Correlation of angiogenesis with other immunohistochemical markers in cutaneous basal and squamous cell carcinomas

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Abstract
The aim of this study was to establish an immunoprofile of squamous cell carcinoma (SCC) and basal cell carcinoma (BCC), and to explore their relationship to tumor cell angiogenesis. For SCCs, the histological grade of differentiation was also taken into account. The angiogenesis was evaluated in 38 randomly selected cases of SCCs and 17 BCCs, respectively, using the antibodies vascular endothelial growth factor (VEGF-A) and COX-2, while the microvessel density (MVD) was evaluated with the CD31. Results: In SCCs, maspin cytoplasm to nuclear shift was an indicator of a deeper tissue invasion and dedifferentiation in the invasion front. The poorly differentiated cases, compared to G1/G2-SCCs, expressed more frequent the markers p16 (30.77% vs. 8%) and VEGF-A (53.85% vs. 32%), regardless the MVD. However, the p16 positivity was more frequent in BCCs than SCCs (52.94% vs. 15.79%). All of the p16-positive carcinomas were located in the head and neck area. DOG-1 marked 21.05% of SCCs and 5.88% of BCCs, being directly correlated with COX-2 positivity. Eccrine glands and hair follicles also expressed DOG-1. Conclusions: In cutaneous SCCs located in the head and neck area, sun-dependent p16/VEGF interaction seems to be responsible by tumor dedifferentiation, whereas maspin cytoplasm to nuclear shift might indicate a high degree of invasiveness. This is the first report about DOG-1 positivity in BCCs and eccrine glands, the significance of this pattern being unknown.

Keywords: epithelial skin tumors, maspin, DOG-1, p63, COX-2, VEGF.

Introduction

Knowing to be the most common primary tumors of the skin [1] and also the most common human solid malignant tumors [2], basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are intensively studied in literature but several aspects about their biological behavior, prognosis and therapy, are so far to be elucidated. With regarding to SCCs, sun-exposure is incriminated to be the major risk factor [3] but the underlying cancer biology that could optimize the therapeutic management is not properly characterized.

In this paper, we intended to set up an immunoprofile of SCC and BCC and to characterize the possible relationship of angiogenesis with other immunohistochemical (IHC) markers. Some of the used antibodies are described in the next paragraphs. The angiogenesis was counted using the endothelial marker CD31 but also other two markers, vascular endothelial growth factor (VEGF-A) and cyclooxygenase-2 (COX-2), respectively, that are known to have pro-angiogenic properties and can be quantified in the cytoplasm of the tumor cells.

To better understand the complexity of angiogenesis and its role in progression of cutaneous carcinomas, the particularities of maspin (Serpin B5) expression in SCC and BCC were also analyzed. Maspin is a serine protease that seems to play role in tumor cells proliferation, apoptosis, and angiogenesis, in several malignant tumors such as carcinomas of the breast, prostate, gastrointestinal tract, and melanomas [1, 4]. Depends on the tumor type, maspin can have even pro-apoptotic/antiangiogenic or pro-angiogenic function [1, 4]. However, few aspects are known about its role in carcinogenesis and prognosis of epithelial skin tumors and about the significance of its cytoplasmic or nuclear immunoreactivity in these malignancies.

The cell cycle regulatory tumor suppressor protein p16INK4A is known to inhibit D-type cyclin-dependent kinases that regulate the activity of retinoblastoma (Rb) gene [5]. It is involved in the carcinogenesis of several malignancies [6] and p16 IHC-overexpression is frequently associated with interaction of the tumor cells with human papilloma viruses (HPV), especially the beta-HPV that could play roles in the carcinogenic pathway [7]. This HPV-p16 correspondence was not found in all of the published studies [7].

DOG-1, a calcium-activated chloride channel also known as anoctamin-1 (ANO-1), ORAOV-2, TAOS-2, FLJ10261, is considered to be a highly sensitive and specific marker for gastrointestinal stromal tumors (GISTs) [8–10]. However, unusual patterns of expression were also reported in a broad range of other mesenchymal tumors such as schwannoma, malignant peripheral nerve sheath tumor, and also melanomas [8]. We also observed DOG-1 positivity in lipomas, myxoid and pleomorphic liposarcomas, and dermatofibrosarcomas [11]. Regarding to epithelial tumors, inconstant positivity was reported in carcinomas of the esophagus, gut, liver, pancreas, salivary glands, urinary bladder, endometrium, and breast [8, 9]. No data regarding DOG-1 immunoreactivity of BCCs have been postulated.
Materials and Methods

Patients and tumor samples selection

The study was carried out on 55 randomly selected patients with skin epithelial carcinomas that underwent surgical excision during the period between 2010 and 2013. The study was conducted at the Department of Pathology, University of Medicine and Pharmacy of Tîrgu Mureș, Romania. Signed written informed consent of patients was obtained for each case. All cases were primary tumors; no recurrences even metastases have been included.

In each of the cases, the patient’s age and gender and also localization, microscopic type, and stage of the tumor were analyzed. The angiolymphatic invasion and associated skin ulceration were also taken into account. All SCCs were invasive-type carcinomas; no in situ carcinomas have been included.

Immunohistochemical analysis

In each of the 55 cases, formalin-fixed paraffin-embedded tissue sections were cut at 5 μm; the IHC stains were performed using the manufacturer’s instructions (Table 1).

Table 1 – Main characteristics of antibodies used for the immunohistochemical stains

<table>
<thead>
<tr>
<th>Antibody (manufacturer)</th>
<th>Clone</th>
<th>Dilution</th>
<th>Antigen retrieval</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maspin (Novocastra, Newcastle-upon-Tyne, UK)</td>
<td>EAW24</td>
<td>1:25</td>
<td>Incubation with citrate buffer (pH 6.0) – 60 minutes at 100°C</td>
<td>• external – renal tubes; • internal – hair follicles, sebaceous and sweat glands.</td>
</tr>
<tr>
<td>CD31/PECAM-1 (LabVision, Fremont, CA, USA)</td>
<td>JC/70A</td>
<td>1:25</td>
<td>Incubation with high pH-solution (pH 10.0) – 30 minutes at 100°C</td>
<td>• external – placenta; • internal – blood vessels – endothelial cells.</td>
</tr>
<tr>
<td>VEGF-A (LabVision)</td>
<td>VG1</td>
<td>1:50</td>
<td>Incubation with high pH-solution (pH 10.0) – 30 minutes at 100°C</td>
<td>• external – renal tubes; • internal – normal mature vessels – endothelial cells.</td>
</tr>
<tr>
<td>COX-2 (Novocastra)</td>
<td>monoclonal</td>
<td>1:100</td>
<td>Incubation with citrate buffer (pH 6.0) – 60 minutes at 100°C</td>
<td>• external – brain; • internal – lymphocytes.</td>
</tr>
<tr>
<td>DOG-1 (Novocastra)</td>
<td>K9</td>
<td>1:50</td>
<td>Incubation with citrate buffer (pH 6.0) – 60 minutes at 100°C</td>
<td>• external – renal tubes; • internal – eccrine glands.</td>
</tr>
<tr>
<td>p16 (Santa Cruz Biotechnology, Dallas, Texas, USA)</td>
<td>JC8</td>
<td>1:50</td>
<td>Incubation with citrate buffer (pH 6.0) – 60 minutes at 100°C</td>
<td>• external – H-SIL; • internal – eccrine glands and hair matrix cells.</td>
</tr>
</tbody>
</table>

Novolink™ Polymer detection system (Novocastra, Newcastle-upon-Tyne, UK) was used for processing of the cases and DAB (3,3’-diaminobenzidine) solution (Novocastra) for developing. The slides were counterstained with Mayer’s Hematoxylin (Novocastra). For negative controls, incubation was done with omission of specific antibodies. For DOG-1 assessment, the hair follicles and eccrine glands were used as internal control, their positivity being not revealed yet in the literature (Figure 1).

Figure 1 – DOG-1 expression in the eccrine glands (A), adenoid-type basal cell carcinoma (B), and squamous cell carcinoma (C and D), with increased intensity in the peripheral immature cells (D).
The IHC assessment was performed with Nikon 800E optical microscope, with digital photo camera. The cut-off point for cytoplasmic positivity of VEGF-A, COX-2, and DOG-1 was defined as 10%. The cut-off point of p16 nuclear expression, with or without cytoplasmic positivity, was established at 10%. Regarding to maspin expression, the cases were evaluated as negative or positive, using the same 10% cut-off value; in the positive cases, cytoplasmic and nuclear expressions were assigned, independently by the percentage of immunoreactive cells [1, 4, 11].

The tumor vascularization was evaluated with CD31 using digital pictures performed inside the tumor clusters and the image analysis software ImageJ (NIH). The endothelial area was recorded by counting the positive vessels in the highly vascularized (“hot-spot”) areas, at 200× high-power fields. We batch-measured the percentage of positive endothelial area versus total area of the microscopic field [12]. The ulcerated and inflammatory areas were not taken into account for assessment of angiogenesis.

Statistical analysis

The statistical data were handling using the GraphPad InStat 3 statistical software and a descriptive analysis. A p-value <0.05 with 95% confidence interval was considered statistically significant. The frequencies and percentages specific for the analyzed parameters and means and standard deviations were used for continuous variables. The Student’s t-test, ANOVA and Fischer’s tests were used for univariate analysis.

Results

Clinicopathological features

The median age of the 55 patients was 65.97±15.01 years (range, 23–92 years), with a male:female ratio of 1:1.04 (21 males and 28 females). Selected cases included 38 SCCs and 17 BCCs. Compared to the SCCs, the BCCs occurred at older ages (p=0.04), more frequent (p=0.006) in females (65% of the BCCs and 45% of SCCs were females [10/28 (35.71%) positive cases] vs. males, five of the 27 (18.52%) cases displaying p16 (p=0.01). On the other hand, the COX-2 positivity was more frequent in tumors diagnosed in males [25/27 (92.59%) positive cases] vs. females, 22 of the 28 (78.57%) cases displaying COX-2 (p=0.007).

Squamous cell carcinomas

The 38 SCCs were classified as well to poorly differentiated, as follows: G1 (six cases), G2 (19 cases), G3 (13 cases). Regarding the immunoprofile of the SCCs, the p16 expression was identified in only six of the 38 cases, all of them being carcinomas of the face (Table 3).

<table>
<thead>
<tr>
<th>Marker</th>
<th>Squamous cell carcinoma (n=38)</th>
<th>Basal cell carcinoma (n=17)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>6 (15.79%)</td>
<td>32 (84.21%)</td>
<td>9 (52.94%)</td>
</tr>
<tr>
<td>Maspin</td>
<td>37 (97.37%)</td>
<td>1 (2.63%)</td>
<td>15 (88.24%)</td>
</tr>
<tr>
<td>VEGF</td>
<td>15 (39.47%)</td>
<td>23 (60.53%)</td>
<td>(29.41%)</td>
</tr>
<tr>
<td>COX-2</td>
<td>33 (86.84%)</td>
<td>5 (13.16%)</td>
<td>14 (82.35%)</td>
</tr>
<tr>
<td>DOG-1</td>
<td>8 (21.05%)</td>
<td>30 (78.95%)</td>
<td>1 (5.88%)</td>
</tr>
<tr>
<td>Endothelial area (CD31)</td>
<td>11.88±3.92</td>
<td>9.85±2.45</td>
<td>0.09</td>
</tr>
</tbody>
</table>

*Two-sided Fisher’s exact test – contingency table (two rows two columns).

Maspin immunoreactivity was emphasized in the cytoplasm of normal keratinocytes, in all of the examined cases. Most of the SCCs displayed only cytoplasm maspin (n=26) but also a mixed expression in both cytoplasm and nuclei was seen in some of the analyzed cases (n=11). It is necessary to mention that all of the six well-differentiated (G1) SCCs and also all cases diagnosed in stage pT1 displayed maspin cytoplasmic positivity, without associated nuclear expression. No differences occurred between immunostaining pattern of maspin, COX-2 and DOG-1 in cases diagnosed as moderately (G2) or poorly differentiated (G3) carcinomas (Table 4).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>G1+G2 (n=25)</th>
<th>G3 (n=13)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor stage (pT)</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>pT1</td>
<td>16 (64%)</td>
<td>4 (30.77%)</td>
<td></td>
</tr>
<tr>
<td>pT2</td>
<td>9 (36%)</td>
<td>9 (69.23%)</td>
<td></td>
</tr>
<tr>
<td>p16 – positive cases</td>
<td>2 (8%)</td>
<td>4 (30.77%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Maspin – positive cases</td>
<td>24 (96%)</td>
<td>13 (100%)</td>
<td>0.12</td>
</tr>
<tr>
<td>VEGF – positive cases</td>
<td>8 (32%)</td>
<td>7 (53.85%)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Table 4 – Clinicopathological and immunohistochemical characteristics of squamous cell carcinomas upon the histological grade of differentiation
In both G2 and G3 cases maspin mixed expression was especially seen in the invasion area (Figure 2). The maspin negative case was also negative for VEGF, COX-2, DOG-1, and p16. All of the cases with maspin mixed expression were also VEGF positive, while the maspin cytoplasmic immunoreactivity did not correlate a specific VEGF pattern.

Comparing the values of endothelial areas, independently by the microscopic tumor type (SCCs and BCCs were included), no correlations were observed between maspin subcellular localization and MVD \( (p=0.41) \), although the values were as follows: 7.87±3.20 for maspin negative cases, 11.34±3.23 for cases with only-cytoplasmic staining pattern, and 11.43±4.64 for cases with mixed maspin expression. Because only three of the cases were maspin negative, this absence of correlation is not a reliable result.

All of the eight cases that were diffusely marked by DOG-1 were also marked by COX-2 and displayed maspin cytoplasmic positivity and p16 negativity. The immunoreactivity of DOG-1 and COX-2 was more intense in the immature areas (Figure 2).

Comparing the well (G1) and moderately differentiated (G2) vs. poorly-differentiated (G3) SCCs, it was noted that most of the cases diagnosed as G3 presented a deepest level of tissue infiltration and displayed more frequent p16 and VEGF positivity, while the G1 cases were rather p16 negative.

### Basal cell carcinomas

Compared to SCCs, the immunoreactivity of p16 protein was more frequent in BCCs \( (p<0.0001) \), all of the positive cases being located in head and neck area; maspin and DOG-1 were less expressed (Table 3).

In BCCs, maspin positivity was mostly seen in the tumor cell cytoplasm (11 cases). All of them had a nodular architecture. The maspin positivity in both cytoplasm and nuclei was observed in only four cases; all of them had an adenoid-architecture. The two maspin-negative cases displayed the same immunoprofile of the above-mentioned negative SCC. In the DOG-1 positive-BCC, simultaneous maspin and COX-2/p16 positivity was seen.

Regards to angiogenesis, although both VEGF and COX-2 were less expressed in BCCs compared to SCCs and the endothelial area was also lower, the difference did not proved to be statistically significant (Table 3).

### Discussion

To better understand the behavior of cutaneous tumors

<table>
<thead>
<tr>
<th>Parameter</th>
<th>G1+G2 ((n=25))</th>
<th>G3 ((n=13))</th>
<th>(P)-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-2 – positive cases</td>
<td>21 (84%)</td>
<td>12 (92.31%)</td>
<td>0.12</td>
</tr>
<tr>
<td>DOG-1 – positive cases</td>
<td>6 (24%)</td>
<td>2 (15.38%)</td>
<td>0.15</td>
</tr>
<tr>
<td>Endothelial area ((CD31))</td>
<td>11.31±3.80</td>
<td>12.94±4.09</td>
<td>0.27</td>
</tr>
</tbody>
</table>

and the therapeutic possibilities, exploration of their sun-related histogenesis and progression [13] could be an important tool. The previous studies proved that, in human keratinocytes the ultraviolet B irradiation can activate the CDKN2A (cyclin-dependent kinase inhibitor 2A) gene that encodes the p16 protein [3, 5, 6, 14]. As result, p16 overexpression is revealed not only in most of the in situ or invasive SCCs (60–75% of the cases) but also in keratoacanthomas, seborrhoeic keratosis, Bowen disease and actinic keratosis; the normal skin being p16-negative [5, 14, 15]. In our material, although most of the cases were localized in sun-exposed areas, activation of p16 protein was restricted to below one quarter of the SCCs, all of the p16 positive-cases being localized on the face. On the other hand, in head and neck areas, activation of CDKN2A in keratinocytes, seems not only to promote carcinogenesis but also to induce tumor dedifferentiation. These facts prove that p16 is indeed necessary but not sufficient to induce tumor progression [10] and dedifferentiation.

In addition, other markers such as maspin might be implicated in behavior of SCC, its role being dependent upon the subcellular localization. Most of the well-differentiated tumors diagnosed in early stages showed in the present material a cytoplasmic positivity, with a nuclear shift in the invasion front and dedifferentiated areas, respectively. It is important to note that the maspin nuclear positivity also associated a high VEGF expression, especially in the poorly differentiated tumors diagnosed in advanced stages [16]. These facts might prove the possible role of VEGF/maspin/p16 interaction in dedifferentiation of SCCs, with increased risk for local recurrence and/or metastasis. This interaction seems to not be involved in the progression of SCCs of the lips [16]. Although increased rate of neovascularization was described in parallel to progression of SCCs [17, 18], we did not notice a significant association.

In other studies, hormonal responsive element in the maspin gene promoter was supposed to be implicated in progression of SCCs [1], although the present study did not reveal sex-dependent maspin immunoreactivity. However, androgen receptors, that could be expressed by the sebaceous glands, keratinocytes, hair follicles, eccrine and apocrine sweat glands [1, 19] could interact with COX-2 and probably DOG-1 pathway, independently by p16. In head and neck SCCs cell lines, DOG-1 positivity was reported to stimulate cell movement and proliferation and to be associated with a high risk of distant metastases and poor outcome [20, 21]. Because a significant higher DOG-1 amplification have been reported in the SCCs of the hypopharynx and larynx compared to the oral activity [9], based on the p16 negativity in all of the DOG-1 positive SCCs of the skin, we suppose that DOG-1 could have a sun-independent role in carcinogenesis of SCCs.

Regard to therapeutic possibilities, in the most recent studies in the field, it was proved that a high serum level of maspin could indicate a higher sensitivity of the tumor cells to cisplatin [22]. This therapeutic implication is far to be elucidated because there are not published data about the relation between subcellular histological expression and maspin serum level.

In line to the previous published studies, most of the BCCs were nodular-type and occurred in the sun-exposed areas [23] of the head and neck, being probably related on the p16-sun-dependent activation [6], in a larger proportion than in SCCs (53% vs. 16%). The adenoid variant can express DOG-1, the meaning of this positivity being necessary to be elucidated in the future.

Conclusions

In SCCs of the skin, the tumors located in the head and neck area present two mechanisms of carcinogenesis. First, a p16-sun-dependent pathway might be involved in VEGF-dependent tumor dedifferentiation. Second, a DOG-1/COX-2 interaction seems to be responsible by the sun-independent carcinogenesis of SCCs that can be influenced by androgens. With regard to the therapy, inhibition of angiogenesis of SCCs of the skin could be more efficient using the anti-maspin drugs while the DOG-1 positive cases could respond to the anti-COX-2 drugs.

Conflict of interests

The authors declare that they have no conflict of interests.

References


