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Analysis of GLUT-1, GLUT-3, and angiogenic index in syndromic and non-syndromic keratocystic odontogenic tumors

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Abstract: The aim of this study was to evaluate the immunoexpression of glucose transporters 1 (GLUT-1) and 3 (GLUT-3) in keratocystic odontogenic tumors associated with Gorlin syndrome (SKOTs) and non-syndromic keratocystic odontogenic tumors (NSKOTs), and to establish correlations with the angiogenic index. Seventeen primary NSKOTs, seven recurrent NSKOTs, and 17 SKOTs were selected for the study. The percentage of immunopositive cells for GLUT-1 and GLUT-3 in the epithelial component of the tumors was assessed. The angiogenic index was determined by microvessel count. The results were analyzed statistically using the nonparametric Kruskal-Wallis test and Spearman's correlation test. High epithelial immunoexpression of GLUT-1 was observed in most tumors ($p = 0.360$). There was a higher frequency of negative cases for GLUT-3 in all groups. The few GLUT-3-positive tumors exhibited low expression of this protein in epithelial cells. No significant difference in the angiogenic index was observed between groups ($p = 0.778$). GLUT-1 expression did not correlate significantly with the angiogenic index ($p > 0.05$). The results suggest that the more aggressive biological behavior of SKOTs when compared to NSKOTs may not be related to GLUT-1 or GLUT-3 expression. GLUT-1 may play an important role in glucose uptake by epithelial cells of KOTs and this process is unlikely related to the angiogenic index. GLUT-1 could be a potential target for future development of therapeutic strategies for KOTs.

Keywords: Odontogenic Cysts; Odontogenic Tumors; Glucose Transporter Proteins, Facilitative.

Introduction

Keratocystic odontogenic tumor (KOT), which is classified by the World Health Organization (WHO) as a benign cystic neoplasm,[1] is an important odontogenic tumor because of its potentially aggressive biological behavior characterized by its tendency to recur and of its association with Gorlin syndrome in some cases.[2,3,4]

Compared to KOTs associated with Gorlin syndrome, non-syndromic KOTs (NSKOTs) have been suggested to exhibit a less aggressive behavior

characterized by lower growth and infiltration capacity and lesser tendency to recur.[5,6,7] Studies investigating extracellular matrix composition[8] and the expression of proteases[9,10] and proteins involved in cell cycle regulation and bone remodeling,[6,11] as well as those on the characteristics of fibroblasts found in the tumor stroma,[11] emphasize the existence of a more aggressive biological behavior of KOTs associated with Gorlin syndrome when compared to NSKOTs.

The expression of proteins involved in the transport of glucose and in the formation of new blood vessels is important for increasing the uptake of nutrients by metabolically active cells.[12] Among the different proteins involved in glucose uptake into the intracellular medium, glucose transporters 1 (GLUT-1) and 3 (GLUT-3) have been identified in normal human tissues as well as in malignant neoplasms.[13,14] In the latter, the expression of these GLUTs has been associated with a more aggressive biological behavior of the tumor characterized by the presence of lymph node metastases, disease recurrence, and lower survival rates of the patients.[12,15,16,17] Despite these important findings, we found only few studies in the English literature (PubMed database) that investigated the expression of GLUTs in odontogenic lesions.[18,19,20]

The formation of new blood vessels from pre-existing vascular structures, a process called angiogenesis, involves the participation of different intracellular signaling pathways with proangiogenic and antiangiogenic functions.[21,22] This complex process, which can be measured by determining the angiogenic index with antibodies against endothelial cell epitopes such as CD34, has been recognized as an important event in the development and progression of odontogenic cysts and tumors, including KOTs.[23,24,25] Although a crucial process in tumor progression, angiogenesis may not accompany the growth of tumor cells, with the consequent emergence of hypoxic areas in the tumor, a fact particularly highlighted in the case of malignant tumors.[26,27] In this respect, neoplastic cells increase the uptake and metabolism of glucose in order to adapt to and survive under the hypoxic conditions of the microenvironment.[12,26]

Based on these findings, the objective of the present study was to evaluate the immunohistochemical expression of GLUT-1 and GLUT-3 in NSKOTs and KOTs associated with Gorlin syndrome (SKOTs) and to establish correlations with the angiogenic index in order to provide a better understanding of the differences in the biological behavior of these tumors.

Methodology

Forty-one paraffin-embedded KOT specimens, including 17 primary NSKOTs, seven recurrent NSKOTs and 17 SKOTs, obtained from the archives of the Laboratories of Oral Pathology of the University of Fortaleza (Unifor) and of the Department of Dentistry, State University of Paraíba (UEPB), were selected for this study according to the criteria described below.

The sample size was defined based on the number of cases available in the archives of the reported laboratories. In all cases, the histopathological diagnosis was made according to the Third WHO Classification of odontogenic tumors.[28] All patients with Gorlin syndrome were diagnosed according to the criteria proposed by Evans et al.[29] and had multiple KOTs. The patients with NSKOTs had single tumors and were submitted to clinical and radiographic examination to exclude the presence of other manifestations of Gorlin syndrome. Only KOT cases with sufficient biological material for immunohistochemistry were included in the sample. KOTs identified as secondarily inflamed after histopathological examination were excluded. The study was approved by the local ethics committee (protocol number 631.261).

Immunohistochemistry

The material fixed in 10% formalin and embedded in paraffin was cut into 3-µm thick histological sections and mounted on glass slides prepared with organosilane adhesive. The tissue sections were deparaffinized and immersed in 3% hydrogen peroxide to block endogenous peroxidase, followed by washing in phosphate-buffered saline (PBS). The clones, dilution, and antigen retrieval condition for the anti-GLUT-1, anti-GLUT-3 and anti-CD34 antibodies are shown in Table. The sections were incubated with the primary antibodies in a moisture chamber. The sections were then washed twice in PBS and treated with the polymer complex

Table. Catalog number/clone, specificity, manufacturer, dilution, antigen retrieval, and incubation of primary antibodies.

Catalog number/clone	Specificity	Manufacturer	Dilution	Antigen retrieval	Incubation
GTX 15309	GLUT-1	GeneTex Inc., San Antonio, TX	1:400	Citrate, pH 6.0, Pascal, 3 min	60 min
GTX 15311	GLUT-3	GeneTex Inc., San Antonio, TX	1:400	Citrate, pH 6.0, Pascal, 3 min	60 min
QBEnd-10	CD34	Dako, Carpinteria, CA	1:50	Tris-EDTA, pH 9.0, Pascal, 3 min	18 h

(Advance™ HRP, Dako, Carpinteria, CA, USA) at room temperature. Peroxidase activity was visualized by immersing the sections in diaminobenzidine (Liquid DAB+, Dako, Carpinteria, CA, USA), which resulted in a brown reaction product. Finally, the sections were counterstained with Mayer's hematoxylin and mounted with a coverslip.

Erythrocytes and any inflammatory cells inside blood vessels were used as positive internal controls for GLUT-1 and GLUT-3, respectively. For the anti-CD34 antibody, the positive control consisted of pyogenic granuloma sections. As negative control, the samples were treated as described above, except that the primary antibody was replaced with a solution of bovine serum albumin in PBS.

Immunostaining analysis

After processing of the histological sections and immunohistochemical treatment, each specimen was analyzed under a light microscope (Leica DM 500, Leica Microsystems Vertrieb GmbH, Wetzlar, Germany) by two previously trained examiners who were unaware of whether the case was a syndromic or non-syndromic primary or recurrent tumor.

GLUT-1 and GLUT-3 expression was analyzed in the epithelial component of the tumors using a method adapted from Nonaka et al.[23] The epithelial component of the tumors was analyzed throughout its extension at 100× magnification and the percentage of positive cells was established using the following scores: 0 (negative), 1 (≤ 25% positive cells), 2 (26%–50% positive cells), 3 (51%–75% positive cells), and 4 (≥ 76% positive cells).

The angiogenic index was determined by microvessel count (MVC) using a method adapted from Maeda et al.[30] At 100× magnification, five fields showing the highest anti-CD34 immunoreactivity were selected immediately beneath the epithelial lining, and microvessels were quantified in each field at 200× magnification. The values obtained per field were summed to establish the total number of microvessels. The latter was used to calculate the mean number of microvessels per case. Single immunopositive cells, as well as clusters of immunopositive cells, were defined as microvessels, irrespective of the presence of a conspicuous lumen. Additionally, groups of single endothelial cells that could represent different sections of the same microvessel were defined as distinct microvessels.[31]

Statistical analysis

The results were analyzed statistically using the Statistical Package for the Social Sciences (version 17.0; SPSS, Inc., Chicago, USA). The nonparametric Kruskal-Wallis test was used to compare the median GLUT-1 immunoexpression scores in the epithelial component between primary NSKOTs, recurrent NSKOTs, and SKOTs. The results of the epithelial expression of GLUT-3 were only submitted to descriptive statistical analysis because of the small number of immunopositive cases.

For distribution analysis of the MVC data, the Kolmogorov-Smirnov test was applied, revealing absence of a normal distribution. Therefore, the median angiogenic indices were compared between primary NSKOTs, recurrent NSKOTs, and SKOTs using the nonparametric Kruskal-Wallis test. Possible correlations between GLUT-1 immunoexpression scores and the angiogenic index of the tumors were evaluated by Spearman's correlation test. A 5% significance level ($p < 0.05$) was adopted for all tests used in this study.

Results

GLUT-1 and GLUT-3 immunoexpression

Positive immunoexpression of GLUT-1 was observed in the epithelial component of all cases.

In the SKOT group, cases classified as score 4 were the most frequent (n = 10; 58.8%) (Figure 1A), followed by scores 3 (n = 4; 23.5%), 1 (n = 2; 11.8%), and 2 (n = 1; 5.9%). There was a predominance of score 4 cases among primary NSKOTs (n = 11; 64.7%), followed by scores 3 (n = 5; 29.4%) and 2 (n = 1; 5.9%) (Figure 1B). In the group of recurrent NSKOTs, cases classified as scores 4 (n = 3; 42.9%) and 2 (n = 2; 28.6%) were slightly more

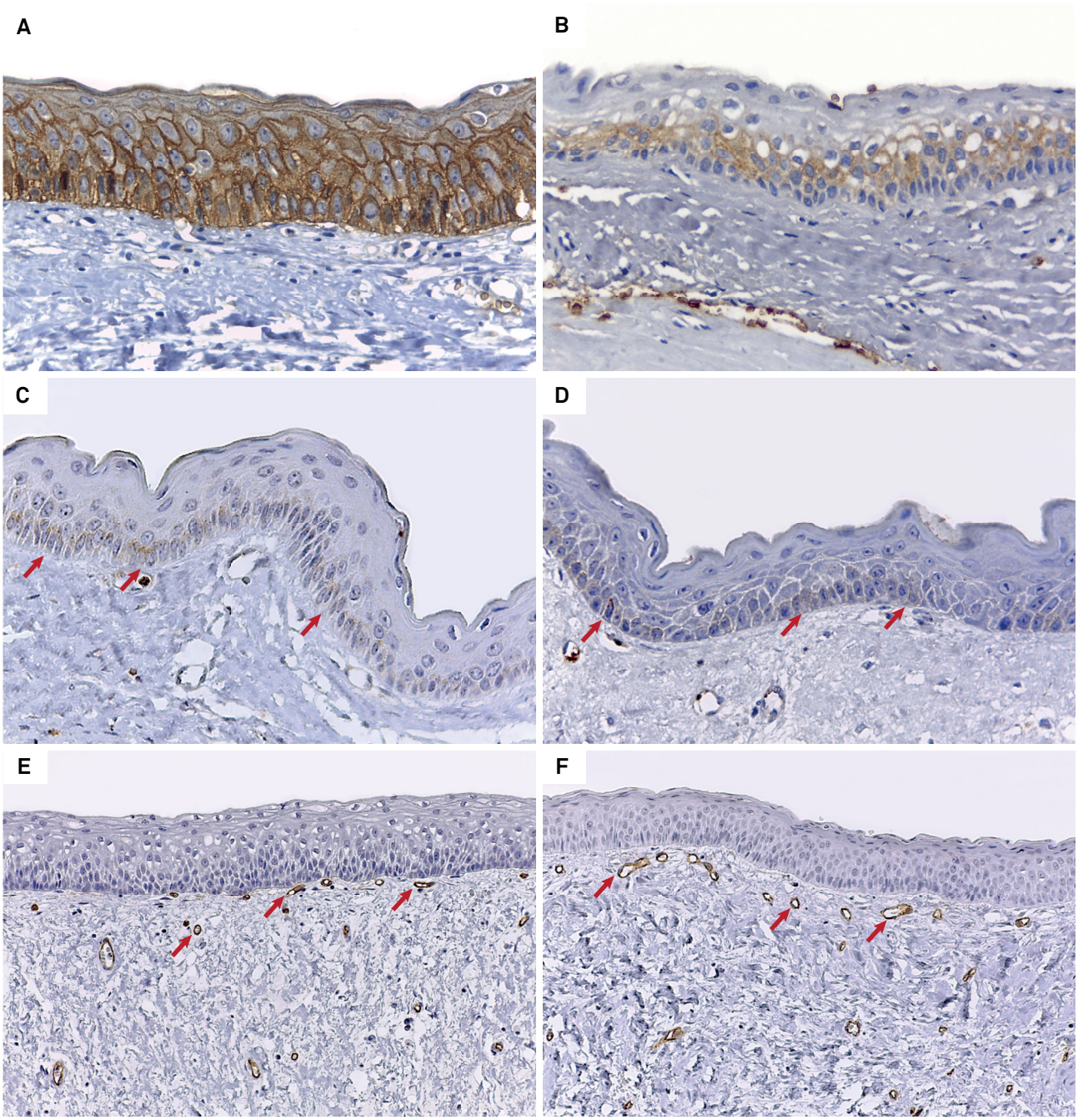

Figure 1. A) Epithelial immunoexpression of GLUT-1 in more than 76% of cells (score 4) in SKOT (Advance, 400×). B) Immunoexpression of GLUT-1 in 26%-50% of epithelial cells (score 2) in primary NSKOT (Advance, 400×). C) Epithelial immunoexpression of GLUT-3 (arrows) in less than 25% of cells (score 1) in recurrent NSKOT (Advance, 400×). D) Immunoreactivity to GLUT-3 (arrows) in less than 25% of epithelial cells (score 1) in SKOT (Advance, 400×). E) Vessels immunoreactive to anti-CD34 antibody (arrows) in SKOT (Advance, 200×). F) Variably sized vessels labeled by anti-CD34 antibody (arrows) in primary NSKOT (Advance, 200×).

frequent when compared to cases classified as scores 3 (n = 1; 14.3%) and 1 (n = 1; 14.3%). The nonparametric Kruskal-Wallis test revealed no significant difference between groups (p = 0.360) (Figure 2).

Analysis of the epithelial immunoexpression of GLUT-3 revealed a higher frequency of negative cases in all groups. In the few GLUT-3-positive cases, expression of this protein in the epithelial component was low and all cases were classified as score 1. Positive GLUT-3 immunostaining was observed in only three (17.6%) of the 17 primary NSKOTs and in two (28.6%) of the seven recurrent NSKOTs (Figure 1C). Only four (23.5%) of the 17 SKOTs exhibited GLUT-3 immunoexpression in the epithelial component (Figure 1D).

Angiogenic index

Anti-CD34 immunostaining was observed in single endothelial cells, in clusters of endothelial cells without a distinct vascular lumen, and in vessels with conspicuous lumens. Analysis of the angiogenic index based on MVC revealed a mean number of 59.38 (range: 37.8–83.0) vessels in SKOTs (Figure 1E). The mean vessel number was 62.81 (range: 41.6–88.6) in primary NSKOTs (Figure 1F). Finally, a mean number of 65.88 (range: 34.0–104.4) vessels was observed in recurrent NSKOTs. The nonparametric Kruskal-Wallis test revealed no significant difference between groups (p = 0.778) (Figure 3).

No significant correlations were observed between GLUT-1 immunoexpression scores and the angiogenic index in SKOTs (p = 0.064; r = 0.459), primary NSKOTs (p = 0.303; r = 0.265) or recurrent NSKOTs (p = 0.984; r = -0.009).

Discussion

Similarly to other tumors, KOTs are characterized by the loss of heterozygosity in tumor suppressor genes such as *P16*, *TSLC1*, *FHIT*, and *PTCH*.[2,32] Mutations in the *PTCH* gene, which are related to the development of Gorlin syndrome,[7,33] have been identified as the most important genetic alteration in KOTs.[33] Although NSKOTs and SKOTs apparently share the same pathogenesis – which consists of the aberrant activation of the SHH signaling pathway as a result of mutations in the *PTCH* gene,[34,35] studies have suggested a lower growth and infiltration capacity, as well as a lesser tendency to recur, for non-syndromic tumors.[5,6,7]

The expression of proteins involved in glucose transport and angiogenesis is important for increasing the uptake of nutrients by metabolically active cells.[12] In this respect, GLUT-1 and GLUT-3 are important proteins involved in the transport of glucose into the intracellular medium. The overexpression of these proteins has been associated with a more aggressive biological behavior of different malignant tumors,

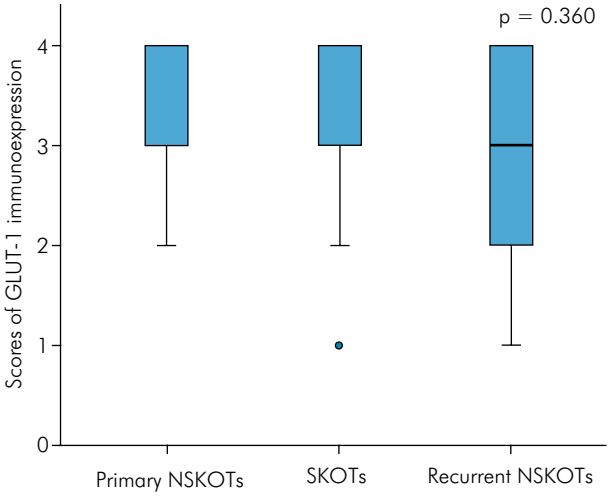

Figure 2. Box plot chart illustrating the scores for GLUT-1 immunoexpression according to KOT groups.

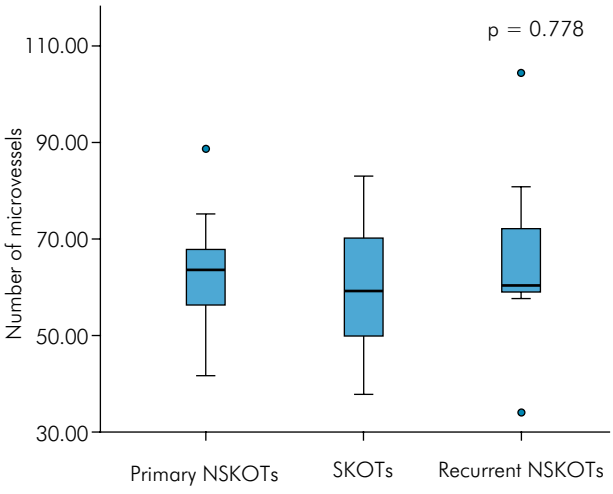

Figure 3. Box plot chart illustrating the number of microvessels according to groups of KOT.

including carcinomas of the oral cavity, larynx, lung, and breast, which is characterized by the presence of lymph node metastases, disease recurrence, and lower survival rates of the patients.[12,15,16,17]

In the present study, positive immunoexpression of GLUT-1 in the epithelial component was observed in all KOT cases, with a higher frequency of score 4 (≥ 76% positive cells). These findings indicate a high expression of GLUT-1 in the epithelial component of these tumors and suggest an important role of this protein in glucose uptake by epithelial tumor cells. On the other hand, no significant differences in GLUT-1 immunoexpression were observed between NSKOTs and SKOTs, suggesting that the expression of this glucose transporter is not implicated in the differences in the biological behavior of these tumors.

With respect to GLUT-3, a higher frequency of negative cases was observed in all KOT groups. Furthermore, the few GLUT-3-positive cases exhibited low expression of this glucose transporter in the epithelial component, classified as score 1 (≤ 25% positive cells). Similar findings have been reported in a study with dentigerous cysts, ameloblastomas, and KOTs.[20] Taken together, these findings suggest that the use of GLUT-3 for glucose uptake is a rare mechanism in KOTs, which is used by a small number of cells in the epithelial component of these tumors. Furthermore, the results indicate that the differences in the biological behavior of NSKOTs and SKOTs are not related to GLUT-3 expression.

There are only few studies[18,19,20] investigating the expression of GLUTs in odontogenic lesions (PubMed database). Rumayor et al.[18] examined the immunohistochemical and ultrastructural characteristics of ghost cells in calcifying cystic odontogenic tumors, pilomatrixomas, and craniopharyngiomas and observed restricted GLUT-1 immunoexpression in viable cells immediately adjacent to ghost cells. According to these authors, since cell metabolism probably slows down during the transformation of ghost cells, GLUT-1 expression in these transitional cells could be related to hypoxia.

On the basis of the findings of Rumayor et al.,[18] higher GLUT-1 and GLUT-3 immunoreactivity would be expected for cells of the superficial epithelial layers of KOTs since they are more distant from connective tissue vascularization and thus in a microenvironment that is more prone to hypoxia. However, all KOTs evaluated in the present study exhibited predominance of GLUT-1 and GLUT-3 immunostaining in deeper layers of the epithelial component and immunoreactivity to these proteins tended to be absent in cells of the superficial layers. This pattern of GLUT expression in KOTs may be related to the greater need of glucose uptake by metabolically active cells located in deeper layers of the epithelial component of these tumors. Also within this context, the predominant expression of GLUT-1 and GLUT-3, but particularly of GLUT-1, in deeper epithelial layers may be one of the reasons for the potentially aggressive behavior of these tumors. Taken together, these findings can improve the current knowledge of molecular pathways involved in KOTs. In addition, GLUT-1 could be suggested as a potential target for future development of therapeutic strategies for KOTs.

In line with this suggestion, Sánchez-Romero et al.[19] observed high GLUT-1 immunoexpression in the epithelial cells of ameloblastic carcinomas, followed by ameloblastomas and tooth germs. Moreover, the expression of this glucose transporter was significantly higher in solid ameloblastomas than in unicystic ameloblastomas. According to these authors, GLUT-1 overexpression could be related to aggressiveness in both ameloblastomas and ameloblastic carcinomas.

In view of the limited diffusion capacity of oxygen and glucose from blood vessels,[36] angiogenesis is an essential event for tumor development and progression.[21,22] However, the formation of new blood vessels may not accompany the growth of neoplastic cells and may result in the emergence of hypoxic areas in the tumor.[26,27] In this respect, hypoxia-inducible factor 1 (HIF-1) plays a key role in the adaptation of neoplastic cells to hypoxia by promoting transcriptional activation of a repertoire of genes, such as those encoding GLUT-1 and vascular endothelial growth factor (VEGF).[22,26,37] The latter has been described as an important proangiogenic factor both under physiological and pathological conditions.[23,37]

Taken together, the results reported herein emphasize the important relationship between hypoxia, glucose uptake into the intracellular medium,

and angiogenesis, particularly in tumors. In agreement with this suggestion, studies on endometrial adenocarcinomas and ovarian carcinomas have shown a positive correlation between GLUT-1 expression and microvessel density in the tumors.[37,38] On the other hand, Kitamura et al.[39] reported an inverse correlation between tumor glycolytic metabolism and angiogenesis in hepatocellular carcinomas. Kubo et al.[40] observed an association between GLUT-1 immunoexpression and low microvessel density in osteosarcomas and questioned the relationship between glycolytic metabolism and angiogenesis in some malignant tumors. In the present study, no significant correlations were observed between GLUT-1 expression and the angiogenic index in KOTs.

Considering the pattern of GLUT-1 and GLUT-3 immunoexpression in the epithelial component of KOTs observed in the present study, the lack of correlation between GLUT-1 expression and the angiogenic index is an expected result. As identified in the present study, GLUT-1 immunoexpression predominated in the deeper layers of the epithelial component of KOTs. Since these epithelial cells are closer to the connective tissue vascularization, they are found in a microenvironment that is less prone to hypoxia. Accordingly, Sánchez-Romero et al.[19] observed a predominance of a prostromal expression pattern of GLUT-1 in ameloblastomas, which may be induced by alternative pathways to hypoxia. Taken together, these findings support the suggestion that, in the epithelial component of KOTs, high GLUT-1 expression is not related to a process of adaptation to hypoxia, but to the existence of cells with increased metabolic demands.

Conclusion

The results of this study suggest that the more aggressive biological behavior of KOTs associated with Gorlin syndrome when compared to NSKOTs is not related to GLUT-1 or GLUT-3 expression. High GLUT-1 expression indicates an important role of this protein in glucose uptake by epithelial cells of KOTs and that this process is unlikely related to the angiogenic index. The predominant expression of GLUT-1 in deeper epithelial layers may be related to the existence of cells with increased metabolic demands in KOTs. Taken together, these findings contribute to the knowledge of the underlying mechanisms involved in the biological behavior of KOTs and support GLUT-1 as a potential target for future development of therapeutic strategies for these tumors.

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