Loss of Inositol Polyphosphate 5-Phosphatase Is an Early Event in Development of Cutaneous Squamous Cell Carcinoma

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Abstract
Cutaneous squamous cell carcinoma (SCC) occurs commonly and can metastasize. Identification of specific molecular aberrations and mechanisms underlying the development and progression of cutaneous SCC may lead to better prognostic and therapeutic approaches and more effective chemoprevention strategies. To identify genetic changes associated with early stages of cutaneous SCC development, we analyzed a series of 40 archived skin tissues ranging from normal skin to invasive SCC. Using high-resolution array-based comparative genomic hybridization, we identified deletions of a region on chromosome 10q harboring the INPP5A gene in 24% of examined SCC tumors. Subsequent validation by immunohistochemistry on an independent sample set of 71 SCC tissues showed reduced INPP5A protein levels in 72% of primary SCC tumors. Decrease in INPP5A protein levels seems to be an early event in SCC development, as it also is observed in 9 of 26 (35%) examined actinic keratoses, the earliest stage in SCC development. Importantly, further reduction of INPP5A levels is seen in a subset of SCC patients as the tumor progresses from primary to metastatic stage. The observed frequency and pattern of loss indicate that INPP5A, a negative regulator of inositol signaling, may play a role in development and progression of cutaneous SCC tumors. Cancer Prev Res; 3(10): 1277–83. ©2010 AACR.

Introduction
More than 1,000,000 nonmelanoma skin cancers are diagnosed annually in the United States, making these the most common type of cancer and the fifth most costly cancer type in the Medicare population (1). The vast majority of nonmelanoma skin cancers are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). Unlike BCC, where distal spread is exceedingly rare, SCC can metastasize with appreciable frequency. Current clinical prognostic algorithms are suboptimal, and therapeutic options for aggressive disease are inadequate. Incomplete understanding of the molecular mechanisms leading to the development and progression of SCC has hindered development of accurate prognostic markers and targeted therapies, as well as more effective early chemopreventive strategies.

Development of genomic technologies in recent years has provided unparalleled opportunities for rapid and detailed study of cancer on the molecular level. Use of high-resolution, genome-wide approaches such as array-based comparative genomic hybridization (aCGH) has significantly affected understanding of cancer and facilitated better disease classification (2) and development of novel diagnostic and therapeutic approaches (3–5). Thus, application of high-resolution aCGH has potential to expand the small but growing body of knowledge focused on genomic characterization of cutaneous SCC (6–13) and identify novel, clinically relevant molecular targets. INPP5A belongs to a large family of inositol polyphosphate 5-phosphatases (14). This 40-kDa membrane-associated type I inositol phosphatase has preferential substrate affinity for inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate (15–18), functioning mostly as a signal-terminating enzyme with implication for several cellular processes, including proliferation. Loss of INPP5A may be linked to cancer development and progression. Deletions in the general chromosomal region encoding INPP5A on chromosome 10q26 are associated with brain tumors (19, 20), and decreased inositol polyphosphate 5-phosphatase activity is associated with several
human leukemias (21, 22). In addition, reduction of INPP5A expression using the antisense approach has led to transformation in cell culture as well as tumor growth in mice (17), suggesting a potential tumor suppressor role for INPP5A.

Herein, we perform genome-wide survey of gene copy number changes in skin tissues and identify frequent deletions of the INPP5A gene in human SCC tumors. In addition, we show that marked decrease of INPP5A protein levels is observed in most cutaneous SCCs. This event occurs early in the development of SCC, as it can be detected even at the stage of actinic keratosis (AK), a common precursor to SCC. However, progressive reduction of INPP5A levels is seen in a subset of SCC patients as the tumor progresses from primary to metastatic stage. Loss of INPP5A, therefore, could play an important role in development and progression of cutaneous SCC.

Materials and Methods

Tissues

Tissue samples analyzed in this study were formalin-fixed, paraffin-embedded (FFPE), archived specimens obtained under the Institutional Review Board-approved protocols at the Arizona Cancer Center, University of Arizona (Tucson, AZ); Southern Arizona Veterans Affairs Health Care System (Tucson, AZ); Loyola University Medical Center (Chicago, IL); and Mayo Clinic. The study was conducted according to the Declaration of Helsinki Principles.

Array CGH

To obtain genomic DNA for aCGH, microscopic examination by pathologist was used to select the areas for harvest and DNA extraction. In all samples, only regions that showed >50% lesional content were harvested. aCGH profiling was done using a method developed by the authors (23). Briefly, DNA was extracted from FFPE tissue blocks using the DNaseasy tissue kit (Qiagen). Normal pooled lymphocyte DNA (Promega) was used as a reference. A total of 5 μg of sample genomic DNA and 1 μg of reference genomic DNA were fragmented using the thermolabile recombinant shrimp DNase (TS-DNase; Affymetrix) to achieve an average DNA fragment length of 200 to 600 bp. Fragmented sample and reference DNA were labeled with Cy5 and Cy3 fluorescent dUTP, respectively, using the Bioprime Array CGH Genomic Labeling System (Invitrogen). Hybridizations were done on Agilent 44K feature microarrays for aCGH (Agilent Technologies) per the manufacturer's specifications and scanned on an Agilent DNA Microarray scanner, followed by image analysis with Feature Extraction software (Agilent Technologies) and data visualization with DNA Analytics software (Agilent Technologies) using the aberration calling algorithm ADM-1 (24).

Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) was carried out using a centromeric probe to chromosome 10 (Abbott Molecular) and INPP5A-directed probes (bacterial artificial chromosomes RP11-500B2 and RP11-288G11; BACPAC Resource Center) to either metaphase spreads or sections prepared from FFPE blocks. FFPE slices were prepared for hybridization using the Paraffin Pretreatment Kit II (Abbott Molecular). Slides were examined and photographed on a Zeiss Axioptot equipped with interference filters (Chroma) and a CoolSnap HQ2 digital camera (Photometrics). The FISH evaluation was semi-quantitative. Whenever the tissue was of sufficient size, 100+ nuclei were examined. However, in cases where a lesion of interest was small (e.g., AK lesions), all available lesional nuclei (i.e., <100) were examined.

Immunohistochemistry

FFPE tissue blocks were sectioned on glass slides at 5-μm thickness and baked for 60 minutes at 60°C. Slides were subsequently subjected to heat-induced epitope retrieval using a proprietary citrate-based retrieval solution for 20 minutes. The tissue sections were incubated for 30 minutes with anti-INPP5A mouse monoclonal antibody (clone 3D8; Novus Biologicals). The sections were visualized with the Bond Polymer Refine Detection kit (Leica Microsystems, Inc.) using diaminobenzidine chromogen as substrate.

Statistics

The two-tailed Fisher's exact test was used to compare the staining patterns between the cohorts of primary SCC tumors that have subsequently metastasized with those that have not. P values of <0.05 were considered statistically significant.

Results and Discussion

The INPP5A gene is frequently deleted in cutaneous SCC tumors

Genomic instability in cancer commonly leads to gross DNA ploidy changes as well as focal gene copy number aberrations. These genetic events contribute to development and progression of cancer by providing inappropriate proliferative stimuli or eliminating essential growth-regulating mechanisms, as illustrated by amplification of oncogenes and deletion of tumor suppressor genes, respectively. Identification of such changes in cancer has elucidated pathogenic molecular mechanisms that could be exploited for clinical benefit (3, 25). In cutaneous SCC, a global picture of genomic aberrations is starting to emerge (6–13), and high-resolution analysis of SCC genomes for identification of focal, clinically relevant gene copy number aberrations is warranted.

To identify novel genes and molecular mechanisms associated with cutaneous SCC development and progression, we analyzed a series of archived skin tissues spanning a range from normal skin to invasive SCC. To this end, we used a high-resolution oligomer aCGH method that we recently optimized specifically for use on archived FFPE tissues. This approach is capable of detecting gene copy...
INPP5A deletions.

aCGH was done using the DNA from a spectrum of 40 FFPE skin tissues, including normal skin (n = 12), precancerous lesions of AK (n = 5), in situ SCC lesions (SCCis; n = 2), and invasive SCCs (SCC; n = 21). A total of 458 copy number aberrations were identified in the examined samples, 267 (58%) of which were amplifications and 191 (42%) were deletions. We observed an increase in the overall frequency of gene copy number aberrations per sample in proportion to the increasing malignant characteristics of the examined tissue, with invasive SCCs harboring, on average, the highest number of aberrations per genome (Fig. 1). Detailed genomic distribution of aberrations as well as their overall percent penetrance in SCC lesions is illustrated in Supplementary Fig. S1.

Examination of the genomic regions characterized by recurrent copy number changes across samples, as detected by aCGH, identified several previously reported regions of aberrations, including amplifications of 1p, 3q, 8q, 14q, and 20q, as well as deletions on 3p and 9p (Supplementary Fig. S1; refs. 9, 11, 12, 26). However, the most prevalent copy number aberration was deletion of the q-ter region of chromosome 10, an area harboring the INPP5A gene (Fig. 2A). aCGH detected loss of the INPP5A gene in 1 of 2 examined SCCis lesions and in 5 of 21 (24%) examined invasive SCC tumors, but in none of the examined AK lesions or normal skin (Table 1). To verify the accuracy of aCGH calls, we did FISH for INPP5A in two of five samples that showed INPP5A deletions by aCGH. Both cases showed clear INPP5A loss, whereas control tissues showed no detectable loss of FISH signal (Fig. 2B).

Observed deletions of INPP5A represent a highly selected, nonrandom genetic event in SCC. Most of 191 deletions identified among SCC samples occur only once, whereas a smaller proportion are observed as recurrent deletions, affecting more than one sample. The region of INPP5A is the single most frequently deleted segment in the interrogated SCC genomes, as well as the only recurrent deletion detected in five independent SCC samples. The core INPP5A deletion, characterized as the smallest area of overlap among the aberrations harboring INPP5A deletions (Fig. 2A), covers a genetic segment containing 587,219 bp, of which 91,861 bp are in the INPP5A gene itself. In addition to INPP5A, this segment contains three genes: GPR123, KNDC1, and VENTX. However, INPP5A is the only gene in this cluster repeatedly affected by the copy number transition (the edge of the aberration), being affected in three of five samples harboring deletion of this region. Taken together, these data strongly suggest that INPP5A gene deletions are highly selected genetic events, rather than nonspecific bystander events in the context of the overall genomic instability of the SCC genome.

INPP5A protein level is frequently reduced in primary SCC tissues

Genomic aberrations detected by aCGH, such as gene deletions, can indicate a "tip of the iceberg" phenomenon, where loss of a gene on the DNA level is seen in a subset of tumors, whereas in remaining cases the implicated gene may be deregulated by other mechanisms, including those affecting its mRNA and protein products. To evaluate whether the genetic loss of INPP5A observed with aCGH might be similarly indicative of a more general phenomenon of INPP5A loss in SCC, we examined INPP5A protein levels in an independent cohort of FFPE skin tissues by immunohistochemistry using a monoclonal antibody to INPP5A. We evaluated a total of 71 archived SCC tumors and compared them with the matched normal skin from the same patient using the histologically normal epidermis, immediately adjacent to the SCC tumor as control. Stained slides were evaluated using a standard scoring system based on the intensity of staining (0-3), with score of 0 representing no staining and score of 3 as intense staining. If a relative difference in signal was observed between tissues being compared, it was recorded as a change in INPP5A protein level.

**Fig. 1.** Distribution of gene copy number aberrations in examined tissues. aCGH was done, and a total number of genomic aberrations were calculated for each examined sample. The bars represent individual samples, which are clustered according to the tissue type, from left to right, including normal skin (NL), AK, SCCis, and invasive SCC. Within each cluster, individual samples are sorted from left to right, according to the number of gene copy aberrations per sample. Asterisk designates samples harboring INPP5A deletions.
Detection of INPP5A by immunohistochemistry showed mainly diffuse cytoplasmic signal. A comparison of INPP5A staining intensity between SCC tissues and matched normal epidermis identified three general staining patterns. The most prevalent pattern of expression, observed in 51 of 71 (72%) examined tissues, manifested as a relative reduction of INPP5A in SCC tissues when compared with matched normal skin (Fig. 3A). Only 20 of 71 (28%) examined tissues showed no difference in INPP5A staining between the SCC and matched normal skin. Importantly, no single case was observed where INPP5A staining was more intense in SCC tumor than in matched normal skin, further highlighting the specificity of the observed pattern (Table 2, top; Fig. 3B). Notably, 10 SCC lesions examined in this cohort were classified by pathology as SCCis. Six of 10 of these SCCis lesions showed reduced INPP5A immunohistochemical signal, indicating no significant difference to the frequency detected in primary SCC in general. The observed reduction of INPP5A signal in SCC tissues is tumor specific, as the history of sun exposure and the extent of sun damage are comparable between the SCC lesion and the examined, adjacent normal epithelium used as a control. Taken together, a significantly higher frequency of INPP5A loss at the protein level compared with loss at the DNA level indicates that gene deletions may represent only one mechanism of INPP5A suppression, whereas a sizable proportion of SCC tumors likely achieve the same effect through deregulation of INPP5A by other mechanisms.

**Table 1. Frequency of INPP5A gene deletions as detected by CGH**

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>INPP5A deletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0/12</td>
</tr>
<tr>
<td>AK</td>
<td>0/5</td>
</tr>
<tr>
<td>SCCis</td>
<td>1/2</td>
</tr>
<tr>
<td>SCC</td>
<td>5/21 (24%)</td>
</tr>
</tbody>
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**Fig. 2.** Identification of INPP5A deletions by aCGH and validation by FISH. A, recurrent deletions involving chromosome 10q26.3 were identified in SCC tissues by aCGH. Log2 scale provided above each panel depicts DNA copy number change ("0" indicates no net change; negative values indicate deletions), with gray boxes depicting deletions. The gray box on the right indicates the area of overlap, shared among the five samples harboring deletions in this region. Black bars on the right depict individual genes, including INPP5A. B, a representative FISH assay is shown with INPP5A signal in red and centromeric chromosome 10 signal in green. Right, two copies of INPP5A are seen in normal keratinocytes (top right) but only one copy in SCC (bottom right). Normal metaphase spread (left) is provided as a reference.

**Fig. 3.** Detection of INPP5A protein loss in primary SCC tissues relative to the matched, normal epidermis. Immunohistochemistry for INPP5A was done on FFPE tissues, and relative intensity of INPP5A staining was compared between the SCC tissue and the adjacent, histologically normal epidermis. A, bottom, primary skin SCC tissue with low level of INPP5A staining; top, matched, adjacent normal epidermis from the same patient. B, a representative case is shown to further illustrate a relative difference in INPP5A staining between the primary SCC lesion and the adjacent normal epidermis. Scale bar, 50 μm.
INPP5A loss is an early event in SCC development

To more precisely evaluate the timing of the reduction of INPP5A level in the development of cutaneous SCC, we examined a series of 26 AKs, the earliest step in SCC development. Using immunohistochemistry, as described above, we compared INPP5A protein levels between the AK lesions and adjacent normal epidermis. A relative reduction of INPP5A in AK lesions was seen in 9 of 26 (35%) examined tissues, whereas 17 of 26 (65%) examined tissues showed no difference in INPP5A levels between the AK and normal epidermis (Table 2, middle).

We next asked whether the observed reduction of INPP5A protein in AKs is caused by genetic loss at the DNA level, such as seen in a subset of SCC tumors. To this end, we carried out FISH analysis and detected no INPP5A gene deletion in any of the examined cases (data not shown). Although a small lesion size and limited number of lesional nuclei available for analysis in some of the studied AKs call for cautious interpretation of these results, it is important to note that no single lesion showed evidence of a clonal population with uniform loss of INPP5A FISH signal, even in cases where such clonal loss was suggested by immunohistochemical data. This absence of perturbations on DNA level in AKs is not surprising given the relative paucity of gene copy number aberrations at early stages of disease detected by aCGH (Fig. 1) and likely indicates that deregulation of INPP5A expression in these precursor lesions occurs mainly on mRNA or protein level.

The less frequent reduction of INPP5A levels in AK than in SCC lesions (35% versus 72%) is also informative and may reflect selection that favors progression of AK lesions with low INPP5A to the next stage of disease. Interestingly, a pattern of INPP5A loss in a subset of AKs, occurring in the form of strikingly demarcated regions of low INPP5A signal, is suggestive of clonally expanding populations of affected cells within epidermis (Fig. 4). As SCCs often arise within the preexisting lesions of AKs, this focal loss of INPP5A in AKs might represent an early step toward a full oncogenic transformation along the spectrum of evolving epidermal neoplasia.

Loss of INPP5A in association with progression to metastatic disease

The above data implicate deregulation of INPP5A levels as an early event in the development of keratinocyte neoplasia, which may provide a selective advantage in progression from AK to SCC. To assess a potential role of INPP5A loss in the process of tumor maintenance and progression, we queried whether reduction of INPP5A level is associated with the subsequent biological step in SCC progression and development of metastatic disease. To this end, we evaluated INPP5A protein levels in a selected cohort of 17 patients with cutaneous SCC tumors that have subsequently metastasized, and where both primary tumor tissue and matched regional metastatic tissue were available for examination. Immunohistochemical analysis of these paired tissues detected further reduction of INPP5A levels in the transition from primary to metastatic SCC in 6 of 17 (35%) examined tissue pairs (Table 2, bottom). Although the remaining 11 of 17 (65%) studied

Table 2. Detection of INPP5A protein levels at successive stages of SCC progression

<table>
<thead>
<tr>
<th>INPP5A staining intensity</th>
<th>Frequency</th>
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<tbody>
<tr>
<td>Primary SCC compared with matched normal skin</td>
<td></td>
</tr>
<tr>
<td>Normal skin &gt; primary SCC</td>
<td>51/71 (72%)</td>
</tr>
<tr>
<td>Normal skin = primary SCC</td>
<td>20/71 (28%)</td>
</tr>
<tr>
<td>Normal skin &lt; primary SCC</td>
<td>0/71 (0%)</td>
</tr>
<tr>
<td>AK compared with matched normal skin</td>
<td></td>
</tr>
<tr>
<td>Normal skin &gt; AK</td>
<td>9/26 (35%)</td>
</tr>
<tr>
<td>Normal skin = AK</td>
<td>17/26 (65%)</td>
</tr>
<tr>
<td>Normal skin &lt; AK</td>
<td>0/26 (0%)</td>
</tr>
<tr>
<td>Primary SCC compared with matched metastatic SCC tissues</td>
<td></td>
</tr>
<tr>
<td>Primary SCC &gt; Met</td>
<td>6/17 (35%)</td>
</tr>
<tr>
<td>Primary SCC = Met</td>
<td>11/17 (65%)</td>
</tr>
<tr>
<td>Primary SCC &lt; Met</td>
<td>0/17 (0%)</td>
</tr>
</tbody>
</table>
pairs show no further loss of INPP5A levels in transition from primary to metastatic disease, it is important to note that no single case was identified where INPP5A staining was stronger in the metastatic tissue than in the primary SCC tumor, further highlighting the specificity of the observed INPP5A loss in SCC progression. These data suggest that reduction of INPP5A levels, although an early event in development of SCC, may also play a role in progression of SCC from primary to metastatic disease in a significant subset of aggressive primary SCC tumors. Future exploration of this question on a larger number of tissue specimens is warranted.

As we evaluated INPP5A staining in this cohort of 17 patients with metastatic SCCs, we noted the presence of normal epidermis immediately adjacent to the primary SCC tissue in 13 of 17 examined primary SCCs. It is interesting to note that a relative difference in INPP5A staining between the SCC tumors and adjacent normal epidermis in these 13 patients shows strikingly high frequency of INPP5A loss in SCC tumors. Twelve of 13 (92%) of these aggressive primary SCC tumors showed loss of INPP5A staining when compared with the adjacent, normal epidermis. This is in contrast to the above-described pattern of INPP5A loss observed in randomly selected primary SCC tumors, where 51 of 71 (72%) SCC tumors showed reduction of INPP5A protein levels by immunohistochemistry (Fig. 3A; Table 2). Thus, higher frequency of INPP5A loss in primary SCC tumors that have shown an aggressive clinical course (i.e., subsequent development of metastases) may indicate more aggressive primary disease and suggest a potential prognostic value of INPP5A levels in assessing the risk of progression in primary SCC tumors. Although this is an exploratory study and the sample sets are not powered to provide robust statistical quantification (comparison by Fisher’s exact test did not reach statistical significance), the observed patterns strongly highlight a trend that merits further exploration on a larger sets of matched, clinically annotated specimens.

In summary, we identify loss of INPP5A as an early event in development of cutaneous SCC. The gene itself is deleted in a significant proportion of SCC tumors, and its protein levels are reduced in the majority of SCC tumors. More frequent reduction of INPP5A levels in aggressive primary SCC tumors, as well as further reduction in metastatic disease, points to a potential role of INPP5A in the development and progression of cutaneous SCC. Our findings support the previously reported observations that implicate INPP5A as a novel tumor suppressor in other human cancers (17). Understanding the precise mechanism(s) of INPP5A loss in SCC and exploring the connection between INPP5A and uncontrolled cellular proliferation in skin cancer may provide novel insights into relevant mechanisms of epithelial carcinogenesis and facilitate development of clinically applicable prognostic markers, therapeutic strategies, as well as novel chemopreventive approaches.

Disclosure of Potential Conflicts of Interest

Authors disclose no potential conflicts of interest.

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