Pathology – Research and Practice 212 (2016) 101–105

Contents lists available at ScienceDirect

Pathology – Research and Practice

journal homepage: www.elsevier.com/locate/prp

Original article

Increasing demonstration of angiogenic markers in skin neoplastic lesions

Carla Mendonça de Almeida[a,1], Sabina Ferreira de Jesus[a,1], Fabiano de Oliveira Poswar[a,b], Emisael Stênio Batista Gomes[a], Carlos Alberto de Carvalho Fraga[a], Lucyana Conceição Farias[a], Sérgio Henrique Souza Santos[c], John David Feltenberger[d], Alfredo Maurício Batista de Paula[a], André Luiz Sena Guimarães[a,*]

[a] Department of Dentistry, Universidade Estadual de Montes Claros, Montes Claros, MG, Brazil
[b] Department of Medicine, Universidade Estadual de Montes Claros, Montes Claros, MG, Brazil
[c] Institute of Agricultural Sciences, Universidade Federal de Minas Gerais, Montes Claros, MG, Brazil
[d] Texas Tech University Health Science Center, Lubbock, TX, USA

ARTICLE INFO

Article history:
Received 17 September 2015
Received in revised form 2 November 2015
Accepted 3 December 2015

Keywords:
Basal cell carcinoma
Squamous cell carcinoma
Actinic keratosis
Skin cancer
Membrane-type matrix metalloproteinase 1
Matrix metalloproteinase-2

ABSTRACT

Background: Skin cancer represents the most common worldwide malignancy. Angiogenesis is an important factor in tumor growth and metastasis. Given these facts, the purpose of the current study was to compare the levels of angiogenic proteins in the context of the most common malignant and premalignant skin lesions.
Methods: Immunohistochemistry of CD31, HIF1A, VEGFR1 and VEGFR2 was performed in basal cell carcinoma (BCC), actinic keratosis (AK) and squamous cell carcinoma of the skin (SCCS).
Results: SCCS presented with increased levels of HIF1A, VEGFR1 and VEGFR2 in comparison to AK. In addition, SCCS also demonstrated increased levels of HIF1A to BCCLR or BCCHR. BCC presented with more vessels than AK. However, no correlation was observed among CD31, HIF1A, VEGFR1 and VEGFR2.
Conclusions: SCCS presented with higher levels of HIF1A, VEGFR1 and VEGFR2, while BCC demonstrated an increased number of vessels in relation to AK. These data suggest that antiangiogenic therapy might be useful for skin cancer treatment.

Introduction

Skin cancer represents the most common malignancy worldwide [12,13]. Some forms of malignant skin neoplasia, such as cutaneous T cell lymphoma, Karposi's sarcoma, Merkel cell carcinoma, sebaceous cell carcinoma, atypical fibroxanthoma and microcystic adnexal carcinoma, comprise only about 1% of all forms of skin malignancies [1]. On the other hand, non melanoma skin cancers (NMSC), such as basal cell carcinoma (BCC) and squamous cell carcinoma of skin (SCCS), are responsible for about 70% and 16% of the cases, respectively [27].

BCC appears without a precursor lesion [1,13,20] and might be locally rapidly destructive. BCC metastasis are rare [13]. BCC can be histologically classified into nodular, micronodular, superficial, sclerosing/morpheic or infiltrative subtypes [20]. Nodular and Superficial BCC are considered less aggressive, with lower recurrence rates than other subtypes [4]. Actinic keratosis (AK) is the potentially malignant form of the SCCS, and about 8 to 20% of AK cases will be associated with malignant transformation [3,20]. The overall metastatic rate of SCCS is estimated to be 3 to 10% depending on tumor location, underlying medical conditions, cell differentiation and size [13]. There is a clear positive association between solar UVR and all types of skin cancer [28]. It is important to highlight that according to field cancerization theory [29] and recent evidence [32], tumoral adjacent skin affected by UV might have molecular alterations in stroma that could induce multiple primary epithelial tumors. Cases of NMSC, particularly SCCS, can be attributed to occupational exposure, as well as recreational exposure. Intermittent exposure, which can occur occupationally, has been found to induce melanoma [35]. Considering the molecular mechanisms that are involved with the most prevalent skin cancers, evidence suggests that that BCC etiology is highly dependent on deregulation of the hedgehog signaling pathway [7,22,26], whereas for SCCS etiology, p53-regulated pathways are of outstanding importance [15,21].

* Corresponding author. Tel.: +55 38 988313705; fax: +55 38 3224.
E-mail address: andreluizguimaraes@gmail.com (A.L.S. Guimarães).
[1] Authors equally contributed.

http://dx.doi.org/10.1016/j.prp.2015.12.004
0344-0338/

Human skin is not directly vascularized and is subject to different degrees of oxygenation in different layers, being lower in the basal layer. As expected, the constitutive expression of the Hypoxia-Inducible Factor 1α (*HIF1A*) gene in the basal layer is higher than in other layers [23]. In the case of melanocytes, which are present in basal layer, a greater expression of HIF1A protein is related to an increased potential for cell proliferation, even in normoxia [23]. On the other hand, the opposite phenomenon occurs with basal layer keratinocytes. Indeed, increased protein levels of HIF1A were associated with lower occurrences of malignant conversion in murine models of skin carcinogenesis [23]. Recently, HIF1A was observed to have an important role in cancer development and metastasis in Head and Neck squamous cell carcinoma [2,11], which has different etiologic factors compared to SCCS. In addition, evidence has demonstrated that angiogenesis is important not only for tumor invasion [8], but also for metastasis [14].

Considering the differences in clinical prognoses and molecular alterations related to the genesis of AK, BCC and SCCS, the main purpose of the current study was to compare the levels of protein related to angiogenesis in the context of the most common malignant and premalignant skin lesions.

Methods

Samples and histopathological analysis

This retrospective study was approved by the Institutional Review Board, and the corresponding document identification number is 13672009. A total of 71 paraffin-embedded, formalin-fixed samples, comprised of 27 BCC, 25 AK and 19 SCCS, were obtained in the institutional pathology laboratory. As inclusion criteria, all patients had BCC, AK or SCCS. Patients with preoperative treatment, as well as other histological types, were excluded. Hematoxylin and Eosin staining (H&E) was performed in 5 μm sections for diagnosis confirmation and pathological grading in a conventional light microscope. BCC samples were graded using the World Health Organization criteria [20,24], and subsequently divided into low risk groups, such as the nodular and the superficial types, or high risk, including infiltrating types, according to the aggressivity of their locally invasive behavior [4,33]. SCCS samples were graded using both Broders' [5] and Bryne's [6] classifications. For AK, a three-tiered keratinocytic intraepithelial neoplasia (KIN) diagnostic system was used [25]. The kappa test revealed good intra-observer concordance with the morphological analysis ($\kappa = 0.877$, $p = 0.000$).

Immunohistochemical reactions

Tissue expression of HIF-1α, VEGFR1, VEGFR2, CD31 was evaluated in 3 μm sections using an immunostaining protocol as described before [30]. Briefly, the sections were deparaffinized with xylene and rehydrated with alcohol solutions. Antigen retrieval was performed in an autoclave at 121 °C for 10 min on citrate buffer. Endogenous peroxidase activity was blocked with two baths of 0.3% hydrogen peroxide for 15 min. Mouse monoclonal antibodies for HIF1A (clone HIF-1α 67, Sigma-Aldrich, St. Louis, USA), VEGFR1 (clone RR9S, Santa Cruz Biotechnology, CA, USA), VEGFR2 (clone A-3, Santa Cruz Biotechnology, CA, USA), CD31 (clone 1A10, Novocastra Lab, New Castle, UK) were then incubated at 4 °C overnight. Detection of the primary antibody was performed through incubations with a secondary biotinilated link and streptavidin–biotin–peroxidase complex using LSAB™ kit (Dako CA, USA), followed by incubation with the chromogen diaminobenzidine. Phosphate-buffered saline was used as a wash buffer between incubations. Counterstaining was performed using Mayer's Hematoxylin. The samples were then dehydrated, and slides were mounted in ERV-mount. Liver samples were used as positive controls. Negative controls were performed by replacing the primary antibody with universal negative control mouse (Dako, Carpinteria, California, USA).

Assessment of immunoreactivity

For staining quantification, 10 fields of tumor parenchyma and stroma were photographed at 400× magnification in a Carl Zeiss (Axioskop 40 microscope and Axiovision software. Cell counting was then performed in the software ImageJ (http://rsbweb.nih.gov/ij/). Positive and negative cells in the 10 fields were summed, and the proportions of positive cells in the parenchyma and stroma were used to quantify the staining. The immunohistochemistry of HIF1A, VEGFR1 and VEGFR2 was evaluated in the tumor cells for SCCS, BCCHR and BCCLR and epithelial dysplasia in AK.

For MVD analyses, the sample was inspected at low microscopic magnification (40×) in order to become familiar with its size and shape and to identify the most appropriate fields for counting (*hot spots*). The microvessels were identified as stained endothelial cells, or transversally sectioned tubes with a single layer of endothelial cells either with or without a thin basement membrane. If two or more CD31-positive foci appeared to belong to a single continuous vessel, this was counted as one microvessel. Within each *hot spot*, five high-powered microscopic fields were studied, providing a total of 15 analyzed fields per sample. The median MVD was used as a threshold to define the values for low expression (MVD < median value) and high expression (MVD > median value). The kappa test revealed good intra-observer concordance with the immunohistochemical analysis ($\kappa = 0.999$, $p = 0.000$) for all markers.

Statistical analysis

The non-parametrical Kruskal–Wallis test was used to assess differences in staining patterns between lesions. For correlation analyses, the Pearson test was used. The kappa statistical analysis (κ) was applied in order to assess intra-observer reproducibility as it pertains to morphological and immunohistochemical analyses. A p value of less than 0.05 was considered significant. All data was typed into SPSS 18.0 for Windows (SPSS Inc., Chicago, IL, USA) and submitted to specific statistical analysis.

Results

Qualitative evaluation of immunohistochemistry

The patterns of the immunohistochemical markers are displayed in Fig. 1. The expression of HIF1A occurred in both nuclei and cytoplasm. The HIF1A intensity was strong and homogenous in SCCS. Both BCCLR and BCCHR presented heterogeneous patterns of staining and intensity. The weaker expression of HIF1A was present only in the lower epithelial layers of AK.

The expression of VEGFR1 was mainly cytoplasmic; however, occasionally, SCCS cells also presented with stained nuclei. In addition, SCCS presented with intense and homogeneous VEGFR1 staining. In BCCLR and BCCHR, VEGFR1 staining was weak and heterogeneous in comparison to SCCS. AK demonstrated VEGFR1 in all epithelial layers; however, the staining was more intense in regions closer to the dermis.

Among all groups, cells presented VEGFR2 with cytoplasmic staining.

In neoplastic lesions, most of the MVD hotspots were located in the tumor stroma, while the AK hotspots were closer to the epithelium. The staining was strong in all lesions.