Expression of a Retinoid-inducible Tumor Suppressor, Tazarotene-inducible Gene-3, Is Decreased in Psoriasis and Skin Cancer

Madeleine Duvic, Bharati Helekar, Claudia Schulz, Mimi Cho, Dan DiSepio, Carina Hager, Dominique DiMao, Parul Hazarika, Brooke Jackson, Joan Breuer-McHam, John Young, Gary Clayman, Scott M. Lippman, Roshandra A. S. Chandraratna, Nancy A. Robinson, Anne Deucher, Richard L. Eckert, and Sunil Nagpal

Section of Dermatology, Department of Internal Medical Specialties [M. D., B. H., C. S., M. C., C. H., D. DiM., P. H., B. J., B. M., J. Y.], Department of Head and Neck Surgery [G. C.], Division of Cancer Prevention [S. M. L.], M. D. Anderson Cancer Center, Houston, Texas 77030; Allergan Research, Irvine, California 92612 [D. DiS., R. A. S. C., S. N.]; and Department of Physiology/Biophysics, Case Western Reserve, Cleveland, Ohio 44106 [N. A. R., A. D., R. L. E.]

ABSTRACT

Tazarotene-induced gene-3 (TIG-3), isolated from human keratinocytes treated with the retinoic acid receptor-selective retinoid Tazarotene, is homologous to H-rev, a class II tumor suppressor. TIG-3 gene localized to chromosome 11q23, a site of loss of heterozygosity in several malignancies. Retinoids influence epidermal differentiation and are used to treat and prevent skin cancer. Therefore, we studied TIG-3 mRNA expression in psoriasis and in basal and SCCs by in situ hybridization and a quantitative QT-RT-PCR assay. Psoriasis lesions had significantly lower staining (median, 3) than paired normal control skin (median, 4; P = 0.012). TIG-3 mRNA was significantly higher in normal control skin (P = 0.001), in paired adjacent skin (median, 3; P = 0.007), and in overlying epidermis (median, 3.0; P = 0.0001) than in 21 SCC specimens as a group (median, 1.5).

Aggressive SCCs (median, 1.0) were lower in TIG-3 mRNA staining than nonaggressive SCCs (median, 1.5; P = 0.007). Three aggressive tumors had no TIG-3 mRNA staining. TIG-3 protein as shown by immunohistochemistry was highest in the suprabasal epidermis of normal skin, just under the stratum corneum, and was decreased in basal and squamous cell carcinomas, similar to the mRNA staining. Reduction in TIG-3 mRNA expression in psoriasis and basal carcinomas and loss in some aggressive SCCs support the hypothesis that TIG-3 may function as a tumor suppressor in both normal and malignant epidermal differentiation.

INTRODUCTION

The protective skin barrier arises from a tightly controlled genetic differentiation program resulting in keratinocyte apoptosis and stratum corneum formation (1, 2). Despite the fact that most keratinocytes undergo apoptosis, malignancies of keratinocytes (basal, SCC, or basosquamous cell carcinomas) commonly arise and are the most common malignancies in humans (3). The molecular basis of some squamous carcinomas is related to mutations in p53 and in H-ras, induced by UV light (4, 5). Mutations in genes of the sonic-hedgehog or patched pathway have been implicated recently in the formation of BCCs, although phenotypic variation is complex (6–9). Many other genes may contribute to the pathogenesis of skin cancers because of the complexity of the epidermal differentiation pathway and the need for chronic repair of DNA damage (10–12).

Benign epidermal hyperplasia may also result in the presence of a cutaneous inflammatory reaction, such as psoriasis, in genetically susceptible individuals (13). The genetic programs controlling epidermal differentiation, although only partially understood, are known to be regulated in part by steroid hormones, including vitamin A (retinoids) and vitamin D (deltooids; Ref. 14). Retinoids exert their effects in part through binding to differentially expressed RAR or retinoid X receptors (15–17). Retinoids and their receptors also interact with specific DNA transcription factors, including AP1, AP2, CBP300, and nuclear factor-interleukin 6, thought to regulate epidermal differentiation (18–21). The cytokine-inducible nuclear transcription factor, nuclear factor-κB, has also been implicated in control of epidermal proliferation (22). Topical retinoid treatment is known to alter the expression of epidermal keratin and other differentiation markers (23).

Retinoids are active clinically in psoriasis (24–26) and for both chemoprevention and therapy of squamous carcinomas and oral leukoplasia (27). Tazarotene, a new synthetic RAR β,γ-
selective retinoid, is effective topically for treatment of psoriasis and acne (28–30). Tazarotene treatment is associated with decreased expression of epidermal genes overexpressed in psoriasis, including type I keratinocyte transglutaminase, epidermal growth factor receptor, MRP-8, and scalpelafin (29, 31, 32). In addition, Tazarotene reduces the expression of inflammatory markers including intercellular adhesion molecule-1 and HLA-DR on cells in psoriasis lesions and increases the reexpression of profilaggrin, a marker of terminal differentiation (31, 33). Recently, a clinical study has suggested that topical Tazarotene may induce remissions of basal cell carcinomas (34).

To understand the mechanism of action of Tazarotene and its effect on epidermal gene expression, novel TIG cDNAs were isolated by differential display PCR from mRNA collected from treated or mock-treated human keratinocytes (35–37). TIG-3 (RANTES-3; Ref. 38), a cDNA of 736 bp, was induced 4-fold in treated human keratinocyte cultures. The cDNA isolated predicted an Mf 18,000 protein of 164 amino acids with 52% homology to H-rev 107, a known class II tumor suppressor (39). H-rev 107 is down-regulated by ras transfection into rat fibroblasts, up-regulated in fibroblasts that spontaneously revert after H-rev 107 is down-regulated by ras transfection into rat fibroblasts, and is induced by IFN-γ in astrocytes (40, 41). TIG-3 has been expressed with an inducible promoter causing growth retardation in Ecr-293 kidney cell lines (38). Similar studies done in a SCC line show growth inhibition by TIG-3 sense constructs in vitro. These studies suggest that the function of TIG-3 is as a tumor suppressor (38). Of interest, TIG-3 maps to chromosome 11q23 that has been reported previously to contain a tumor suppressor (42, 43).

Cell culture studies have shown that TIG-3 is up-regulated by all RAR-selective retinoids including Tazarotene and was inducible in keratinocytes and in the spontaneously transformed keratinocytes, the HaCat cell line (38). Psoriasis is a disease characterized by rapid epidermal proliferation in response to T-cell inflammation, which is inhibited by topical Tazarotene (28). Thus, we hypothesized that: (a) TIG-3 expression may also be associated with growth inhibition in normal as well as inflamed epidermis; and (b) loss of TIG-3 expression could result in epidermal malignancies. We examined the level and pattern of expression of TIG-3 mRNA and protein as a putative tumor suppressor in normal skin, compared with psoriasis and BCCs and SCCs.

**MATERIALS AND METHODS**

**Patient Specimens.** Skin biopsy specimens were taken from unaffected, normal skin and from 29 psoriasis patients' lesions at baseline prior to their use of an investigational 0.1% Tazarotene gel and at 3 and 14 days of treatment. Aggressive (n = 11) and nonaggressive (n = 10) SCCs and BCCs (n = 6) of the skin and paired normal skin specimens from overlying and/or adjacent normal skin, as available, were obtained from patients undergoing surgery in Dermatology Section and the Department of Head and Neck Surgery, M. D. Anderson Cancer Center (Table 1). Additional specimens from BCCs, SCCs, and normal adjacent skin for mRNA isolation were taken from Moh's surgery cases and snap frozen at −70°C (Table 2). All patients signed informed consent under approved protocols.

Specimens were fixed in 4% paraformaldehyde at 4°C for 16–24 h, processed in alcohol, and embedded in paraffin as described previously (1, 11). Sections of 4 μm were mounted on sialinized, nuclease-free glass slides (CEL Associates, Houston, TX) for in situ hybridization.

**Preparation of Riboprobes.** Plasmid (pAGN-TIG 3) containing a 600-bp 3′ cDNA from TIG-3 was inserted in reverse orientation in EcoRI in a PCRII vector. One μg of cDNA template was linearized with NotI or HindIII and transcribed using either Sp6 or T7 polymerase to yield antisense and sense riboprobes, respectively. The riboprobes were transcribed in the presence of UTP-digoxigenin using a Genius 4 kit, according to the manufacturer's instructions (Boehringer-Mannheim, Indianapolis, IN) as described previously (44). The empirical concentration of the probes was estimated using a dot blot method involving serial dilutions with a Dig DNA labeling and detection kit (Boehringer Mannheim).

**ISH and Detection of Riboprobes.**ISH was performed according to a modified procedure of Xu et al. (44). Deparaffinized and rehydrated tissue sections were washed in PBS (pH 7.2), followed by 0.2 N HCl at room temperature for 10 min. They were treated with protease K in 10 mM Tris and 2 mM CaCl2 (pH 8.0) for 20 min at 37°C. After three washes in PBS, sections were postfixed with 4% paraformaldehyde for 5 min at room temperature, washed three times in PBS, and acetylated at pH 8.0. After a final PBS wash, sections were dehydrated in ethanol and air-dried.

Prehybridization was carried out in a humidification chamber in 50% deionized formamide, 2× SSC solution, 2× Denhardt’s solution (0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, and 0.2% BSA), 10% dextran sulfate, 400 μg/ml yeast tRNA, 250 μg/ml salmon sperm DNA, and 20 mM DTT in diethyl pyrocarbonate-treated water. Sense or antisense riboprobes added at 400 ng/ml were hybridized for 4 h at 42°C. Posthybridization washes were in 2× SSC and 0.1% SDS at room temperature for 1 h and 40 min, followed by another wash with 0.1× SSC and 0.1% SDS for 20 min at room temperature. Probe binding was detected using a Fab fragment of anti-digoxigenin antibody and chromogen substrate solution (45 μl of nitroblue tetrazolium and 35 μl of 5-bromo-4-chloro-3-indoly-phosphate solution in 10 ml of buffer 3 containing 10 mM Tris, 0.1 M NaCl, and 50 mM MgCl2 pH 9.5). The color reaction was for 1 h.

**Analysis of in Situ Hybridization.** The sections were graded for intensity by three blinded observers using a semiquantitative scale (11, 31). 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 data did not fit a Poisson distribution and therefore were analyzed using nonparametric comparisons. Median scores were compared using Wilcoxon signed ranks or Mann-Whitney with an SPSS software package.

**IHC.** IHC was performed on paraffin-embedded tissue sections from all tumors studied by ISH by methods described previously (11, 31). TIG-3 rabbit polyclonal antibody raised to recombinant TIG-his tag protein was used as the primary antibody.
Table 1  TIG-3 mRNA hybridization in SCC tumors compared with normal overlying or adjacent skin specimens

<table>
<thead>
<tr>
<th>Site/Metastasis</th>
<th>Tumor no.</th>
<th>Differentiation</th>
<th>TIG-3 levels compared with tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Well</td>
<td>Moderate</td>
</tr>
<tr>
<td>Scalp/skull, multiple tumors</td>
<td>96-41288A7</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Face, multiple tumors</td>
<td>96-51845A11</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Dermis/parotid, L, V</td>
<td>96-35269A21</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Parotid/LN</td>
<td>97-38951E16</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Non-aggressive squamous cell carcinomas

| Elbow                           | 96-05316A1 | +              | –        | –    | 1.2-fold increase | 1.4-fold increase |
| Retromauricular                  | 96-51337A2 | +              | –        | –    | 2-fold increase   | No change        |
| Forehead                        | 97-00964D2 | –              | –        | –    | No change         | 1.5-fold increase |
| Ear                             | 96-46239A6 | –              | +        | –    | 1.5-fold increase | 1.5-fold increase |
| Forehead                        | 97-09809A6 | –              | +        | –    | 2-fold increase   | 2.5-fold increase |
| Forehead                        | 97-20557A3 | –              | +        | –    | 1.2-fold increase | 1.6-fold increase |
| Nose                            | 96-35773A6 | –              | –        | +    | 1.5-fold increase | 1.5-fold increase |
| Forehead                        | 97-5342A22 | –              | –        | +    | 1.5-fold increase | 2-fold increase  |
| Forehead                        | 97-02450A1 | –              | –        | +    | 1.5-fold increase | 1.5-fold increase |

a NA, not available; LN, lymph node; PN, perineural invasion; L, lymphatics; V, vasculature.

RESULTS

TIG-3 mRNA and Protein Is Expressed Highly in the Suprabasalar Epidermis and Adnexa of Normal Skin. If TIG-3 acts as a tumor suppressor and controls normal epithelial differentiation, then it would be expressed in normal epidermis. The intensity of the antisense riboprobe staining was strongest at a dilution of 1:1000 and was detected with horse antirabbit secondary antibody at 1:100 using a Vector Stain ABC kit (Vector Labs, Burlingame, California). Preimmune rabbit serum (1:100) was used as a negative control for TIG-3 antibody staining.5

QT-RT-PCR mRNA Analysis. Total RNA was extracted from normal and tumor specimens (Table 2) using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD). Contaminating DNA was removed by DNase I treatment in 4 mM MgCl2 at 37°C for 30 min and then terminated at 72°C for 5 min. The PCR primers (45F-CAAGAGGCCCACCCGGAG and 111R TATAACGGGCCAGTTGCTCAT) and fluorescent oligonucleotide probe (65T-CCTGATTGAGATTTTCCGCCTTGGC) for the TIG-3 QT-RT-PCR assay were designed from the published sequence, accession no. AR060228 (38), using the Primer Express software (Perkin-Elmer). RNA samples were analyzed in triplicate with a control that lacked reverse transcriptase (–RT control). Samples were run in parallel with 10-fold dilutions of mRNA from tumor line 22B HNSCC (Ref. 45; 600 ng to 600 pg) and assigned arbitrary number of molecules to generate a standard curve (Ci versus arbitrary RNA template molecules). The number of template molecules in the samples were calculated by interpolation of the Ct against the 22B HNSCC RNA standard curve. 36B4 mRNA was also measured by QT-RT-PCR and used to normalize as a standard, using the primers described previously (46).

by in situ hybridization throughout the epidermis, hair follicles, and sebaceous glands of normal control skin (Fig. 1, A and B). In thinner epidermis of normal skin (Fig. 1, A and C), all layers stained strongly with the antisense probe. The sense probe showed only faint background staining intensity (Fig. 1). Where the epidermis was thicker, staining intensity was more pronounced in the suprabasal layers than in the basal layers in normal skin (Fig. 1, H and I).

Immunoreactive TIG-3 protein staining in the upper layers of normal skin (Fig. 1, D and E) was similar to the pattern of the mRNA seen by in situ hybridization (Fig. 1, A and B). TIG-3 protein was more restricted than the mRNA because it was most intense in the most well-differentiated layers of the normal suprabasal epidermis, just under the stratum corneum (Fig. 1, D and E). The hair follicles, sebaceous glands, and small blood vessels of the dermis also stained prominently with TIG-3 antibody. The immunoreactivity was cytoplasmic in appearance in keratinocytes. Preimmune rabbit serum gave only background reactivity in normal skin (Fig. 1F).

**TIG-3 mRNA Expression Is Significantly Decreased in Psoriatic Epidermis Compared with Paired Normal, Uninvolved Skin.** Normal, uninvolved skin from psoriasis patients was similar in intensity and pattern of mRNA staining to normal controls without skin cancer in mRNA in situ hybridization intensity (Fig. 1G). Psoriasis lesions prior to treatment (Fig. 1H) were severalfold thicker than normal skin because of epidermal acanthosis and increased numbers of suprabasal keratinocytes. The intensity of staining for TIG-3 antisense probe was reduced in the basal layers of psoriasis lesions (Fig. 1H) compared with normal control skin (Fig. 1G). As shown in Fig. 2, normal, uninvolved paired skin from 30 psoriasis patients (median, 4.0) had significantly more intense TIG-3 antisense staining than the basal and suprabasal layers of psoriasis lesions (median, 3.0; P = 0.012, Wilcoxon signed ranks test). Suprabasal TIG-3 staining was more intense in paired, uninvolved normal skin specimens compared with baseline psoriasis lesions in 16 of 30 patients’ lesions examined, was less intense in 4, and did not differ by more than 1 degree in 10 patients.

Pooled mRNA had been analyzed previously from lesions in this clinical study from the 15 responding patients who had >50% decrease in plaque elevation by 8 weeks (38). In the responders, topical Tazarotene gel application was associated with increased levels of total TIG-3 mRNA transcripts as compared with untreated lesions by a quantitative assay (38). In the analysis done by considering responding and nonresponding patients together, the median staining intensities after Tazarotene treatment for 3 or 14 days, as judged by ISH, were not significantly different from baseline (Fig. 2).

**TIG-3 Expression Is Significantly Decreased in Both Basal and SCCs Compared with Adjacent and Overlying Normal Skin.** BCCs and SCCs studied by ISH were derived from sun-exposed areas (except one case) from subjects between 50 and 80 years of age (Table 1). All except 2 patients were males. SCCs of the skin were defined as aggressive if they were >2 cm in size, had neural invasion, and/or were metastatic to regional lymph nodes. Aggressive tumors studied extended into deeper structures, including auricle, periauricular soft tissues, parotid glands, intraorbital nerves, scalp, nose, and intraoral lymph nodes. As indicated in Table 1, some tumors did not have paired overlying or adjacent skin available to study. Nonaggressive tumors were restricted to scalp and superficial facial tissues and were <2 cm in size.

We examined the median intensity of TIG-3 mRNA in situ staining comparing normal skin from psoriasis patients as well as overlying and adjacent paired normal skin from skin cancer patients to their tumors (Fig. 2). As shown in Figs. 2–4, the median staining intensity of the TIG-3 antisense probe was decreased in BCCs, basosquamous carcinomas, and SCCs when compared with normal skin from psoriasis patients and from paired tumor patients. Psoriasis control normal skin and paired adjacent normal suprabasal layers of skin had similar staining (median, 4.0). Both were significantly higher in TIG-3 mRNA staining than basal cell carcinomas (median, 1.33; P = 0.045, Mann-Whitney; n = 6). BCCs studied by ISH showed reduction in TIG-3 mRNA staining in the tumor that was similar to the normal overlying basal cell layer (Fig. 3A). IHC staining intensity in the suprabasal layer of adjacent skin (Fig. 3B) was higher than in the basal layer of normal skin and in basal cell carcinomas shown in Fig. 3, D and E. Normal skin showed no specific reactivity with preimmune serum and secondary antibody (Fig. 3C).

The staining intensity of the TIG-3 antisense riboprobe by ISH was also significantly higher in normal skin specimens from all sources, compared with all SCCs as a group. As shown in Fig. 2, TIG-3 mRNA staining was stronger in normal skin from psoriasis controls (median, 4.0; P = 0.001), in normal skin overlying SCCs (median, 3.0; P = 0.0001), and in the adjacent skin to SCCs (median, 3.0; P = 0.007) compared with staining intensity in

---

**Table 2** Specimens from adjacent normal skin and tumors for QT-RT-PCR RNA measurements

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age/sex</th>
<th>Diagnosis</th>
<th>Sites of biopsies</th>
<th>TIG-3 levels: normal vs. tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>65 WM</td>
<td>BCE</td>
<td>Left back</td>
<td>Similar to NL skin</td>
</tr>
<tr>
<td>2</td>
<td>62 WM</td>
<td>Well-differentiated SCC</td>
<td>Nasal root</td>
<td>Tumor &gt; NL</td>
</tr>
<tr>
<td>3</td>
<td>70 WM</td>
<td>BCE</td>
<td>Left forehead</td>
<td>Both low</td>
</tr>
<tr>
<td>4</td>
<td>77 WF</td>
<td>Benign lichenoid keratosis</td>
<td>Right leg</td>
<td>Similar to NL control</td>
</tr>
<tr>
<td>5</td>
<td>61 WF</td>
<td>BCC</td>
<td>Right upper cheek</td>
<td>Normal &gt; tumor</td>
</tr>
<tr>
<td>6</td>
<td>66 WM</td>
<td>SCC in situ</td>
<td>Right upper cheek</td>
<td>Both low</td>
</tr>
<tr>
<td>7</td>
<td>71 WM</td>
<td>BCE</td>
<td>Left preauricular</td>
<td>N1 skin low</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BCE</td>
<td>Posterior neck</td>
<td>N1 skin low</td>
</tr>
</tbody>
</table>

*NL, normal; W, white; M, male; F, female.*
21 aggressive and nonaggressive SCC specimens as a group (median, 1.5). Examples of aggressive SCCs shown in Fig. 4 had either overlying or adjacent normal skin. Aggressive SCCs were \( 2c \) m and had neural invasion or metastasis to parotid gland or regional lymph nodes. For this reason, they were not always available with normal adjacent or overlying skin (Table 1).

All SCC specimens were studied both by ISH and by IHC. There is reduced TIG-3 mRNA signal in SCC tumors compared with overlying normal skin with higher expression in adjacent skin. All tumors showed correlation in the levels of mRNA staining and TIG-3 protein reactivity. (Examples of specimens studied by both techniques are found in Fig. 4, B and D). Thus, TIG-3 mRNA and protein staining were similar, as shown.

The variation in staining intensities found in normal skin overlying both aggressive and nonaggressive SCC tumors, from which the tumors arose, was greater than the variation seen in normal control skin as well as in adjacent skin (Fig. 2). Fig. 4 shows examples of overlying normal skin with high TIG-3 (B and D), moderate TIG-3 (A), and low TIG-3 (E, L) mRNA staining. Pseudoepitheliomatous hyperplasia found in two tumors (D and E) could be either high or low in staining intensity. Adjacent skin at a distance from tumors (Fig. 4, G–J and K–N) showed very strong TIG-3 mRNA and protein staining as a rule, but the intensity was decreased at the site where tumors arose (Fig. 4, G–J and K–N). The mRNA and protein staining patterns were similar, as shown.

**TIG-3 Expression Is Less in Aggressive SCCs Compared with Nonaggressive SCC Tumors.** The median staining intensity in 11 aggressive SCCs (median, 1.0) was also decreased compared with 10 nonaggressive SCCs (median,
Ten of 11 aggressive SCCs versus 7 of 10 nonaggressive SCCs showed decreased TIG-3 mRNA ISH signals compared with paired normal skin. All tumors studied showed reduced TIG-3 mRNA and protein staining compared with normal control donor skin specimens. Three aggressive SCCs and a metastatic SCC lesion to the lymph node had no staining present for TIG-3 mRNA, suggesting that complete loss of gene expression may have occurred. In three nonaggressive SCCs, overlying skin and tumor were similarly both reduced (compared with normal skin). Two of these three tumors were interpreted by the pathologist as well-differentiated, and the third was poorly differentiated.

**Development of QT-RT-PCR Assay to Measure TIG-3 mRNA Transcripts.** A quantitative fluorescent real time QT-RT-PCR assay (47) was developed to measure TIG-3 mRNA transcripts in paired patient specimens (Table 2). The cDNA for 36B4, encoding a ribosomal protein, was used to normalize levels of TIG-3 mRNA, expressed as a ratio. Levels of TIG-3 mRNA transcripts were determined from two samples of normal, non-sun-exposed abdominal skin from patients without cancer and from 7 skin tumors with paired adjacent skin specimens taken at the time of Moh’s surgery (Table 2; Fig. 5). TIG-3 mRNA levels from triplicate samples of reverse-transcribed mRNA were normalized to 36B4. Two specimens from normal abdomen skin of patients without skin cancer (Fig. 5, A and B) were similar and showed highest TIG-3 mRNA levels, similar to the levels measured in HaCat keratinocytes (not shown). High levels of TIG-3 mRNA were also found in one very well-differentiated SCC (patient 2) and in a lichenoid actinic keratosis with epidermal acanthosis (patient 4). A small, well-localized BCE on the infraorbital cheek had reduced TIG-3 transcripts compared with the normal adjacent skin specimen (patient 5). Similar to the in situ findings, three patients had low TIG-3 levels in both tumor and adjacent normal skin. Patient 7, with lowest TIG-3 in normal adjacent skin, also had multiple facial basal cell carcinoma occurring in the setting of severe, extensive photodamage over the whole area.

**DISCUSSION**

TIG-3 (RANTES-3) is a newly described, retinoid-inducible putative tumor suppressor that was isolated from Tazarotene-
treated keratinocytes. TIG-3 has been shown to inhibit cellular proliferation when expressed and is associated with retinoid responsiveness in malignant cell lines in vitro (38). This study shows for the first time that TIG-3 mRNA and protein expression are significantly reduced in basal and SCCs arising from sun-exposed skin, compared with normal skin. Lower TIG-3 mRNA and loss of expression are seen in aggressive SCCs compared with nonaggressive SCCs in this study.

The highest levels of TIG-3 mRNA by ISH and TIG-3 protein by IHC are found within the normal suprabasal epidermis, hair follicles, and sebaceous glands. TIG-3 in normal skin may be, therefore, associated with terminal keratinocyte differentiation and growth arrest occurring in the suprabasal layers. In hyperproliferative epidermis, as in psoriasis lesions and overlying SCCs, TIG-3 mRNA by ISH and protein by IHC are reduced relative to skin of normal thickness.

Treatment of hyperproliferative psoriasis lesions with topical Tazarotene gel induces 4-fold increased expression of TIG-3 mRNA in epidermis of responding patients compared with pretreatment levels (38). In psoriasis lesions, inflammation is present that affects epidermal differentiation, and the skin is able to normalize after topical retinoid therapy (28, 33). Up-regulation of TIG-3 expression may be part of the normalization process, and low TIG-3 expression may contribute to the increased rate of epidermal proliferation that characterizes psoriasis (48).

Recently, topical Tazarotene gel treatment has also been shown to induce clinical remission of 47% of basal cell carcinomas treated over a period of months (34). Whereas loss of TIG-3 may be an important event leading to skin cancer, expression of TIG-3 in suprabasal epidermis may help to regulate normal terminal differentiation. TIG-3 mRNA levels are highest in normal skin and differed significantly between non-
Fig. 4 Expression of TIG-3 mRNA by ISH and protein by IHC is reduced in paired adjacent and overlying normal skin compared with SCCs. Specimens were studied as in Fig. 1 by either ISH or IHC. A, moderately well-differentiated, aggressive SCC in dermis with normal overlying skin by ISH (×40). B, moderately well-differentiated, aggressive SCC (34065) in dermis with normal overlying skin by ISH (×40). C, same tumor B by IHC with normal epidermis to left, perpendicular to the base of photo and tumor nodules to the left (×40). D, moderately well-differentiated aggressive SCC (38303) from face studied by ISH. Tumor is throughout the dermis with pseudoepitheliomatous hyperplasia in overlying normal skin (×40). E,
aggressive and aggressive SCCs. Therefore, with further investigation, TIG-3 may become the first molecular marker of aggressiveness and of retinoid action during treatment or chemoprevention studies. These new data support the rational of using retinoids for both prevention and treatment of skin cancer.

TIG-3 is homologous to a class II tumor suppressor found in H-ras revertant lines, H-rev 107 (40, 49). TIG-3 mapped to chromosome 11q23, which has long been hypothesized to contain a tumor suppressor (38). This region has shown loss of heterozygosity and poor prognosis in cancers of the head and neck, melanoma, cervix, breast, lung, and ovarian malignancies and also leukemias, lymphomas, and rhabdomyosarcomas (38, 42, 43, 50–54). With respect to head and neck tumors, 25% showed loss of heterozygosity at 11q23 with a significant association with persistent or recurrent disease after radiation (55). The expression of TIG-3 mRNA in most aggressive SCC tumors is reduced by at least 50% compared with paired specimens of adjacent or overlying normal skin by in situ hybridization studies, suggesting that loss of heterozygosity may be present. All 11 aggressive SCC tumors have markedly reduced TIG-3 expression levels compared with overlying or adjacent normal skin. In three aggressive skin tumors, one a metastasis to node, TIG-3 mRNA expression is absent. Loss of TIG-3 protein in aggressive SCC is also confirmed by IHC. Analysis of the TIG-3 promoter, coding sequences, and gene sequences will be required to understand the basis of this finding.

Loss of heterozygosity in tumors reflects deletion of a large portion of the genomic DNA containing one allele and should be associated with a 50% reduction of the mRNA transcripts. Complete inactivation of a tumor suppressor requires that the second allele also acquire a second mutation within the tumor tissue. It is possible that inactivation of both copies of TIG-3 may be associated with the development of more aggressive skin cancers, and this would agree with studies demonstrating loss of chromosome 11q23 in head and neck SCCs (55). In contrast, mutations may be subtle and may arise without consistently altering mRNA levels (56). Loss of TIG-3 expression may also result from mutations or small deletions in the retinoid response elements (38) or hypermethylation of the gene’s promoter. More direct measures of mRNA levels, such as the QT-RT-PCR assay we report, and sequence information are ultimately required to understand the importance of tumor suppressors in carcinogenesis. ISH localized mRNA expression within tumor specimens, but it is only semiquantitative.

Basal cell carcinomas, generally nonaggressive, localized tumors arising from differentiated hair follicle keratinocytes, express levels of TIG-3 similar to the basal layer of normal epidermis. In keeping with the hypothesis that TIG-3 is a tumor suppressor, basal layers have less TIG-3 mRNA and protein than more differentiated epidermal layers. A quantitative, sensitive QT-RT-PCR assay was developed to measure TIG-3 transcripts within specimens. By this assay, normalized TIG-3 mRNA levels are 2–4-fold higher in normal non-sun-exposed skin from controls without cancer than in normal skin adjacent to three basal cell carcinomas and one SCC. One BCC and one SCC showed only slightly lower levels, and one very well-differentiated, nonaggressive SCC was 3-fold increased in tumor compared with normal skin. In these studies, there was no opportunity to study mRNA transcripts in aggressive SCCs and no mechanism to control for the relative amount of tumor and normal skin contributing material to total mRNA transcript pools. Microdissection may be required to dissociate tumor specimen from normal skin. Increased TIG-3 mRNA levels may be measured from a large tumor mass, even if the relative levels are low, in comparison with normal skin.

Overlying normal skin has more variable expression of TIG-3 compared with adjacent skin specimens; therefore, we also used normal skin specimens from psoriasis patients as controls. Skin overlying tumors may have low TIG-3 mRNA by ISH and low protein by IHC. In some SCCs where the point of tumor origin was present, the normal skin at the tumor origin has low TIG-3 staining. These observations, although preliminary,
do suggest that loss of TIG-3 in normal skin may be associated with the development of SCC tumors. The QT-RT-PCR assay showed that TIG-3 mRNA transcripts are low in both the perilesional photodamaged skin and in several basal cell carcinomas. One patient with SCC had low TIG-3 mRNA both in normal skin and tumor by QT-RT-PCR. If TIG-3 is lost or reduced in overlying normal skin, there could be a field effect, as reported for loss of p53 both in sun-damaged skin and resulting tumors (57, 58).

Mutations in p53 are reported in 90% of SCCs and 50% of basal cell carcinomas and result from UV light-induced pyrimidine-cytosine photoproducks (57). Whether these types of changes and this kind of frequency will also be found in skin cancers with respect to TIG-3 is not yet determined.

Heterogeneity of mRNA expression is present in some tumors, especially when there was mixed differentiation. This was confirmed using IHC showing high TIG-3 within areas of keratinization (keratin pearl formation). Heterogeneity in TIG-3 expression and ability to be induced by retinoid treatment might help to explain the clinical heterogeneity in response to retinoids.

It is well known that retinoids may prevent or retard the growth of some SCCs, although the mechanisms are not well established (27, 59, 60). Whereas loss of heterozygosity at the chromosome 11q23 region has been found in 25% of recurrent SCCs of the head and neck, no specific gene has been implicated until now (55). On the basis of these new data, TIG-3 is a lead candidate tumor suppressor explaining loss of heterozygosity at this locus. Irreversible loss or decrease in TIG-3 mRNA and protein in SCCs may be associated with more aggressive tumors, loss of retinoid response, and ultimately tumor progression or invasion. Mutations in TIG-3 that alter its function could also occur without influencing the mRNA levels. It should be noted that the expression of TIG-3 is significantly lower in aggressive compared with nonaggressive SCCs, unlike DNA repair or p53 mutations (58, 61). TIG-3 is thus a possible biomarker for distinguishing between nonaggressive and aggressive biological behavior. Further studies involving the regulation of TIG-3 by retinoids and elucidating the structure of the TIG-3 protein and the identification of key functional mutations will be important to understand the role of TIG-3 in squamous carcinogenesis and epidermal differentiation.

ACKNOWLEDGMENTS

We acknowledge the participation of Dr. Margaret Kripke, Dr. Sarah Strom Lori Buschman, Dr. Adan El-Nagaar, Dr. Stephen Mays, Dr. Kendra Woods, and Annette Basey in obtaining and/or processing the clinic specimens and lines for analysis. Dr. Peter Davies, Department of Integrative Biology, University of Texas Medical School, was responsible for the creation of the QT-RT-PCR assay.

REFERENCES


Expression of a Retinoid-inducible Tumor Suppressor, Tazarotene-inducible Gene-3, Is Decreased in Psoriasis and Skin Cancer

Madeleine Duvic, Bharati Helekar, Claudia Schulz, et al.