Expression of heparanase in basal cell carcinoma and squamous cell carcinoma*

Maria Aparecida Silva Pinhal1,2
Alessandra Scorse Costa1
Rodrigo Lorenzetti Serrano1

Maria Carolina Leal Almeida2
Thérèse Rachell Theodoro1,2
Carlos D’Apparecida Santos Machado Filho1

DOI: http://dx.doi.org/10.1590/abd1806-4841.20164957

Abstract: **Background:** Heparanase is an enzyme that cleaves heparan sulfate chains. Oligosaccharides generated by heparanase induce tumor progression. Basal cell carcinoma and squamous cell carcinoma comprise types of nonmelanoma skin cancer. **Objectives:** Evaluate the glycosaminoglycans profile and expression of heparanase in two human cell lines established in culture, immortalized skin keratinocyte (HaCaT) and squamous cell carcinoma (A431) and also investigate the expression of heparanase in basal cell carcinoma, squamous cell carcinoma and eyelid skin of individuals not affected by the disease (control). **Methods:** Glycosaminoglycans were quantified by electrophoresis and indirect ELISA method. The heparanase expression was analyzed by quantitative RT-PCR (qRT-PCR). **Results:** The A431 strain showed significant increase in the sulfated glycosaminoglycans, increased heparanase expression and decreased hyaluronic acid, comparing to the HaCaT lineage. The mRNA expression of heparanase was significantly higher in Basal cell carcinoma and squamous cell carcinoma compared with control skin samples. It was also observed increased heparanase expression in squamous cell carcinoma compared to the Basal cell carcinoma. **Conclusion:** The glycosaminoglycans profile, as well as heparanase expression are different between HaCaT and A431 cell lines. The increased expression of heparanase in Basal cell carcinoma and squamous cell carcinoma suggests that this enzyme could be a marker for the diagnosis of such types of non-melanoma cancers, and may be useful as a target molecule for future alternative treatment. **Keywords:** Carcinoma, basal cell; Carcinoma, squamous cell; Glycosaminoglycans; Hyaluronic acid; Neoplasms; Polymerase chain reaction; Skin neoplasms

INTRODUCTION

According to data from the National Cancer Institute (INCA), nonmelanoma skin cancer is the most common cancer in Brazil. It corresponds to 33% of all malignant tumors in the country, and presents low mortality and high cure rates when detected early on. Of the types of nonmelanoma skin cancer, basal cell carcinoma (BCC) is the most common, responsible for 70% of the diagnoses, while squamous cell carcinoma (SCC), is responsible for approximately 25% of the cases. These tumors present differences in behavior, growth, and metastatic capacity.1 Both BCC and SCC present good prognoses, especially if detected in their initial stages.2

The BCC consisting of cells that resemble epidermal basal cells is the least aggressive of the types of skin cancer.3 BCC is a tumor with a low degree of malignancy, with the capability of local invasion, tissue destruction, recurrence, and a limited potential of metastasis.4 BCC is formed by the atypical proliferation of squamous cells, of an invader nature, which can provoke metastasis.5 SCC presents a considerable potential for recurrence, which is associated with the size of the tumor, degree of histological differentiation, depth of the lesion, perineural invasion, state of the patient’s immune system, and anatomic detection.6

Received on 21.07.2015.
Approved by the Advisory Board and accepted for publication on 17.11.2015.
1 Work conducted at the Faculdade de Medicina do ABC (FMABC) and the Universidade Federal de São Paulo (UNIFESP), São Paulo, SP, Brazil.
2 Financial Support: FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo); CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnologia); CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior)
Conflict of Interest: None.
3 Faculdade de Medicina do ABC (FMABC), Santo André, SP, Brazil.
4 Universidade Federal de São Paulo (UNIFESP), São Paulo, SP, Brazil.
©2016 by Anais Brasileiros de Dermatologia

An Bras Dermatol. 2016;91(5):595-600.
Individuals that develop BCC present a high risk of developing new foci of basal carcinomas, as well as other types of skin cancer, such as melanomas and SCCs.  

Exposure to ultraviolet radiation is the main risk factor associated with the genesis of BCC and SCC, which is evident due to its greater occurrence when exposed to sunlight. Studies suggest that the exposure to chronic UVB radiation activates heparanase, leading to the degradation of the heparin sulfate in the basal membrane and the increase in the interaction between the growth factor of the epidermis and the dermis.

The skin itself contains a large quantity of hyaluronic acid (HA), dermatan sulfate (DS), heparan sulfate (HS), and keratan sulfate (KS), which modulate adhesion, migration, and cell proliferation processes.  

The sulfated glycosaminoglycan include chondroitin sulfate (CS), DS, KS, heparin (HEP), and HS, while the hyaluronic acid represents a non-sulfate GAG class. The GAG can interact with distinct proteins, including chemokines, cytokines, growth factors, enzymes, and adhesion molecules, promoting the regulation of diverse biological functions.

Proteoglycans are macromolecules made up of a protein skeleton linked to GAG strains. Proteoglycans are present on the cell’s surface, intracellular granules, and the extracellular matrix, which can regulate cytokines, angiogenic factors, and growth factors. The effects of proteoglycans in various cell mechanisms in general are modulated by interactions with GAG strains or by interactions with the protein skeleton. Proteoglycans play an important role in the organization of collagen fibers and participate in biological phenomena, such as differentiation, maintenance, and organization of the extracellular matrix. HS proteoglycans are essential components of the extracellular matrix and basal membrane, responsible for the integrity of the membrane and the barrier function. HPSE has the capacity to cleave proteoglycan strains, facilitating invasion and metastasis of the tumor cells, generating oligosaccharides that increase the activity of angiogenic factors, cytokines, and growth factors, thus inducing cell proliferation, migration and inflammatory responses. The composition of the extracellular matrix is associated with the infiltration of metastatic tumor cells and inflammatory cells.

The present study sought to compare the profile of GAGs and the mRNA expression of the HPSE enzyme in SCC cell strains (A431) and non-neoplastic strains (HaCaT), as well as investigate the HPSE expression in BCC and SCC samples from surgical resections in order to compare the results between such groups and control tissues from skin obtained from plastic surgery, by means of Blepharoplasty, analyzing possible correlations between the HPSE expression and the occurrence of BCC and SCC.

METHODS

Patients

This study analyzed 30 patient skin tissue samples, with no restrictions as to race, age, or gender. To evaluate the mRNA expression of HPSE, the quantitative RT-PCR (qRT-PCR) method was applied. The samples were obtained from the Surgery Ward of the Dermatology Department of the ABC Medical School, retrieved from dermatological surgeries that had been previously recommended by this institution’s outpatient service. The samples were divided into three groups, 10 samples of SCC, 10 samples of BCC, and 10 samples of non-neoplastic skin tissue received from blepharoplasty plastic surgery, which were used as the control tissues (CTR). The collection of tissue samples were performed using a 2 mm punch and all samples in this study were stored in liquid nitrogen for processing. The procedures described in this study were approved by the Research Ethics Committee from the ABC Medical School, registered under protocol number 041/2011.

Cell strains

This study used cell strains from human keratinocytes (HaCaT) and human SCC cells (A431). The strains defined as HaCaT and A431 were cultivated in a DMEM sterile culture medium, containing 10% bovine fetal serum (FBS) and antibiotics (100 µg/mL of streptomycin and 100 UI/mL of penicillin).

Enzymatic degradation

The definition of galactosaminoglycans (CS and DS) was obtained after enzymatic degradation with specific lyases; chondroitinase AC, which specifically degrades chondroitin sulfate; and chondroitinase ABS, which degrades CS and DS. The identification of the GAGs that have been synthesized and secreted into the culture medium was conducted by means of electrophoresis in a 0.55% agarose gel in a 1,3-diaminopropane acate (PDA), 0.05M, pH 9.0, 100 Volts, for one hour, in a cooler at 4°C (Dietrich 1976). After electrophoresis had been performed, the GAGs were precipitated in agarose gel in a 0.1% cetyltrimethylammonium solution (Cetavlon) for 2 hours. The gel was dried under ventilation and heat, and was later stained with a 0.1% toluidine blue stain in 1% acetic acid and 50% ethanol. The excess stain was removed by rinsing with a bleaching solution (1% acetic acid, 50% ethanol). The stained gel, dried at room temperature, was submitted to radioautography by exposure to an X-ray. The sensibilized film was then submitted to scanning in a Cyclone™ device (Packard Instrument Company, Meriden, CT, USA), showing the S-GAGs by scintillations per minute (spm)

HA dose

The fluorimetric method was applied to determine the HA described by Martins et al.  

The quantification of HA from each sample was determined in values expressed by the µg of HA / µg of total proteins.

mRNA extraction, cDNA synthesis, and HPSE expression

The skin samples were submitted to mRNA extraction using the RNAspin kit (GE Healthcare®), following manufacturer instructions. The reverse transcription was performed by applying the protocol described by the manufacturer as of 5µg of total RNA. The mixture was incubated at 70°C, for 10 minutes. Next, 4 µL of 5X buffer solution, 2 µl of dithiothreitol 0.1 mM of Promega®, 1 µl of deoxyxynucleotide triphosphate, 10 mM of Promega®, and 1 µl of reverse transcriptase enzyme (Promega®) was added to the mixture. This solution was incubated for 10 minutes at 25°C, 50 minutes at 42°C, and 10 minutes at 70°C to obtain the cDNA.
qRT-PCR

The qRT-PCR method allows for the definition of the relative mRNA expression of HSPE, which was achieved by using the sense oligonucleotide primer 5’TGGCAAGAAGGTCTGGTTAGGAGA 3’ and antisense oligonucleotide primer 5’ GCAAGGTGTCCGATAGCAAGG 3’. The amplification was performed using the SYBR® Green PCR Master Mix Reagent (Applied Biosystems, Carlsbad, California, USA), according to the following modified protocol: 1.5 µL of forward primer at 1.5 µM, 1.5 µL of reverse primer at 1.5 µM, 3 µL of cDNA 1:10, and 6 µL of SYBR® Green Master Mix 2X. The mRNA expression of HSPE was presented in relation to the geometric average of the endogenous constitutive gene expression (-ΔCt): ribosomal protein 18S L13A (RPL13a), sense oligonucleotide primer 5´TTGAGGACCTCT GTGTATTTGTCAA3´, antisense oligonucleotide primer 5´CCTGGAGGAGAAGAGGAAAGAGA3´, and the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense oligonucleotide primer 5´TCGACAGTCAGCCGATCTTCTTT3´ and antisense oligonucleotide primer 5´GCCCAATAGCACCACCGTGA3´. All of the trials were conducted in triplicate. The ABI PRISM 7000 SDS technological platform was used under the following thermocycling conditions: 95ºC for 10 minutes, 45 cycles at 95ºC for 30 seconds and at 60ºC for 1 minute, resulting in an approximate reaction time of 2 hours.

Statistical Analysis

The statistical analysis was performed using the Prism5 program for Windows (GraphPad Prism® Software Inc., CA, USA). All variables were considered to be non-parametric, in accordance with the Kolmogorov-Smirnov test. When comparing the two groups, the Mann-Whitney test was applied, and to compare more than two groups, the Kruskal Wallis test was applied, followed by the Dunn post-hoc test. For the analyses, the quantitative variables were described by average and standard deviation, while the significance level was set as p < 0.05.

RESULTS

This study began by investigating the presence of sulfated GAG, by electrophoresis, in HaCaT and A431 cell strains. According to that illustrated in graph 1, we observed that the HaCaT and A431 cells presented a compound of electrophoretic migration that resembled CS/DS and another band corresponding to HS.

The definition of the type of CS and/or DS was determined after enzymatic degradation with specific lysis, chondroitase AC, and chondroitase ABC, as presented in graph 2. The analysis of the enzymatic digestion with chondroitase illustrated the presence of DS, which had been synthesized and secreted into a culture medium of both cell strains, HaCaT and A431 (Graph 2)

Graph 3 shows the results obtained from the quantification of DS and HS, synthesized by the HaCaT and A431 cell strains. As observed in graph 3, the HS expression was significantly greater in the A431 cells, when compared to the HaCaT cells, respectively: 119030 ± 20775 cpm / µg total protein and 21731.25 ± 831.25 cpm / µg total protein, for the cell fraction (p = 0.0022, non-paired t test) and 94835 ± 18669 cpm / µg total protein 2787 ±50 cpm / µg total protein, for the HS secreted into the medium (p < 0.0001, non-paired t test). The DS values also presented significant...
mRNA expression of HPSE obtained from qRT-PCR, as described in the Methods section. The values represent the relative expression of HPSE, using endogenous genes as the control (GAPDH, glyceraldehyde-3-phosphate-dehydrogenase, and RPL13a, ribosomal protein). HaCat, non-neoplastic human skin cell strain, and A431, human SCC neoplastic cell strain. The values represent the average and standard deviation of trials performed in triplicate. *P = 0.0048

Graph 3: Quantification of GAG sulfates in HaCaT and A431 cells

HS, heparan sulfate; DS, Dermatan sulfate. Cell fractions, quantification of GAGs synthesized by cells and the extracellular matrix (*P = 0.0022 and **P = 0.0007, non-paired t test). Medium, GAGs secreted into the culture medium (*P < 0.0001 and **P = 0.0013, non-paired t test).

Graph 4: Quantification of hyaluronic acid (HA) synthesized by HaCaT and A431 cells

HA, Hyaluronic acid. Cell fraction, quantification of HA synthesized by cells and the extracellular matrix; Medium, HA secreted into the culture medium. (*P = 0.0308, non-paired t test).

Graph 5: Heparanase Expression in different cell strains

Graph 6 illustrates that SCC and BCC, as compared to the blepharoplasty samples, presented an increased mRNA expression of the HPSE enzyme. A significant difference was observed between the mRNA of the HPSE when compared to the control and SCC samples, respectively: (0.01827 ± 0.02204)(and SCC samples, respectively: (0.02251 ± 0.2921), applying the Mann-Whitney test with p < 0.0001. In addition, the heparanase mRNA was also significantly higher in SCC (0.2251 ± 0.2921), as compared to BCC (0.03881 ± 0.06836), p = 0.0002. Nonetheless, a statistically significant difference between the control samples and patient tissues as regards SCC was identified (p = 0.0024).
Graph 6: Relative mRNA expression of HPSE. The results were obtained by analysis using qRT-PCR, as described in the Methods section. CTR, samples collected from patients that presented no neoplasias (Control); BCC, sample of basal cell carcinoma; and SCC, samples from squamous cell carcinoma. The relative expression of HPSE was obtained using the endogenous genes GAPDH and RPL13a. The strains represent the average of the values of HPSE expression in each group. The values were collected through trials performed in triplicate. CTR versus BCC *P<0.0024; CTR versus SCC **P<0.0001, and BCC versus SCC ***P = 0.0002 (non-paired t test)
strain, as compared to the HaCaT strain, presents a significant increase in HPSE expression. It can therefore be hypothesized that the increase in HPSE expression in the A431 strain may well be related to the increase in this strain’s HS.

BCC and SCC samples present an increase in the mRNA expression of the HPSE enzyme, as compared to skin that has not been affected by such types of nonmelanoma cancer. Such results suggest that the HPSE is possibly linked to cell mechanisms involved in the development of BCC and SCC.

Future studies are warranted to elucidate the mechanisms of cell signaling involved in the increase of the HPSE enzyme expression in the development of BCC and SCC.

REFERENCES

1. Inca.gov.br [Internet]. Tipos de Câncer. Pele não melanoma [acesso 21 Jul 2015] Disponível em: http://www.inca.gov.br/estimativa/2014/sintese-de-resultados-comentarios.asp.

2. Souza RJ, Mattelli AP, Corrêa MP, Rezende ML, Ferreira AC. Estimativa do custo do tratamento de câncer de pele tipo não-melanoma no Estado de São Paulo – Brasil. An Bras Dermatol. 2011;86:657-62.

3. Sampaio APS, Rivitti EA. Dermatologia. 3. ed. São Paulo: Artes Médicas; 2008.

4. Mantese SAO, Berbert AP, Alevi JSM, Menezes JSM, Sandras CMC, Moreira LR. Heparanase expression in B16 melanoma cells and peripheral blood neutrophils before transcription start site induced heparanase silencing through interference with DNA methylation. J Invest Dermatol Symp Proc. 2000;5:55-60.

5. Elkin M, Ilan N, Ishai-Michaeli R, Friedmann Y, Papo O, Pecker I, et al. Heparanase expression in circulating lymphocytes of breast cancer patients depends on the presence of the primary tumor and/or systemic metastasis. Neoplasia. 2007;9:504-10.

6. Edovitsky E, Elini M, Zcharia E, Perez T, Vlodavsky I. Heparanase gene silencing, tumor invasiveness, angiogenesis, and metastasis. J Natl Cancer Inst. 2004;96:1219-30.