brown staining; 3, moderate staining, brown color; 4, strong, deep brown; 5, dark black staining obscuring the architecture.
[c]Staining intensity of ISH: 0, no staining; 1, light blue, faint staining; 2, blue staining; 3, moderate staining, purple color; 4, strong, very deep purple; 5, dark black staining obscuring the architecture.
[d]n/a, not applicable.
[e]p before treatment, IHC, BCC *versus* epidermis; [f]p after treatment, IHC, BCC *versus* epidermis; [g]p before treatment, ISH, BCC *versus* epidermis; [h]p after treatment, ISH, BCC *versus* epidermis; [i]p IHC, BCC, before treatment *versus* after; [j]p ISH, BCC, before treatment *versus* after; [k]p IHC, epidermis, before treatment *versus* after; [l]p ISH, epidermis, before treatment *versus* after.

Table IV. Growth patterns of BCC before and after topical tazarotene treatment

Growth patterns of BCC	No. of BCC lesions/total (%)	
	Before treatment	After treatment
Superficial	4 (21%)	7 (37%)
Nodular	8 (42%)	2 (11%)
Infiltrative or morpheaform	1 (5%)	1 (5%)
Mixed	6 (32%)	6 (32%)
No tumor	–	3 (16%)
Total	19	19

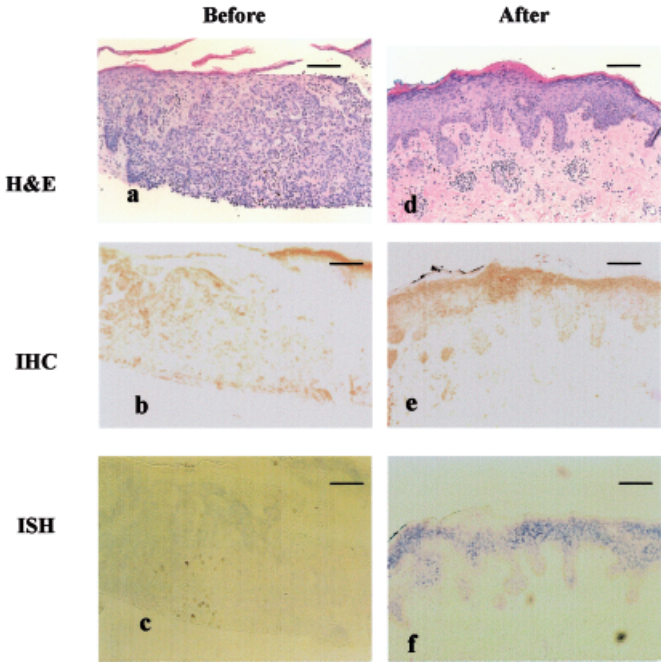

Figure 3. Improvement in BCC histology correlates with increased TIG-3 expression following topical tazarotene. Tumor no. 3 (**Table III**) is shown at baseline (*a, b, c*) and after tazarotene treatment (*d, e, f*). Hematoxylin and eosin staining (*a, d*) is for histology, TIG-3 protein expression is stained by IHC (*b, e*), and TIG-3 mRNA expression is stained by ISH (*c, f*). *Scale bar*: 50 μm.

As shown in **Table II**, local symptoms and side-effects commonly experienced included burning or itching, erythema, and irritation. Ulceration or crusting could not be distinguished from tumor ulceration induced by therapeutic effect. Irritation was reduced by discontinuing the medication for a few days.

Histologic improvement of BCC tumors after topical tazarotene treatment The histology of 19 pairs of BCC from 14 patients was evaluated at baseline and following topical tazarotene treatment (**Table III**). At baseline, BCC were graded as superficial ($n=4$), nodular ($n=8$), infiltrative ($n=1$), or mixed histology ($n=6$). Mixed lesions included superficial plus nodular BCC ($n=3$), nodular plus infiltrative BCC ($n=2$), and nodular with adenoid differentiation ($n=1$). Representative specimens are shown in the upper row of **Fig 1**. Ten of 19 BCC specimens (53%) were histologically improved after treatment with topical tazarotene, compared to baseline. Three BCC (nos. 13, 15, 16) were histologically clear without remaining tumor at resection (**Tables III, IV**). Five nodular BCC (nos. 3, 8, 12, 17, 19) at baseline were judged as having superficial histology after treatment, as shown in **Fig 3** for tumor no. 3. Tumor no. 1 showed a nodular and infiltrative pattern at baseline with nodular and superficial histology after treatment.

One infiltrative BCC of the nasal ala (lesion 5) was completely resolved by clinical examination, but infiltrative tumor cells were still present on histologic examination (not shown). Six lesions (32%) had unchanged histology. Three BCC (16%) had an infiltrative pattern on the deeper resection that had not been appreciated in the more superficial baseline specimen.

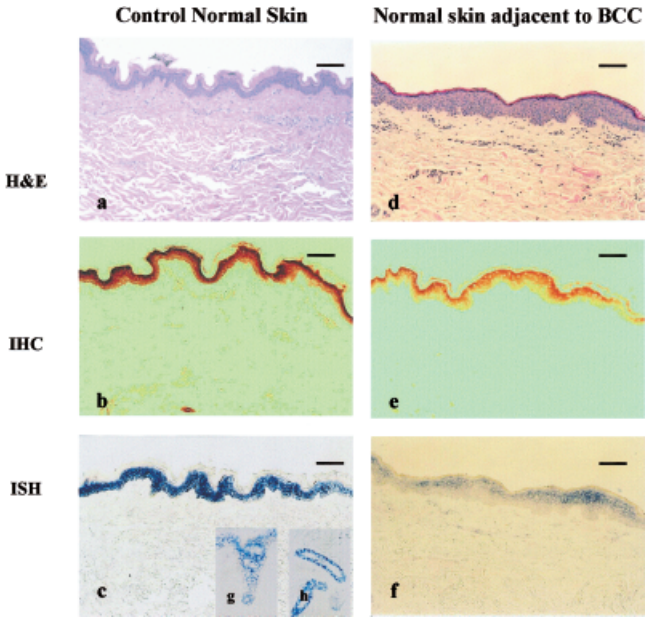

Figure 4. TIG-3 expression of protein and mRNA is lower in the epidermis adjacent to BCC tumors compared to the normal epidermis. IHC staining and ISH were used to detect the presence of TIG-3 protein (*middle row*) and mRNA signal (*bottom row*). *Scale bar*: 50 μm.

TIG-3 protein is significantly reduced in BCC compared to overlying normal epidermis at baseline As shown in **Fig 4**, yellow-brown staining for TIG-3 protein is stronger in the suprabasal layers *versus* the basal layer of normal skin from control subjects ($n=6$). The cell cytoplasm stains homogeneously in keratinocytes, including those of the appendages (not shown). The staining intensity of TIG-3 protein is diminished in both suprabasal and basal layers of the epidermis adjacent to BCC tumors ($n=3$) compared to normal control epidermis.

As shown in **Table III** and **Fig 5**, prior to treatment the mean level of TIG-3 protein in BCC at baseline (1.53 ± 0.69) was 2.17-fold less than that in the paired overlying epidermis (3.32 ± 1.38) ($p<0.001$). Fourteen of 19 BCC (74%) showed reduced levels of TIG-3 protein. One superficial lesion (5%) showed complete loss of TIG-3 protein expression and 13 of 19 BCC (69%) had diminished TIG-3 protein expression compared to overlying normal epidermis. Decreased TIG-3 protein was observed in all histologic types of BCC including two superficial, five nodular, one infiltrative, and five mixed tumors (middle row, **Fig 1**). In five of 19 BCC (26%), TIG-3 protein expression was similar to overlying epidermis but reduced compared to normal skin.

TIG-3 protein is significantly increased in BCC after topical tazarotene treatment TIG-3 protein expression in the overlying epidermis was similar before and after treatment with tazarotene gel (3.32 ± 1.38 before *vs* 3.05 ± 1.18 after, $p=0.399$ by paired *t* test, $n=19$). In contrast, the mean level of TIG-3 protein (2.44 ± 0.81) was higher after therapy than that at baseline (1.53 ± 0.69) ($p<0.001$ by paired *t* test, $n=16$). Eleven of 16 BCC

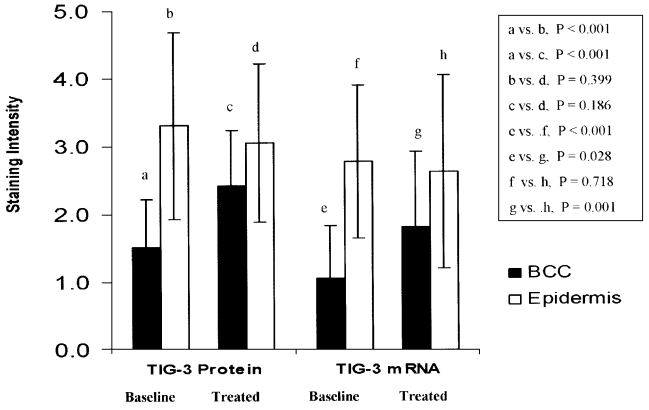

Figure 5. Mean level of TIG-3 protein and mRNA significantly diminishes in BCC at baseline and increases after tazarotene treatment. The intensity of staining for TIG-3 protein by IHC (*left four bars*, *a*, *b*, *c*, and *d*) and the intensity of staining for TIG-3 mRNA by ISH (*right four bars*, *e*, *f*, *g*, and *h*) were graded using a semiquantitative scale of 0–5, as described in *Materials and Methods*. The mean intensities of staining in BCC (*black bars*) were compared with the overlying epidermis (*white bars*) at baseline (*a vs b* and *e vs f*) and after the treatment (*c vs d* and *g vs h*). The mean intensities of staining in BCC (*black bars*) at baseline were compared with those after treatment (*a vs c* and *e vs g*).

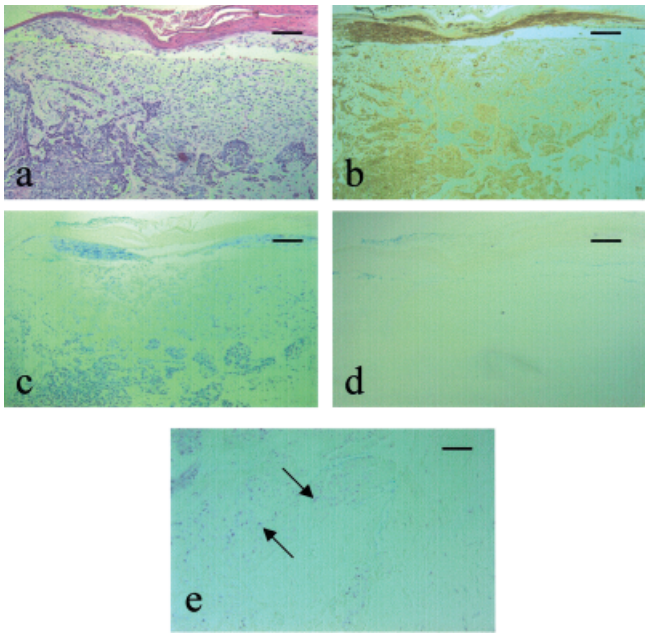

Figure 6. TIG-3 mRNA staining in nuclei seen in BCC lesions with diminished TIG-3 mRNA. Hematoxylin and eosin staining is for histology (*a*); IHC staining and ISH were used to detect the presence of TIG-3 protein (*b*) and mRNA (*c*). The specificity of the binding of the antisense riboprobe was verified by using sense probe as a negative control (*d*). TIG-3 mRNA signal in nuclei was seen in tumor no. 1 BCC with diminished TIG-3 mRNA (*e*). *Scale bar*: 50 μm.

(69%) with residual tumor at excision showed increased TIG-3 protein expression after treatment, whereas five of 16 BCC (31%) had no change. Post-treatment TIG-3 protein expression in BCC was not significantly different from paired, treated, overlying epidermis (**Table III**, **Fig 5**). After treatment, TIG-3 protein was only lower in eight of 16 BCC (50%) compared to overlying epidermis, as opposed to 14 of 19 BCC with lower expression at baseline (74%, $p = 0.021$ by χ^2 test) (**Table III**).

Three of 19 paired lesions showed complete histologic clearing, and in these the overlying treated epidermis was graded as 5 (most intense) in two and 3 (moderate) in the third.

TIG-3 mRNA is significantly reduced in BCC compared to overlying normal epidermis at baseline As evaluated by ISH, TIG-3 mRNA stained blue-purple in the cytoplasm and was homogeneously distributed in normal epidermis and appendages. TIG-3 mRNA was diminished in the basal layer of the epidermis adjacent to tumors, compared to normal control epidermis (**Fig 4**). TIG-3 mRNA in the basal layer of epidermis overlying BCC was less than that in normal control epidermis.

As shown in **Table III** and **Fig 5**, the mean level of TIG-3 mRNA BCC (1.05 ± 0.78) was 2.66-fold less than that in overlying epidermis (2.79 ± 1.13, $p < 0.001$ by paired *t* test) at baseline. TIG-3 mRNA was reduced in 18 of 19 BCC tumors (95%) compared to the overlying epidermis (**Table III**). Four of 19 BCC (21%) had complete absence of TIG-3 mRNA and 14 (74%) had decreased TIG-3 expression compared to overlying epidermis. These changes were irrespective of tumor histology (**Fig 1**, *bottom row*). One superficial BCC of the trunk (lesion 11) had equal TIG-3 mRNA staining to the paired epidermis. As shown for lesion 1 in **Fig 6**, nuclear staining was observed in some tumor cells with decreased or absent cytoplasmic staining.

TIG-3 mRNA significantly increased in BCC after topical tazarotene treatment The mean level of TIG-3 mRNA did not change in overlying normal epidermis from baseline to post-treatment (2.79 ± 1.13 *vs* 2.63 ± 1.42, respectively, $p = 0.718$ by paired *t* test). In BCC TIG-3 mRNA was increased from 1.05 ± 0.78 at the baseline to 1.81 ± 1.11 following treatment ($p < 0.028$ by Student paired *t* test) (**Table III**, **Fig 5**) although TIG-3 mRNA in BCC was still lower than in overlying epidermis (2.63 ± 1.42) ($p = 0.001$) (**Table III**, **Fig 5**). Ten of 16 BCC (63%) were found to have increased TIG-3 mRNA expression, five tumors (31%) had stable TIG-3 mRNA expression, and one superficial BCC had decreased TIG-3 mRNA after treatment. After treatment TIG-3 mRNA was lower in 11 of 16 BCC (69%) compared to paired overlying epidermis, in contrast to baseline where 18 of 19 BCC were lower in TIG-3 mRNA (95%, $p = 0.204$ by χ^2 test) (**Table III**).

In three BCC with complete histologic clearing, the overlying epidermis was graded by ISH as 5 (most intense) in one and 3 (moderate) in two following therapy.

Relationship between TIG-3 expression and clinical and histologic improvement Twelve of 19 pairs of BCC (63%) had clinical responses to treatment (CCR, 1; PR, 5; MI, 6) and seven did not respond (SD, 6; PD, 1). Eleven of 16 BCC (69%) evaluated after therapy had increased TIG-3 protein expression as above. Seven of 11 lesions with upregulated expression of TIG-3 protein after treatment had clinical responses (64%), compared to two of five lesions (40%) without TIG-3 protein upregulation ($p = 0.071$, by Fisher's two-sided exact test). Similarly, 10 of 16 BCC (63%) had increased TIG-3 mRNA as above, and seven of 10 lesions (70%) with increased TIG-3 mRNA had clinical responses, compared with two of six lesions (33%) without TIG-3 mRNA upregulation ($p = 0.059$, by Fisher's two-sided exact test).

Ten of 19 paired lesions (53%) had histologic improvement to treatment as mentioned before (**Table IV**). Six of 11 lesions (54.5%) with upregulated expression of TIG-3 protein after treatment had histologic improvement, compared with one of five lesions (20%) without TIG-3 protein upregulation. There was a significant correlation between the increased expression of TIG-3 protein (but not TIG-3 mRNA) and histologic improvement ($p = 0.020$, by GLM model univariate).

DISCUSSION

Our study analyzed the expression of TIG-3 protein and mRNA levels in paired BCC before and after treatment with 0.1% topical tazarotene gel. Consistent with a pilot study involving only three BCC (Duvic *et al*, 2000), TIG-3 protein and mRNA were selectively lost or decreased in BCC. We also report for the first time that tazarotene application was associated with reversal of the diminished expression of TIG-3 protein and mRNA in human subjects' BCC tumors. These data support the hypothesis that TIG-3 (RARRES-3) is a RAR-induced tumor suppressor and a potential target for therapeutic and chemoprevention *in vivo* for skin cancer as well as tumor cell lines (DiSepio *et al*, 1998; Duvic *et al*, 2000).

The mechanism resulting in diminished TIG-3 expression in BCC is unknown. Reversibility by treatment in psoriasis and skin cancer cells and lesions suggests the importance of modulation of gene transcription rather than genetic deletions (DiSepio *et al*, 1998; Duvic *et al*, 2000). Mutations in TIG-3 coding sequences in squamous cell carcinoma cells and head and neck squamous cell carcinoma cell lines have not been detected (Schulz, manuscript in preparation). Differences have been observed in the methylation patterns of the TIG-3 promoter from squamous carcinoma cell lines, which could affect transcription. This study, conducted *in vivo*, also supports the reversibility of TIG-3 expression in human tumors after topical tazarotene application. Suppressed expression of retinoid receptors, especially RAR-β, is reported in actinic keratosis, squamous cell carcinoma, and premalignant oral lesions, and can be upregulated by isotretinion (Lotan *et al*, 1995; Xu *et al*, 2001). The expression of retinoid receptors in BCC has not been fully explored.

TIG-3 or RARRES-3 was first identified from tazarotene-treated keratinocytes using differential display PCR to amplify and identify mRNA (DiSepio *et al*, 1998). TIG-3 has been shown to be inducible by RAR-selective retinoids in primary human keratinocytes and TIG-3 transfection results in growth inhibition of tumor cells *in vitro* (DiSepio *et al*, 1998; Deucher *et al*, 2000). Although we previously showed that TIG-3 mRNA expression was induced in psoriatic lesions after application of topical tazarotene 0.1% gel for 2 wk (DiSepio *et al*, 1998; Duvic *et al*, 2000), this is the first demonstration that reversal of diminished TIG-3 expression in BCC is associated with topical application of tazarotene.

Peris *et al* (1999) first demonstrated that topical tazarotene gel 0.1% induced clinical remission in 53% of BCC treated for 8 mo, without histologic confirmation in all cases. Our results are similar with 21 of 35 BCC (60%) treated for up to 12 wk showing a clinical improvement or reduction in tumor size; only one tumor progressed. Histologic evaluation of tumors following therapy is important as one tumor with complete clinical clearing was still present microscopically and three tumors called PR were actually clear by histology. Of importance, our study showed a significant correlation between increased TIG-3 protein and histologic improvement and the treatment with tazarotene. It is possible that TIG-3 protein contributes to inhibition of hyperproliferation, induction of differentiation, and the suppression of malignant phenotype, and is thus causally linked to the histologic outcome.

Epidermal carcinogenesis is a complex process that involves multiple, sequential genetic changes in oncogenes and tumor suppressors (Hanahan and Weinberg, 2000; Lacour, 2002). Ultraviolet-light-induced mutations in the tumor suppressor p53 are found commonly in skin cancers and from the adjacent field of normal skin (Brash *et al*, 1996; Ananthaswamy *et al*, 1997). TIG-3 protein and mRNA were detected in normal skin from control subjects, but TIG-3 protein was lost in 5% and decreased in 69% of all BCC, regardless of growth pattern. TIG-3 mRNA was absent in 21% of BCC and diminished in 74% of BCC. One would not expect TIG-3 protein to remain in the absence of mRNA; however, *in situ* hybridization and immunohistochemical techniques are at best only semiquantitative. In addition, retinoids may affect post-transcriptional regulation of epidermal proteins, i.e., keratin 19 mRNA levels (Crowe, 1993). Whereas tazarotene treatment was associated with higher expression of TIG-3 in BCC, the overlying epidermis appeared to have a steady level of expression. But the overlying skin was still diminished in TIG-3 compared to control normal skin, suggesting a field effect. Aggressive squamous cell carcinomas also have diminished TIG-3 expression, with complete loss found in the most aggressive tumors (Duvic *et al*, 2000). The finding of diminished TIG-3 in skin cancer and psoriatic epidermal hyperproliferation may be associated with a field effect in areas where tumors arise, similar to p53 (Brash *et al*, 1996). In this study, 36% and 30% of BCC without defined clinical responses also had increased TIG-3 protein and mRNA levels, respectively. In contrast to the Peris trial of 8 mo, BCC in this trial received treatment for only up to 12 wk, and these lesions might have showed clinical responses with longer treatment.

Tazarotene is known to modulate other genes in addition to TIG-3. Both sporadic and hereditary BCC are associated with mutations in the sonic hedgehog pathway leading to increased expression of a transcription factor, gli-1 (Green *et al*, 1998; Tojo *et al*, 2002). Whether gli-1 can modulate TIG-3 expression is unknown. Tazarotene also induces the suppressor TIG-1, which has been reported to be diminished in prostrate cancer but has not yet been studied in skin cancer (Jing *et al*, 2002).

Tazarotene gel was well tolerated even when applied to biopsy sites. The side-effects were related to irritation at the application site and were observed in a majority of patients. It is possible that retinoid irritation could also induce TIG-3 expression or participate in clinical response of tumors. TIG-3 is homologous to the tumor suppressor H-rev that can be induced by γ interferon (Kuchinke *et al*, 1995). Psoriatic lesions have high γ interferon levels with low TIG-3, however (DiSepio *et al*, 1998; Breuer-McHam *et al*, 2000; Duvic *et al*, 2000). Topical imiquimod therapy clears some BCC by local induction of cytokines (Beutner *et al*, 1999) and tazarotene also regulates the expression of inflammatory cytokines (Esgleyes-Ribot *et al*, 1994; Nagpal *et al*, 1996b; Duvic *et al*, 1997; 1998).

Diminished expression of TIG-3 protein and mRNA was found in all histologic types of BCC and increased expression was found to be associated with clinical and histologic improvement. We speculate that loss of TIG-3 may play a role in skin cancer development as it is growth inhibitory in carcinoma lines *in vitro* (DiSepio *et al*, 1998; Deucher *et al*, 2000). Restoration of TIG-3 protein and mRNA levels could mediate the clinical response to tazarotene (Peris *et al*, 1999) and other RAR retinoid compounds through modulating epidermal differentiation and growth inhibition. TIG-3 may be a useful intermediate biologic marker for retinoid chemoprevention and treatment studies.

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REFERENCES

Ananthaswamy HN, Loughlin SM, Cox P, Evans RL, Ullrich SE, Kripke ML: Sunlight and skin cancer: Inhibition of p53 mutations in UV-irradiated mouse skin by sunscreens. *Nat Med* 3:510–514, 1997

Aneskievich BJ, Fuchs E: Terminal differentiation in keratinocytes involves positive and negative regulation by retinoic acid receptors and retinoid X receptors at retinoid response elements. *Mol Cell Biol* 12:4862–4871, 1992

Balmer JE, Blomhoff R: Gene expression regulation by retinoic acid. *J Lipid Res* 43:1773–808, 2002

Beutner KR, Geisse JK, Helman D, Fox TL, Ginkel A, Owens ML: Therapeutic response of basal cell carcinoma to the immune response modifier imiquimod 5% cream. *J Am Acad Dermatol* 41:1002–1007, 1999

Brash DE, Ziegler A, Jonason AS, Simon JA, Kunala S, Leffell DJ: Sunlight and sunburn in human skin cancer: p53, apoptosis, and tumor promotion. *J Invest Dermatol Symp Proc The* 1:136–142, 1996

Breuer-McHam JN, Ledbetter LS, Sarris AH, Duvic M: Cytokine expression pattern distinguishes HIV associated skin diseases. *Exp Dermatol* 9:341–350, 2000

Chambon P: The molecular and genetic dissection of the retinoid signaling pathway. *Rec Prog Horm Res* 50:317–332, 1995

Crowe DL: Retinoic acid mediates post-transcriptional regulation of keratin 19 mRNA levels. *J Cell Sci* 106:183–188, 1993

Deucher A, Nagpal S, Chandraratna RAS, Di Sepio D, Robinson NA, Dashti SR, Eckert RL: The carboxy-terminal hydrophobic domain of TIG-3, a class II tumor suppressor protein, is required for appropriate cellular localization and optimal biological activity. *Int J Oncol* 17:1195–1203, 2000

Di Iasio MG, Calin G, Tibiltti MG, *et al*: Refinement of the LOH region 1 at 11q23.1 deleted in human breast carcinomas and sublocalization of 11 expressed sequence tags within the refined region. *Oncogene* 18:1635–1638, 1999

DiSepio D, Ghosh C, Eckert RL, *et al*: Identification and characterization of a retinoid-induced class II tumor suppressor/growth regulatory gene. *Proc Natl Acad Sci USA* 95:14811–14815, 1998

Duvic M, Nagpal S, Asano AT, Chandraratna RAS: Molecular mechanisms of tazarotene action in psoriasis. *J Am Acad Dermatol* 37 (2 Part 3):S18–S24, 1997

Duvic M, Asano AT, Hager C, Mays S: The pathogenesis of psoriasis and the mechanism of action of tazarotene. *J Am Acad Dermatol* 39:S129–S133, 1998

Duvic M, Helekar B, Schulz C, *et al*: Expression of a retinoid-inducible tumor suppressor, tazarotene-inducible gene-3, is decreased in psoriasis and skin cancer. *Clin Cancer Res* 6:3249–3259, 2000

Eckert RL, Crish JF, Banks EB, Welter JF: The epiderimis: Genes on, genes off. *J Invest Dermatol* 109:501–509, 1997

Elder JT, Astrom A, Pettersson U, *et al*: Differential regulation of retinoic acid receptors and binding proteins in human skin. *J Invest Dermatol* 98:673–679, 1992

Esgleyes-Ribot T, Chandraratna RA, Lew-Kaya DA, Sefton J, Duvic M: Response of psoriasis to a new topical retinoid, AGN 190168. *J Am Acad Dermatol* 30: 581–590, 1994

Green J, Leigh IM, Poulsom R, Quinn AG: Basal cell carcinoma development is associated with induction of the expression of the transcription factor Gli-1. *Br J Dermatol* 139:911–915, 1998

Hajnal A, Klemenz R, Schafer R: Subtraction cloning of H-rev107, a gene specifically expressed in H-ras resistant fibroblasts. *Oncogene* 9:479–490, 1994

Hanahan D, Weinberg RA: The hallmarks of cancer. *Cell* 100:57–70, 2000

Jing C, El-Ghany A, Beesley C, Foster CS, Rudland PS, Smith P, Youqiang K: Tazarotene-induced gene 1 (TIG1) expression in prostate carcinomas and its relationship to tumorigenicity. *J Natl Cancer Inst* 94:482–490, 2002

Kanjali S, Duvic M: Cutaneous carcinogenesis: Molecular model of cancer development. In: Miller S (ed.) *Cutaneous Oncology*. Cambridge, UK: Blackwell Scientific Publications, 1998; p 19–29

Kraemer KH, DiGiovanna JJ, Moshell AN, Tarone RE, Peck GL: Prevention of skin cancer in xeroderma pigmentosum with the use of oral isotretinoin. *N Engl J Med* 318:1633–1637, 1988

Kruger K, Blume-Peytavi U, Orfanos CE: 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, 1999

Kuchinke W, Hart RP, Jonakait GM: Identification of mRNAs regulated by interferon-γ in cultured rat astrocytes by PCR differential display. *Neuroimmunomodulation* 2:347–355, 1995

Lacour JP: Carcinogenesis of basal cell carcinomas: Genetics and molecular mechanisms. *Br J Dermatol* 146 (s61):17–19, 2002

Lippman SM, Lotan R, Clayman GL: Systemic prevention and treatment of skin cancer. In: Weber RS, Miller MJ, Goepfert H (eds). *Basal and Squamous Skin Cancers of the Head and Neck*. Baltimore: William and Williams, 1996; p 351–355

Lotan R, Xu XC, Lippman SM, Ro JY, Lee JS, Lee JJ, Hong WK: Suppression of retinoic acid receptor-β in premalignant oral lesions and its upregulation by isotretinoin. *N Engl J Med* 332:1405–1410, 1995

Murakami Y, Nobukuni T, Tamura K, *et al*: Localization of tumor suppressor activity important in non-small cell lung carcinoma on chromosome 11q. *Proc Natl Acad Sci USA* 95:8153–8157, 1998

Nagpal S, Patel S, Asano AT, Johnson AT, Duvic M, Chandraratna RAS: Tazarotene-induced gene 1 (TIG1), a novel retinoic acid receptor-responsive gene in skin. *J Invest Dermatol* 106:269–274, 1996a

Nagpal S, Thacher SM, Patel S, *et al*: Negative regulation of two hyperproliferative keratinocyte differentiation markers by a retinoic acid receptor-specific retinoid: Insight into the mechanism of retinoid action in psoriasis. *Cell Growth Differ* 7:1783–91, 1996b

Nagpal S, Patel S, Jacobe H, *et al*: Tazarotene-induced gene 2 (TIG-2), a novel retinoid responsive gene in skin. *J Invest Dermatol* 109:91–95, 1997

Peris K, Fargnoli MC, Chimenti S: Preliminary observations on the use of topical tazarotene to treat basal-cell carcinoma. *N Engl J Med* 341:1767–1768, 1999

Rapini R: Basal cell and squamous cell carcinomas. In: Gross K, Steinman H, Rapini R, (eds). *Mohs Surgery – Fundamentals and Techniques*. St Louis, MO: Mosby, 1999; p 161–192

Rippey JJ: Why classify basal cell carcinoma? *Histopathology* 32:393–398, 1998

Seitz CS, Lin Q, Deng H, Khavari PA: Alterations in NF-κB function in transgenic epithelial tissue demonstrates a growth inhibitory role for NF-κB. *Proc Natl Acad Sci USA* 95:2307–2313, 1998

Stoler A, Kopan R, Duvic M, Fuchs E: The use of monospecific antisera and cRNA probes to localize the major changes in keratin expression during normal and abnormal epidermal differentiation. *J Cell Biol* 107:427–446, 1988

Thacher SM, Krasinski G, Malhotra M, Duvic M, Nagpal S, Chandraratna RAS: Coordinate regulation of keratinocyte differentiation markers by retinoids in cell culture and in psoriasis. *J Invest Dermatol* 106:819, 1996

Tojo M, Kiyosawa H, Iwatsuki K, Kaneko F: Expression of a sonic hedgehog signal transducer, hedgehog-interacting protein, by human basal cell carcinoma. *Br J Dermatol* 146:69–73, 2002

Veal CD, Capon F, Allen MH, *et al*: Family-based analysis using a dense single-nucleotide polymorphism-based map defines genetic variation at PSORS1, the major psoriasis-susceptibility locus. *Am J Hum Genet* 71:554–564, 2002

Webster GF, Guenther L, Poulin YP, Solomon BA, Loven K, Lee J: A multicenter, double-blind, randomized comparison study of the efficacy and tolerability of once-daily tazarotene 0.1% gel and adapalene 0.1% gel for the treatment of facial acne vulgaris. *Cutis* 69 (2, Suppl.):4–11, 2002

Weinstein G, Krueger GG, Lowe NJ, *et al*: Tazarotene gel, a new retinoid, for topical therapy of psoriasis: Vehicle-controlled study of safety, efficacy, and duration of therapeutic effect. *J Am Acad Dermatol* 37:85–92, 1997

Xu XC, Wong WY, Goldberg L, *et al*: Progressive decreases in nuclear retinoid receptors during skin squamous carcinogenesis. *Cancer Res* 61:4306–4310, 2001