The immunoexpression of MMP-1 and MMP-13 in eyelid basal cell carcinoma

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Abstract
Basal cell carcinoma (BCC) is the most frequent human malignancy and at the same time the most frequent periorcular malignancy, representing almost 80% of all non-melanoma skin cancers and 90% of eyelid cancers. The study included 50 cases of eyelid BCC, out of which 41 were nodular BCC (NBCC) and nine were infiltrative BCC (IBCC), with various Breslow scores (BS) and primary tumor (pT) category. We analyzed the immunoexpression of matrix metalloproteinases (MMPs) 1 and 13 in the tumoral epithelial component (TEC) and inflammatory stromal component (ISC) of BCC in relation to the two histopathological parameters. The immunoexpression for MMP-1 was identified in 41 (82%) cases and for MMP-13 in 46 (92%) cases both in the TEC and ISC of both types of BCC. The statistical analysis revealed that both collagenases had positive/high scores significantly associated with advanced BS. For MMP-1, there were statistical associations in TEC related to IBCC and high pT category, while MMP-13 only revealed statistical association in ISC with high pT. The presence of collagenase MMP-1 and MMP-13 expression in a high number of cases, both in TEC and ISC, confirms their intervention in the tumor progression and proposes these MMPs as potential targets in antineoplastic therapy.

Keywords: eyelid basal cell carcinoma, MMP-1, MMP-13.

Introduction
The eyelid is the main location of neoplasms in ophthalmology practice, the structures being involved in 5–10% of all types of skin cancer [1–3]. BCC is the most frequent human malignancy [4] and it is also the most frequent periorcular malignancy, representing almost 80% of all nonmelanoma skin cancers and 90% of eyelid cancers [5–12].

Matrix metalloproteinases (MMPs) are degrading enzymes with an important role in tumor progression through the increase of angiogenesis induces by tumors and the destruction of local tissue architecture and basal membranes to allow tumor invasion and metastasis [13]. Their involvement in tumorigenesis seems to be far more complex than initially considered. Efficient destruction of extracellular matrix (ECM) around the invasive cancer islands involves the interaction between tumor, stromal and inflammatory cells, all of them expressing a distinct set of MMP [13]. Collagenases are the most important secreted proteinases, capable to initiate the native degrading of fibrillar collagen (type I, II, III, V, IX), MMP-1 splits type III collagen, while MMP-8 splits collagen types I and II [14].

Aim
In the present study, we analyzed the expression of MMP-1 and MMP-13 in the tumoral epithelial component (TEC) and inflammatory stromal component (ISC) of nodular BCC (NBCC) and infiltrative BCC (IBCC) in relation with the Breslow score (BS) and primary tumor (pT) category.

Materials and Methods
The study included 50 cases of eyelid BCC diagnosed in the Laboratory of Pathology, Emergency County Hospital of Craiova, Romania, during 2016–2019. The biological material was represented by fragments of surgical excision from patients hospitalized and operated in the Departments of Plastic Surgery and Ophthalmology of the same Hospital. The material was fixed in 10% neutral buffered formalin and then processed by the paraffin embedding technique and Hematoxylin–Eosin (HE) staining. Lesion classification by the histopathological (HP) type and the depth of tumor invasion (BS and pT category) were done according to the literature recommendations [15, 16].

We then performed serial sections on which we performed immunohistochemical analysis using a detection system based on enzyme detection of antigen signal [Labeled Streptavidin–Biotin 2 System–Horseradish Peroxidase (LSAB2–HRP), Dako, code K0675]. For reaction visualization, we used the 3,3’-Diaminobenzidine (DAB, Dako, code 3467) chromogen and for validation, we used external positive (testicle for MMP-1 and liver for MMP-13) and negative controls (by omitting the
primary antibody). Antigen recovery was done under microwave for 20 minutes in citrate buffer, pH 6. We assessed the semi-quantitative expression of MMP-1 (clone EP1247y, 1/50 dilution) and MMP-13 (clone ab84594, 1/50 dilution) in TEC and ISC of NBCC and IBCC compared to BS and pT category. We used a reaction quantification system applied independently by two specialists, based on immunostaining intensity noted with 1 (reduced intensity), 2 (moderate intensity) and 3 (high intensity), calculating an average composite score (CS) for each lesion. The absence of the immunoreaction was noted with a score of 0. In case of inconsistency in the intensity of the immunoreactions, the specimens were reviewed, and a consensus was established. The quantification system used was considered optimal to differentiate between positive/negative cases, the positive cases showing differences in relation to the intensity of the reactions and not to the number of labeled cells.

We made the statistical analysis on the results using tests for evaluating the differences between the immunoreaction scores of the lesion categories, respectively χ² (chi-squared) test, within the Statistical Package for the Social Sciences (SPSS) 10 software. During the study, it were respected the general norms of conduit regarding the research ethics problems and necessities, consisting in the approval of the local Ethics Committee and informed consent of patients.

Results

HP analysis of the 50 cases of BCC revealed 41 cases of NBCC and nine cases of IBCC, with different BS and pT categories. For NBCC, we found 30 cases in pT1 (BS II six cases, BS III 12 cases and BS IV 12 cases) and 11 cases in pT2 (BS III four cases and BS IV seven cases) and for IBCC, we found three cases in pT1 (BS IV) and six cases in pT2 (BS III two cases and BS IV four cases).

The immunoreaction for MMP-1 was identified in 41 (82%) cases and for MMP-13 in 46 (92%) cases, in both TEC and ISC of both types of BCC. In case of ISC, the inflammatory positive chronic infiltrate was represented mainly by mononuclear cells, respectively lymphocytes, plasma cells, macrophages, fibroblasts. For MMP-1, in both types of BCC, we noticed cytoplasm positivity with CS that varies between TEC and ISC, respectively 0.50–1 in TEC and 1.33–2.75 in ISC. The analysis of MMP-13 expression also indicated cytoplasm positivity in both types of BCC with CS values of 0.33–1.75 for TEC and 0.66–2.50 for ISC (Table 1).

| pT category | BS category | BCC type | MMP-1 | MMP-13 |
|-------------|-------------|----------|-------|--------|
|             |             | No. of cases | CS TEC | CS ISC | No. of cases | CS TEC | CS ISC |
|             | BS II       | NBCC 3      | 0.50   | 1.33   | 2          | 0.33   | 0.66   |
|             |             | IBCC 0      | –      | –      | 0          | –      | –      |
|             | BS III      | NBCC 8      | 0.66   | 1.66   | 12         | 1.58   | 2.08   |
|             |             | IBCC 0      | –      | –      | 0          | –      | –      |
|             | BS IV       | NBCC 12     | 1.00   | 2.50   | 12         | 1.75   | 2.16   |
|             |             | IBCC 3      | 1.00   | 2.00   | 3          | 1.66   | 2.33   |
|             | BS III      | NBCC 2      | 0.5    | 1.50   | 4          | 1.50   | 2.00   |
|             |             | IBCC 2      | 1.00   | 2.50   | 2          | 1.50   | 2.50   |
|             | BS IV       | NBCC 7      | 1.00   | 2.71   | 7          | 1.71   | 2.00   |
|             |             | IBCC 4      | 1.00   | 2.75   | 4          | 1.75   | 2.50   |

BCC: Basal cell carcinoma; BS: Breslow score; CS: Composite score; IBCC: Infiltrative basal cell carcinoma; ISC: Inflammatory stromal component; MMP: Matrix metalloproteinase; NBCC: Nodular basal cell carcinoma; pT: Primary tumor; TEC: Tumoral epithelial component.

MMP-1 immunoexpression

MMP-1 immunoexpression analysis in NBCC indicated a moderate/high reaction intensity for ISC and low intensity for TEC (Figure 1A). In the case of TEC, immunoreaction positivity was identified especially in cells on the bounds of tumor cell islands. For ISC, the positivity of the immunoreaction was identified especially in the cells of the peritumoral inflammatory infiltrate.

In IBCC, the CS was low in TEC and high in ISC (Figure 1B). In the case of TEC, the positivity of the immunoreaction was diffusely identified in the tumor cell cords. For ISC, the immunoreaction was identified in the cells of the intratumoral inflammatory infiltrate.

MMP-13 immunoexpression

MMP-13 immunoexpression analysis in NBCC indicated low/moderate CS values in TEC and moderate values in ISC with random distribution on the neoplastic islands and in the intratumoral inflammatory cells (Figure 1C).

For IBCC, we also found mostly moderate CS values independent of BS, also with random distribution in the tumor and ISC (Figure 1D).

Statistical analysis of MMP-1 immunoexpression in TEC indicated the association of high CS with IBCC (p=0.183, χ² test), high pT category (p=0.699, χ² test) and advanced BS (p=0.002, χ² test), with statistically significant association only for the last parameter (Figure 2, A–C). For MMP-1 immunoexpression in ISC, we determined a non-significant statistically relation of IBCC (p=0.207, χ² test) of pT2 category (p=0.163, χ² test) with high CS (Figure 2, D and E). By comparison, high BS was significantly associated with high MMP-1 CS (p=0.012, χ² test) (Figure 2F).

MMP-13 immunoexpression analysis in TEC indicated a relation between positive/moderate CS with IBCC (p=0.600, χ² test) and pT2 category (p=0.321, χ² test), without statistically significance (Figure 3, A and B). For BS, we determined statistically significant differences, moderate values being associated only with BS III and IV (p<0.001, χ² test) (Figure 3C). In ICS, moderate values of CS were associated with NBCC, for IBCC exiting moderate/high values, which was a statistically significant association (p=0.012, χ² test) (Figure 3D). In the case of

| BS category | MMP-1 | MMP-13 |
|-------------|-------|--------|
| BS II       |       |        |
| BS III      |       |        |
| BS IV       |       |        |
| BS III      |       |        |
| BS IV       |       |        |

| BS category | MMP-1 | MMP-13 |
|-------------|-------|--------|
| BS II       |       |        |
| BS III      |       |        |
| BS IV       |       |        |
| BS III      |       |        |
| BS IV       |       |        |
pT category, we found moderate CS in pT1 tumors and moderate/high CS in pT2 tumors, but the correlation was not statistically significant ($p=0.306$, $\chi^2$ test) (Figure 3E).

Moderate/high MMP-13 immunostaining was associated with BS III and IV, which was a statistically significant relation ($p<0.001$, $\chi^2$ test) (Figure 3F).

**Figure 1** – (A) NBCC, BS IV; (B) IBCC, BS IV; (C) NBCC, BS III; (D) IBCC, BS III. Anti-MMP-1 antibody immunostaining: (A and B) ×200. Anti-MMP-13 antibody immunostaining: (C and D) ×200. BS: Breslow score; IBCC: Infiltrative basal cell carcinoma; MMP: Matrix metalloproteinase; NBCC: Nodular basal cell carcinoma.

**Figure 2** – (A) Distribution of cases depending on CS TEC MMP-1 and tumor type; (B) Distribution of cases depending on CS TEC MMP-1 and pT category; (C) Distribution of cases depending on CS TEC MMP-1 and BS; (D) Distribution of cases depending on CS ISC MMP-1 and tumor type; (E) Distribution of cases depending on CS ISC MMP-1 and pT category; (F) Distribution of cases depending on CS ISC MMP-1 and BS. BS: Breslow score; CS: Composite score; ISC: Inflammatory stromal component; MMP-1: Matrix metalloproteinase-1; pT: Primary tumor; TEC: Tumoral epithelial component.
MMP-1 and MMP-13 immunoexpression analysis indicated a significant association of the two immuno-markers in the sense of positive/high CS of MMP-1 and high CS of MMP-13 in TEC ($p=0.002$, $\chi^2$ test) and ICS ($p=0.005$, $\chi^2$ test) (Figure 4, A and B).

Discussions

MMPs are degrading enzymes, responsible for the destruction of tissues in a series of pathologies including cancer, their expression being induced by inflammatory cytokines at the stromal level [17] but also by tumor cells (autocrine and paracrine mechanisms). They are involved in tumor progression by increasing tumor induced angiogenesis and the destruction of local tissue architecture and basal membranes to allow tumor invasion and metastasis [13]. The proteins are coded by a gene family that play role in the development of normal tissues and their remodeling and repair [18, 19].

Varani et al. reported that from the MMP family, MMP-1 is the most abundantly expressed in different skin lesions [20]. In BCC, MMP-1 immunoexpression, unlike other collagenases, such as MMP-3, MMP-11, MMP-2, and MMP-9, is mainly located in stromal cells around malignant cells [20–23].

In the present study, the immunoreaction of MMP-1 was identified in the cytoplasm in 82% of the investigated cases, both in tumor epithelium and in stromal inflammatory cells of both types of NBCC and IBCC BCC. Statistical analysis of MMP-1 immunoexpression in TEC indicated the association of high CS with IBCC, pT category and advanced BS, with statistically significant correlation only for BS. For ISC, statistical analysis of MMP-1 immunoexpression showed an association statistically non-significant of IBCC and pT2 tumors with high CS. By comparison, high BS was significantly associated with high CS.

One study in the literature revealed increased MMP-1 immunoexpression in peritumoral tissue compared to distant epidermal tissue [24]. In another study, different varieties of BCC also expressed MMP-1 in all investigated cases [25]. Shaheen et al. noticed that the immunoexpression of MMP-1 in peritumor area of BCC is significantly higher than inside the tumor, surgical safety
The presence of collagenases MMP-1 and MMP-13 immunoeexpression in a high number of cases, both in TEC and ICS confirms the intervention of the investigated MMPs in tumor progression. Both collagenases indicated positive/high scores that were statistically significantly associated with advanced BS. For MMP-1, statistical associations were present in TEC reported to IBCC and high pT, while for MMP-13, this aspect was present in ICS only compared to high pT. Reaction positivity for MMP-1 and MMP-13 in periocular inflammatory cells indicates the role of inflammation in the tumor progression modulation. Given their role in tumor development, both collagenases can be potential targets in cancer therapy.

Conflict of interests
The authors declare that they have no conflict of interests.

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