ORIGINAL PAPER

RJME
Romanian Journal of
Morphology & Embryology
http://www.rjme.ro/

Assessment of VEGF and EGFR in the study of angiogenesis of eyelid carcinomas

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Abstract

A tumor represents an abnormal tissue growth that can arise from any ocular structure, such as eyelids, muscles or the optic nerve. At the eyelids, there are two main tumor types: basal cell carcinoma and squamous cell carcinoma. Angiogenesis plays a crucial role in growth, invasion and metastasis processes of any tumor. It is well known the fact that without new vessels formation tumors cannot exceed 1–2 mm diameter. Immunohistochemical analysis has been performed on 43 cases of primary carcinomas of the eyelid, diagnosed between 2010 and 2014 in the Laboratory of Pathological Anatomy of the University Emergency County Hospital of Craiova, Romania. Biological material was represented by surgical resection samples, coming from the Clinic of Ophthalmology the anteriorly named Hospital. Within the immuno-histochemical study, we have evaluated epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) expression in a group of 43 cutaneous carcinomas of the eyelid, depending on the type and differentiation grade of the tumor. Of the 43 samples, 23 came from patients with eyelid basal cell carcinoma and 20 came from patients with eyelid squamous cell carcinoma. In our study, EGFR and VEGF immunoexpression was superior for squamous cell carcinomas, compared to basal cell carcinomas, fact that was statistically significant. Regarding squamous cell carcinomas, the immunoexpression of these two markers was superior in moderate/poor differentiated forms, compared to well differentiated forms, fact that was statistically significant. The markers used in this study were found to be associated with the acquisition of aggression and angiogenic phenotypes by analyzed carcinomas.

Keywords: angiogenesis, basal cell carcinoma, squamous cell carcinoma, VEGF, EGFR.

⊟ Introduction

A tumor represents an abnormal tissue growth that can arise from any ocular structure, such as eyelids, muscles or the optic nerve. At the eyelids, there are two main tumor types: basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) [1, 2]. The tumorigenesis process was defined as a proliferative multistep disorder, characterized by uncontrolled cell growth because of progressive accumulation of genetic mutations and chromosomial aberrations *via* variable multistep pathways [3].

Angiogenesis plays a crucial role in growth, invasion and metastasis processes of any tumor. It is well known the fact that without new vessels formation tumors cannot exceed 1–2 mm diameter [4, 5–7].

Vascular endothelial growth factor (VEGF) plays one of the main parts in the angiogenesis process of any neoplasia. VEGF overexpression, as well as increased serum values of this cytokine, have been observed in several malignancies [4, 5]. Vascular endothelial growth factor-A (VEGF-A) is a homodimeric glycoprotein acting as a survival and growth factor for endothelial cells, but it can also determine increased vascular permeability, vaso-dilatation and modifications of immune cells properties, as a result of binding its main receptors: VEGF 21 and 22 receptors. VEGF-A was identified as the main angiogenesis factor for most of the human cancers, including breast,

colon, lung, and prostate cancer [8, 9]. Squamous cell carcinoma also expresses high levels of VEGF-A, especially at the tumor front, which is the main site for angiogenesis induction [8, 10].

Epidermal growth factor receptor (EGFR) represents a key player in epithelial tissue homeostasis. It is a trans-membrane tyrosine kinase receptor, also known as ErbB1 or HER1, which has a vital importance in squamous cell carcinoma development. In normal epithelial tissue, EGFR signaling pathway is involved in epithelial cells proliferation, differentiation and migration [11, 12]. EGFR activation and overexpression is present in various epithelial malignant tumors, including colorectal cancer, lung cancer other than small cell carcinoma and breast cancer [11, 13]. In many carcinomas, EGFR overexpression is associated with aggressive disease, poor prognosis and treatment issues [11, 14]. In cutaneous squamous cell carcinoma, EGFR overexpression, numeric aberrations, genetic amplification and hyperactivity have been reported comparing to normal cutaneous tissue [11, 15–17]. In metastasized cutaneous squamous cell carcinoma, EGFR overexpression is an ordinary event [11, 18].

Aim

The aim of our study is to analyze the angiogenesis in eyelid basal cell and squamous cell carcinomas, as well

as in different forms of differentiations of these tumors *via* VEGF and EGFR immunoexpression.

⊟ Materials and Methods

Immunohistochemical analysis has been performed on 43 cases of primary carcinomas of the eyelid, diagnosed between 2010 and 2014 in the Laboratory of Pathological Anatomy of the University Emergency County Hospital of Craiova, Romania. Biological material was represented by surgical resection samples, coming from the Clinic of Ophthalmology the anteriorly named Hospital. The samples included in our study fulfill the AJCC (*American Joint Committee on Cancer*) basal cell or squamous cell carcinoma diagnosis criteria for eyelid tumors [19]. Our study included only those samples with adequately quantitative material, as well as those samples for which primary processing (*e.g.*, fixation) provided high quality results *via* antigenic sites preservation. Recurrences and cases in which chemotherapy or local radiotherapy were used for other tumors were excluded, in order to ensure the homogeneity of the group.

For the immunohistochemical analysis, 4 μm seriate sections were obtained from the paraffin-embedded samples. These sections were applied on poly-L-lysine coated slides and included in 37^0C thermostat for six hours (Table 1).

Table 1 – *Panel of used antibodies*

Antibody	Clone / Manufacturer	Dilution	Antigen retrieval	Positive control
EGFR	Polyclonal / Sigma	1:1000	No retrieval	Placenta
VEGF	C1 / Dako	1:100	Citrate, pH 6	Kidney

EGFR: Epidermal growth factor receptor; VEGF: Vascular endothelial growth factor.

Negative external controls (primary antibody omission) and positive external controls were used for the immunohistochemical reactions validation. Image acquirement was performed using Nikon Eclipse E600 microscope with camera for image processing and Lucia 5 software.

Staining quantification was performed using a score resulting from multiplying the number of stained cells (P) by the staining intensity (I). Depending on the number of stained tumoral cells, the cases were categorized as follows: 1 (<25% stained cells), 2 (25–49% stained cells), 3 (50–75% stained cells) and 4 (>75% stained cells). Regarding staining intensity, the categories were as follows: 1 (weak), 2 (moderate) and 3 (strong). For the statistical analysis, the reactions immunoexpression was considered as low for scores between 1–4, moderate for scores between 6–8 and high for scores between 8–12.

Quantitative assessment of the immunohistochemical expression of the used antibodies was performed according to score 1 (Table 2). Qualitative assessment of the staining intensity was performed according to score 2 (Table 2).

Table 2 – *Immunohistochemical reactions assessment*

Score 1	0	1+	2+	3+
Positive cells	<10%	10–25%	25–50%	>50%
Score 2	**1**	**2**	**3**	
Staining intensity	Weak	Moderate	Strong	

Statistical analysis used averages, standard deviations and comparison tests (ANOVA, *chi*-square), which were performed within the SPSS 10 automatic soft. Statistical

correlations or differences were significant if *p* was less than 0.05.

The research was conducted in compliance with ethical rules in force and existing medical ethics.

⊟ Results

Within the immunohistochemical study, we have evaluated EGFR and VEGF expression in a group of 43 cutaneous carcinomas of the eyelid, depending on the type and differentiation grade of the tumor. Of the 43 samples, 23 came from patients with eyelid basal cell carcinoma and 20 came from patients with eyelid squamous cell carcinoma.

EGFR immunoexpression was detected in tumor cells in 79% of the cases; the expression was localized at the cells membrane and apical cytoplasmic. Furthermore, the expression was also present in sebaceous and sweat glands and in the basal and intermediate layers of the epidermis.

EGFR immunostaining was present in 73.9% of the analyzed basal cell carcinomas. The number of marked cells was between 10–30% and the staining intensity was mostly weak and rarely moderate, with and average EGFR score of 1.5 (Figures 1A and 3; Table 3). In comparison, EGFR reaction for the squamous cell carcinomas was present in 85% of the cases, with 15–85% marked cells, variable staining intensity and an average EGFR score of 5.7 (Figures 1B and 3; Table 3).

Table 3 – *Average EGFR and VEGF values in eyelid carcinomas*

Carcinoma	Basal cell carcinoma	Squamous cell carcinoma
Average EGFR score	1.5	5.7
Average VEGF score	2	5.3

EGFR: Epidermal growth factor receptor; VEGF: Vascular endothelial growth factor.

VEGF immunostaining was identified in 62.8% of the analyzed carcinomas; the staining was present in the tumor cells cytoplasm. Endothelial cells and some of the stromal elements (lymphocytes, plasmocytes, fibroblasts or macrophages) also presented VEGF immunostaining.

VEGF immunoreaction was present in 43.4% of the analyzed basal cell carcinomas, with 15–32% of marked tumor cells, a weak/moderate staining intensity and an average VEGF score of 2 (Figures 2A and 4; Table 3). Comparatively, the immunoreaction was present in 85% of the studied squamous cell carcinomas, with 10–80% of marked tumor cells, variable staining intensity and an average VEGF score of 5.3 (Figures 2B and 4; Table 3).

EGFR and VEGF analysis depending on the differentiation grade of the squamous cell carcinoma indicated some differences (Table 4).

Table 4 – *Average values of EGFR and VEGF scores depending on squamous cell carcinomas differentiation grading*

Squamous cell carcinoma	WD	MD	PD
Average EGFR score	1.2	2.5	2.7
Average VEGF score	1	6.1	7.1

EGFR: Epidermal growth factor receptor; VEGF: Vascular endothelial growth factor; WD: Well differentiated; MD: Moderate differentiated; PD: Poor differentiated.
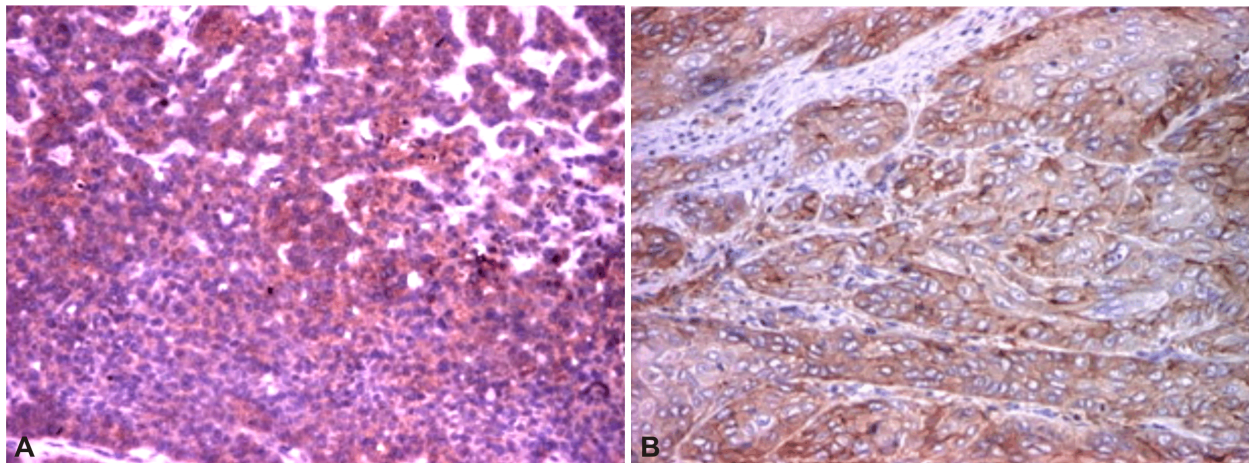
Figure 1 – EGFR expression in eyelid carcinomas: (A) Basal cell carcinoma; (B) Squamous cell carcinoma. EGFR immunostaining, ×100.

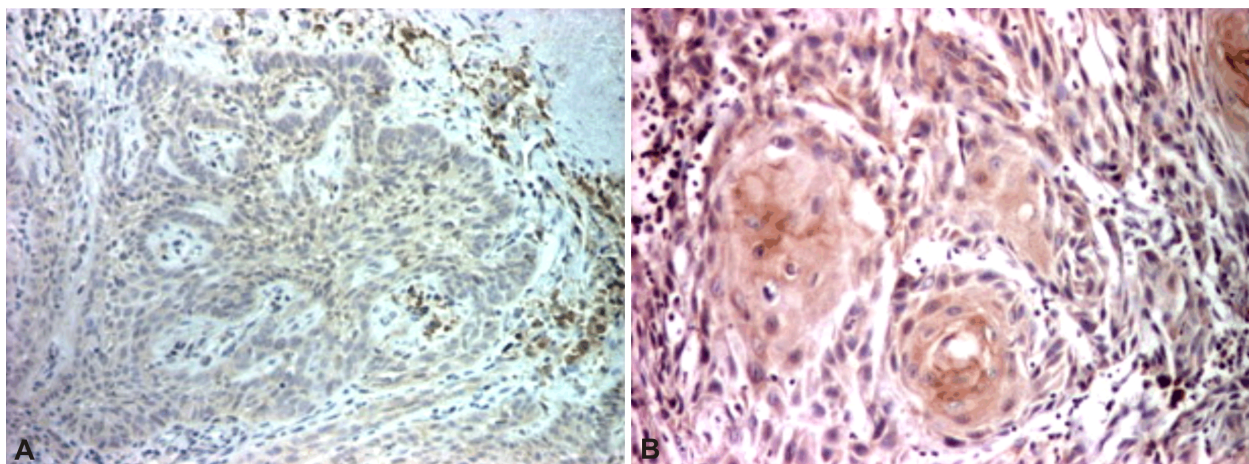**Figure 2 – VEGF expression in eyelid carcinomas: (A) Basal cell carcinoma; (B) Squamous cell carcinoma. VEGF immunostaining, ×100.**
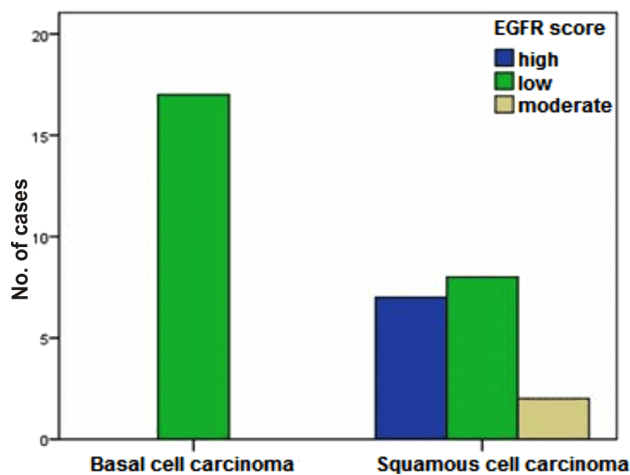

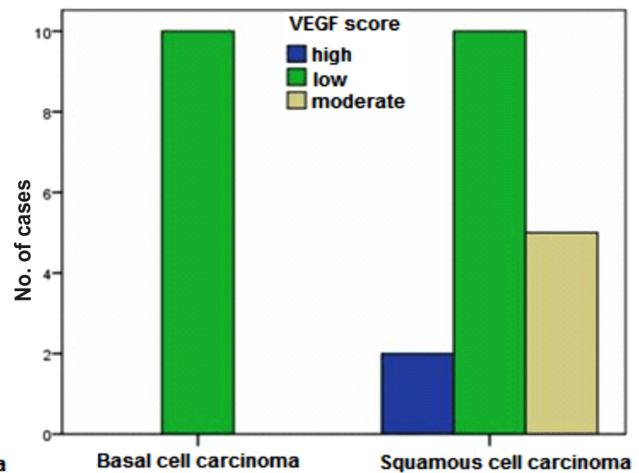**Figure 3 – EGFR score distribution in basal cell and squamous cell carcinomas. EGFR: Epidermal growth factor receptor.**

Figure 4 – VEGF score distribution in basal cell and squamous cell carcinomas. VEGF: Vascular endothelial growth factor.

EGFR immunostaining was present in 66.6% of the well differentiated squamous cell carcinomas, with 10–30% marked cells, weak/moderate staining intensity and an average score of 1.2 (Figure 5A; Table 4). Regarding VEGF immunostaining in well differentiated squamous cell carcinomas, it was present in 66.6% of cases, with 10–25% marked cells, weak staining intensity and an average score of 1 (Figure 5B; Table 4).

In moderate differentiated squamous cell carcinomas, EGFR immunostaining was present in 85.7% of the cases; 25–60% tumor cells were marked, the staining intensity was moderate/strong and the average score was 2.5 (Figure 6A; Table 4). VEGF immunoreaction was present in all cases of moderate differentiated squamous cell carcinoma, with 35–80% tumor cells marked, moderate/strong staining intensity and a 6.1 average score (Figure 6B; Table 4).

EGFR immunostaining was present in all cases of poor differentiated squamous cell carcinoma, with 45–85% stained tumor cells, moderate/strong staining intensity and an average score of 2.7 (Figure 7A; Table 4). Regarding VEGF staining in poor differentiated squamous cell carcinomas, it was present in all analyzed cases, with 45–80% positive tumor cells, moderate/increased staining intensity and an average score of 7.1 (Figure 7B; Table 4).

Statistical analysis indicated no significant differences of the EGFR average score reported to the tumor differentiation grade. However, the analysis of the percentage of staining indicated significant superior values for moderate/poor differentiated squamous cell carcinomas comparing to well differentiated squamous cell carcinomas, [$F_{(2.14)}$= 13.8, p=0.000], ANOVA test (Figure 8). Regarding VEGF, statistical analysis indicated no significant differences of the average scores reported to the tumor differentiation grade. Nevertheless, the analysis of the percentage of staining indicated significant superior values for moderate/poor differentiated squamous cell carcinomas comparing to well differentiated squamous cell carcinomas, [$F_{(1.15)}$= 37.3, p=0.000], ANOVA test (Figure 9).
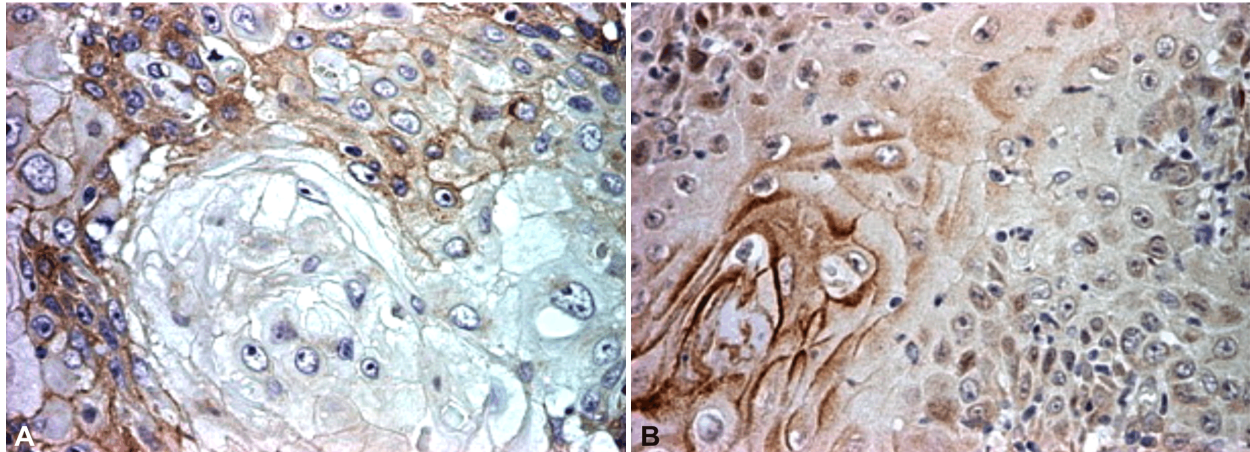

Figure 5 – *Well-differentiated squamous cell carcinoma: (A) EGFR immunostaining, ×200; (B) VEGF immunostaining, ×200.*
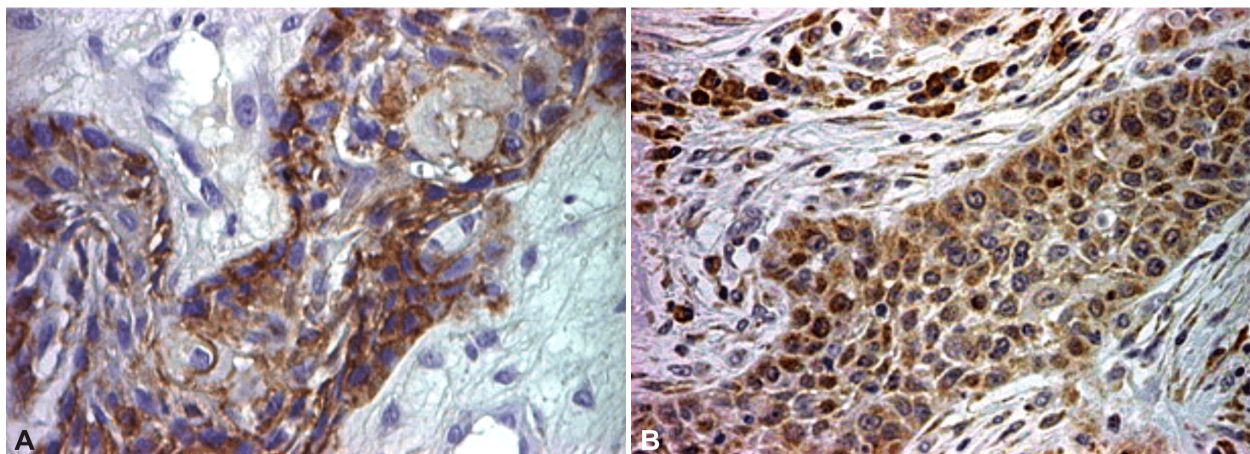

Figure 6 – *Moderate differentiated squamous cell carcinoma: (A) EGFR immunostaining, ×200; (B) VEGF immunostaining, ×200.*
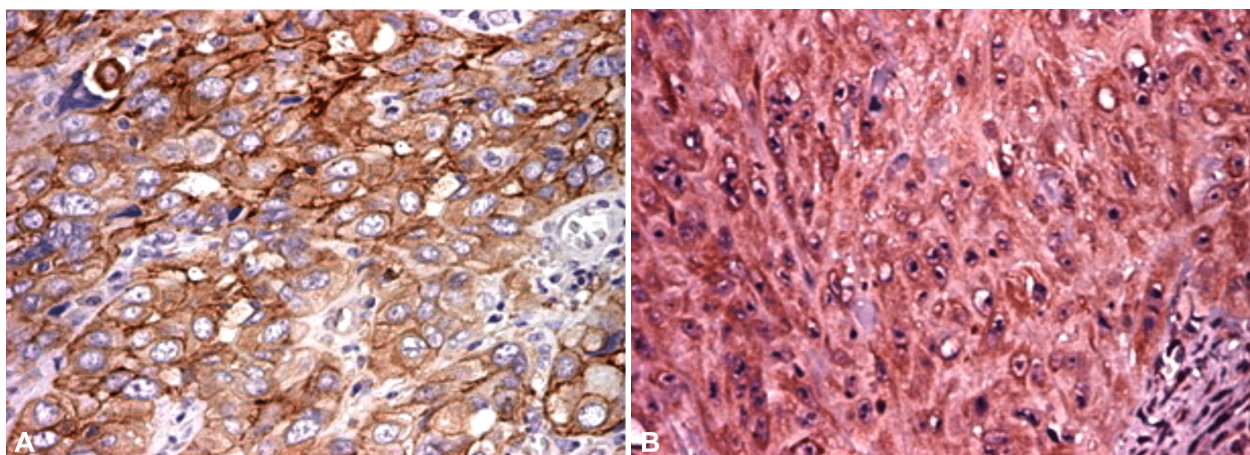

Figure 7 – *Poor differentiated squamous cell carcinoma: (A) EGFR immunostaining, ×200; (B) VEGF immunostaining, ×200.*
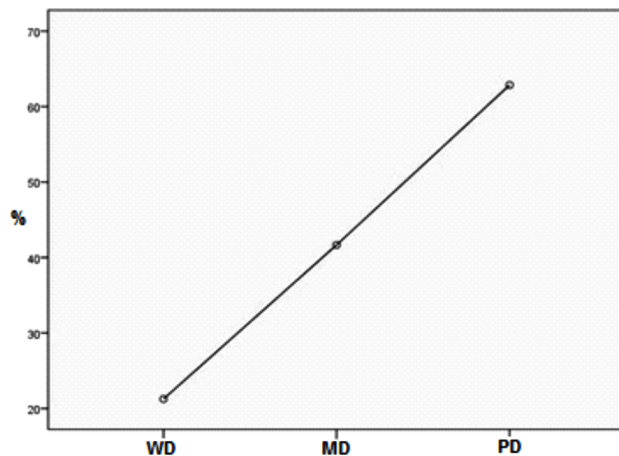
Figure 8 – *EGFR values distribution in squamous cell carcinomas. WD: Well differentiated; MD: Moderate differentiated; PD: Poor differentiated; %: Percent of EGFR positive cells.*

Figure 9 – *VEGF values distribution in squamous cell carcinomas. WD: Well differentiated; MD: Moderate differentiated; PD: Poor differentiated; %: Percent of VEGF positive cells.*

⊟ Discussion

In our study, EGFR and VEGF immunoexpression was superior for squamous cell carcinomas, compared to basal cell carcinomas, fact that was statistically significant. This highlights increased aggressiveness, metastatic potential, as well as an increased angiogenic potential of the squamous cell carcinoma, compared to basal cell carcinoma. Thus, these two types of malignant lesions have different tumor progression patterns, which support the different clinical outcome.

Regarding squamous cell carcinomas, the immunoexpression of these two markers was superior in moderate/poor differentiated forms, compared to well-differentiated forms, fact that was statistically significant.

Our study highlights statistically significant increased VEGF immunoexpression in squamous cell carcinomas, reported to basal cell carcinomas. Furthermore, significant differences were determined between different grades of differentiation of squamous cell carcinomas, increased VEGF values were associated to poor differentiated squamous cell carcinomas.

There is evidence that VEGF plays an important role in cutaneous carcinogenesis [20–23]. In human epidermis, there is a normal expression of VEGF (at low rate, though); well differentiated epidermal cell layers present a higher VEGF expression than poor differentiated epidermal cell layers [23–26]. Various studies proved *via in situ* hybridization and immunohistochemistry techniques that VEGF levels are increased in tumor cells, compared to normal epidermal cells [20–23]. Tumor cells of human basal cell carcinomas present decreased VEGF expression [10, 20–22], while stained cells are present predominantly at the tumor periphery [10, 20]. In contrast, squamous cell carcinomas, which are more aggressive tumors than basal cell carcinomas, present increased and widespread expression, with overexpression in tumor cells, which are localized around inflammatory cells [10, 20, 21]. Moreover, VEGF expression is increased in poor differentiated squamous cell carcinomas comparing to well-differentiated squamous cell carcinomas [10]. Vascular density is also increased in squamous cell carcinomas, especially in advanced squamous cell carcinomas, compared to normal skin, actinic keratosis, basal cell carcinomas or early stage squamous cell carcinomas [20, 22, 23].
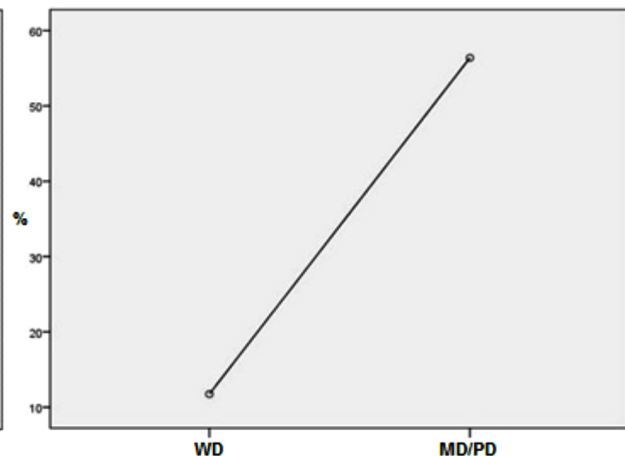
EGFR immunoexpresion revealed by our study was significantly increased in squamous cell carcinomas compared to basal cell carcinomas. Statistically significant increased EGFR values were also observed in moderate/poor differentiated squamous cell carcinomas in comparison with well-differentiated squamous cell carcinomas. This aspect suggests that extended studies are needed in order to validate the EGFR prognosis and therapy potential for eyelid carcinomas.

Other studies report a massive and constant EGFR expression in only one third of the studied cases [24]. In primitive cutaneous squamous cell carcinomas, the staining intensity is frequently weak and can be compared to basal layers of the normal epithelium, except for poor differentiated squamous cell carcinomas, which lose the membrane staining and occur the cytoplasm staining [23–25].

Shimizu *et al.* [18] reports that EGFR staining intensity was negatively correlated to differentiation grade in squamous cell carcinomas. The publication, which studied both primary tumors and metastasis in five patients with cutaneous squamous cell carcinoma, determined EGFR overexpression in four out of five cases. The staining intensity was weaker in primary tumors, which were poor and focal positive, compared to metastasis [18, 24].

Another study highlights that EGFR expression was not necessarily associated with an increased risk of locoregional or distant metastasis and there were no significant EGFR-related differences regarding survival. In cases with locoregional lymph node metastasis, EGFR expression was not strictly correlated to survival time. Though a significant difference in survival decrease was not seen, there was a trend suggesting that EGFR overexpression is correlated to poor prognosis [26].

Lichtenberger *et al.* [27] describe in their study a new VEGF function, besides its well-known angiogenic role. In a study conducted on K5-SOS-dependent mouse skin tumor model, the authors prove that *in vivo* autocrine VEGF is required for epithelial tumor cell proliferation in a cell-autonomous and angiogenesis-independent manner. In human squamous cell carcinoma, there is a similar mechanism, which might be used by human epithelial

tumors with Ras oncogenic pathway signaling. The same authors demonstrate that in the absence of epidermal VEGF and EGFR expression, tumor development was completely inhibited. Thus, a synergistic, tumor-promoting effect of VEGF and EGFR signaling in cancer cells is proved; similar results were obtained using VEGF and EGFR pharmacological inhibitors [27].

⧉ Conclusions

The markers used in this study were found to be associated with the acquisition of aggression and angiogenic phenotypes by analyzed carcinomas. In this regard, EGFR and VEGF prove useful for characterizing the biological behavior of eyelid carcinomas and constitute potential therapeutic targets.

Conflict of interests

The authors declare that they have no conflict of interests.

Author contribution

Andrei-Theodor Bălăşoiu and Raluca Niculina Ciurea equally contributed to the manuscript.

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