Expressions of GLUT-1, PK-M2 and HIF-1α and Mutation Status of BRAF in Odontogenic Keratocysts

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Objective: To investigate the expressions and clinicopathological features of glucose transporter 1 (GLUT-1), pyruvate kinase M2 (PK-M2) and hypoxia-inducible factor 1α (HIF-1α) in odontogenic keratocysts (OKCs), and to investigate the mutation status of v-raf murine sarcoma viral oncogene homolog B1 (BRAF).

Methods: Following a retrospective review of the clinicopathological data of 28 OKC cases, the expressions of GLUT-1, PK-M2 and HIF-1α in these tissue samples were detected through immunohistochemistry. The BRAF mutation statuses of all cases were examined using polymerase chain reaction amplification and direct sequencing.

Results: The expression levels of HIF-1α varied in 96.4% of OKC tissues, and there were higher positive rates of PKM2 (100%) and GLUT-1 (100%) in these tissues. None of the 28 OKC samples carried the BRAF mutation.

Conclusion: The positive expressions of GLUT-1, PK-M2 and HIF-1α indicate that patients with OKCs undergo anaerobic glycolysis to a certain extent, but these processes appear to be irrelevant to clinicopathological features and to the BRAF mutation.

Key words: BRAF gene, GLUT-1, HIF-1α, odontogenic keratocyst, PK-M2


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Odontogenic keratocysts (OKCs) are benign odontogenic epithelial lesions. They originate from the remnants of the dental lamina, including the epithelial rests of Serres or the epithelium of the oral mucosa1. OKCs are of particular interest because of their aggressive behaviour and high recurrence rates2. Notwithstanding the numerous hypotheses regarding the pathogenesis of lesions, their exact aetiology remains unclear. Recent evidence suggests that the genetic basis for OKC development relates to clonality, abnormal cell cycle, proliferation, apoptosis, tumour suppression, matrix metalloproteinase activity and abnormal signalling pathways3,4.

The biological behaviour and molecular pathology of OKCs remain controversial. Despite their high recurrence rates and locally aggressive behaviour1, OKCs are derived from odontogenic epithelial elements; however, the pathogenesis of these odontogenic tumours remains unclear. The 2005 World Health Organisation
(WHO) classification defined an OKC as an odontogenic tumour and named it a keratocystic odontogenic tumour. It was defined as “a benign intraosseous tumour of odontogenic origin, with a characteristic lining of para-keratinized stratified squamous epithelium and potential for aggressive, infiltrative behaviour”5. In the latest edition of the WHO classification, published in 2017, OKCs were classified as odontogenic cysts, owing to the ongoing debate regarding their neoplastic nature6.

The pathogenesis of OKCs remains unclear. Increasing evidence shows that metabolic reprogramming may play a significant role in the occurrence and development of tumours. Even under aerobic conditions, rapidly proliferating tumour cells still use high-rate glycolysis as their main energy supply. This increases the glucose uptake and lactic acid production of tumour cells, thus enabling them to obtain the large numbers of synthetic substances needed for survival and unlimited proliferation. This phenomenon is called the Warburg effect or aerobic glycolysis7. Thus, the energy metabolism needs of tumour cells require a very efficient sugar transport pathway. Hypoxia inducible factor 1 (HIF-1) is a nuclear transcription factor that regulates cell oxygen homeostasis; HIF-1α is the main subunit of its activity regulation. It drives glycolysis to supply energy by mediating the transcription of glucose transporter 1 (GLUT-1) and other genes to maintain the energy metabolism of tumour cells8,9. At the same time, lipid-insoluble glucose can enter malignant tumour cells and participate in further metabolism, though this can only occur with the help of transporters. Thus, GLUT-1 is considered the first rate-limiting factor of glucose metabolism in malignant tumour cells. Tumours are generally highly dependent on anaerobic glycolysis; they require an increased level of glucose uptake, which explains the overexpression of GLUT-1. In all types of cancers, the overexpression of GLUT-1 has been associated with poor differentiation and poor prognosis regarding malignant tumours10,11. Pyruvate kinase M2 (PK-M2) is the rate-limiting enzyme in the last step of the glycolysis pathway. It cannot catalyse this last step and glucose metabolism cannot be carried out, resulting in a large number of intermediates in the pathway. Thus, these intermediates are converted into pentose phosphate, and participate in amino acid synthesis and other pathways for the synthesis of nucleic acids, proteins and other biological macromolecules needed for tumour cell growth. Pyruvate kinase catalyses the conversion of phosphoenolpyruvate to pyruvate. In recent years, researchers have found that PKM2 is closely related to the maintenance of the Warburg effect and tumorigenesis12-14. It has also been shown that replacing PKM2 with its isoenzyme PKM1 in tumour cells can reverse the Warburg effect, reduce lactic acid production, increase oxygen consumption and inhibit cell growth12. HIF-1 has also been shown to be highly expressed in many tumour cells. This may be related to the regulation of the expression of glycolysis-related genes13. PK-M2 can increase the transcriptional level of HIF-1, and there is a positive feedback regulation between HIF-1 and PKM214. Few studies have investigated metabolism regarding OKCs, so in the present study, HIF-1α, GLUT-1 and PK-M2 were investigated regarding glucose metabolism to determine whether the Warburg effect also exists in OKCs.

Gene mutation profiles can greatly affect the pathological understanding and clinical management of OKCs in another important respect. Mitogen-activated protein kinases (MAPKs) are serine/threonine-specific protein kinases that transduce intracellular signals in critical biological events, including cell proliferation, differentiation, survival, death and transformation15. In this vital pathway, the v-raf murine sarcoma viral oncogene homolog B1 (BRAF), a member of the RAF family, functions as a pro-oncogene. BRAF gene mutations lead to continuous activation of the MAPK pathway, which results in abnormal cell proliferation and tumorigenesis16,17. Some recent in vitro and in vivo studies18-19 have reported that the MAPK signalling pathway contributes significantly to the pathogenesis of OKCs. The development of high-throughput deoxyribose nucleic acid (DNA) sequencing methods, such as next-generation sequencing using Illumina sequencing, has advanced studies into the MAPK signalling pathway and their association with OKC. This has revealed the BRAF V600E-mediated activation of the Ras/Raf/MAPK-extracellular signal-regulated kinase (MEK/ERK) pathway18,19. Many recent reports have suggested that high frequencies of gene alterations affecting the MAPK pathway exist in ameloblastomas20; however, previous findings regarding the BRAF V600E mutation in OKC are inconsistent. Brown et al21 reported that none of the 19 OKC cases they analysed harboured the mutation, as assessed using BRAF V600E allele-specific polymerase chain reaction (PCR). Brunner et al22, however, detected a wild-type BRAF variant in a patient with an OKC. França et al18 detected the BRAF V600E mutation in one of the 28 OKC cases they analysed, using TaqMan allele-specific Qpcr. These results appear to suggest that BRAF V600E might not play a central role in OKC pathogenesis. However, Cha et al19 detected BRAF gene mutations in 24 out of 38 cases (63.2%), indicating that this mutation might play an...
important role in the pathogenesis of OKCs.

Mutated BRAF genes play an important role in driving metabolic reprogramming. The BRAF V600E mutation has been found to significantly upregulate the expression of HIF-1α in melanoma and thyroid papillary carcinoma\textsuperscript{21,24}. In papillary thyroid cancer, BRAF mutations can also induce the overexpression of PKM2 and GLUT-1\textsuperscript{25,26}. BRAF mutations can also induce DNA strand breaks, activate the DNA damage response pathway and upregulate the expression of GLUT-1 in non-transformed epithelial cells. This provides a tumour-promoting phenotype by enhancing glucose metabolism in cells\textsuperscript{27}. The aforementioned studies show that the mechanism of tumour occurrence and development caused by BRAF mutations is related to the glucose pathway. To date, no studies have examined the relationship between BRAF gene mutations and metabolic reprogramming in OKC.

Thus, the purpose of the present study was to examine the expression of HIF-1α, PKM2 and GLUT-1 and determine whether there is metabolic reprogramming in OKC, and to detect the mutation of BRAF to clarify its relationship with metabolic reprogramming in OKC.

Materials and methods

Sample selection

Twenty-eight cases of OKC were included; the patients were diagnosed between 2009 and 2021 at the Department of Oral Pathology, Peking University School and Hospital of Stomatology, Beijing, China. Clinical data, including age, lesion location, disease course, symptoms, histological type, clinical findings and follow-up data, were reviewed. All fresh tissue and paraffin-embedded blocks with diagnoses of OKC were evaluated by two experienced pathologists using hematoxylin and eosin (HE) staining. This study was reviewed and approved by the Ethical Committee of Peking University Health Science Centre (no. PKUSSIRB-201840167).

DNA extraction

DNA was extracted from formalin-fixed paraffin-embedded tissue samples. In total, 24 paraffin-embedded OKC tissue samples were analysed. After the samples were deparaffinised with xylene, genomic DNA was extracted from the paraffin-embedded tissues using a QIAamp DNA Mini Kit (Qiagen, Duesseldorf, Germany), according to the manufacturer’s instructions. The concentration and purity of DNA were measured using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and 70 ng genomic DNA was used for PCRs.

Laser capture microdissection (LCM)

In total, 20 fresh frozen OKC samples were collected by certified medical pathologists. They were embedded in optimal cutting temperature media and HE staining was performed on the collected tissue samples to ensure the accuracy of the cases and determine the amount of epithelial tissue. Frozen sections of OKC tissues were prepared using a CM1950 cryostat (Leica, Wetzlar, Germany). The epithelial tissues of these samples were collected using laser-assisted microdissection 7000 (Leica; Fig 1). Genomic DNA of the frozen epithelial tissues was extracted using the QIAamp DNA Mini Kit according to the manufacturer’s instructions. The concentration and purity of DNA were measured using the NanoDrop 8000 spectrophotometer and 70 ng genomic DNA was used for PCRs.

PCR and direct sequencing

PCR was performed using 50 µl reaction mixture, containing 25 µl Premix Ex Taq (Takara Bio, Kusatsu, Japan), 0.5 µl of each primer (approximately 10 pmol of each primer; primer sequences: forward: 5’-TGCTTTGCTTCGGATAGGAAAATG-3’; reverse: 5’-CCACAAAAATGGATCCAGACA-3’), 22 µl nuclease-free water and approximately 70 ng template DNA. Thermocycling conditions were optimised for each primer pair and the following conditions were used: initial denaturation at 94°C for 5 minutes; 35 cycles of denaturation at 94°C for
30 seconds, annealing at 60°C for 30 seconds, elongation at 72°C for 30 seconds, then final extension at 72°C for 10 minutes. The amplified products were sequenced directly using the primers employed in the original PCR. Sequencing was performed on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems, Waltham, MA, USA). All detected mutations were confirmed via reverse sequencing, and via at least two additional independent PCR experiments.

GLUT-1, PKM2 and HIF-1α immunohistochemistry (IHC)

HE staining and IHC were performed on 3-μm thick tissue sections in all 28 cases. Negative control slides were incubated with a buffer instead of with antibodies. Sections, when available, were stained for HIF-1α, PKM2 and GLUT-1 antibodies, as listed in Table 1; the IHC reactivity for each tumour tissue sample was estimated via light microscopy. The positive rates of all markers were analysed.

Table 1  Positivity and dilution of metabolism-related protein immunoexpression in OKCs.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Pretreatment</th>
<th>Dilution</th>
<th>Company</th>
<th>−</th>
<th>+</th>
<th>++</th>
<th>+++</th>
</tr>
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<tbody>
<tr>
<td>HIF-1α</td>
<td>EDTA HIER</td>
<td>Operating fluid</td>
<td>ZSGB-Bio (Beijing, China)</td>
<td>1 (3.58%)</td>
<td>21 (75.00%)</td>
<td>3 (10.71%)</td>
<td>3 (10.71%)</td>
</tr>
<tr>
<td>PK-M2</td>
<td>EDTA HIER</td>
<td>1:150</td>
<td>OriGene (Rockville, MD, USA)</td>
<td>0</td>
<td>6 (21.43%)</td>
<td>12 (42.86%)</td>
<td>10 (35.71%)</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>EDTA HIER</td>
<td>Operating fluid</td>
<td>ZSGB-Bio</td>
<td>0</td>
<td>1 (3.58%)</td>
<td>10 (35.71%)</td>
<td>17 (60.71%)</td>
</tr>
</tbody>
</table>

HIER, heat-induced epitope retrieval; −, negative; +, weakly positive with less than 33% of epithelial cells positive; ++, moderately positive with 33% to 66% of epithelial cells positive; ++++, strongly positive with more than 66% of epithelial cells positive.

Table 2  Demographic, clinical and pathological features of the studied samples.

<table>
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<th>Case no.</th>
<th>Sex</th>
<th>Age, y</th>
<th>Site</th>
<th>BRAF (exon 15)</th>
<th>HIF-1α</th>
<th>GLUT-1</th>
<th>PK-M2</th>
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<td>+++</td>
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<td>+++</td>
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F, female; M, male; −, negative; +, weakly positive with less than 33% of epithelial cells positive; ++, moderately positive with 33% to 66% of epithelial cells positive; ++++, strongly positive with more than 66% of epithelial cells positive.
Statistical analysis

Categorical variables were expressed as percentages and compared using a Fisher exact test. Normally or near-normally distributed variables were presented as mean ± standard deviation (SD). Differences between repeated measures within a group were analysed using t tests. Statistical analysis was performed using SPSS 19.0. The level of statistical significance was set at \( P < 0.05 \).

Results

The demographic and clinical features of the OKC samples used in this study are shown in Table 2. The 28 patients with OKCs included 16 males and 12 females; 8 cases occurred in the maxilla and 20 in the mandible. The patients’ ages ranged from 3 to 68 years (median age 28.5 years). None of the 28 paraffin tissue samples carried the BRAF gene mutation, and this mutation was also not found in the 20 fresh tissue samples from these 28 cases. Nevertheless, metabolism-related proteins showed a high positive rate, among which the positive rate of HIF-\( \alpha \) was 96.4% and those of GLUT-1 and PK-M2 were both 100.0% (Table 1). Immunohistochemical reactivity was detected for both GLUT-1 and PK-M2 in the cytoplasm of parakeratinized epithelium 5-8 cell layers (Figs 2a, b, d and e). Immunohistochemical reactivity was detected for HIF-1\( \alpha \) in the nuclei and cytoplasm of epithelial cells in the prickle and superficial cell layers, and to a lesser extent within epithelial cells within the basal and parabasal cell layers (Figs 2c and 2f).

Discussion

OKCs are locally aggressive lesions with a high recurrence rate and strong proliferative activity\(^{28-30}\). The majority of malignancies, including prostate cancer, glioma, hepatocellular carcinoma and other human cancers, exhibit GLUT-1, PK-M2 and HIF-1\( \alpha \), which may enable anabolic metabolism to support cell proliferation, neoplasm spread and recurrence\(^{31-33}\). The present study reports that GLUT-1, PK-M2 and HIF-1\( \alpha \) are overexpressed in OKCs; these traits indicate that OKCs have substantial metabolic demands and that metabolic reprogramming may have a defining role in explaining the aggressive clinical behaviour, high growth potential and high recurrence rate of OKCs.

Although several treatment methods have been developed in addition to simple enucleation, surgical approaches are often limited owing to the high recurrence of OKCs, as well as their locally destructive consequences. In recent years, strategies for targeting glycolysis metabolic pathways during the treatment of malignant tumours have attracted much attention through the Warburg effect. Carrying out targeted glycolysis therapy in OKCs is a potentially valuable research direction.
Thus, the question of how to screen and determine the subtypes and functions of glycolytic enzymes with high expressions regarding OKC specificity is worthy of special attention. In addition, due to the heterogeneity and microenvironment variability of OKC cells, the expressions and activities of glycolytic enzymes may change. Furthermore, the therapeutic effects of a single glycolytic enzyme target may not be particularly effective. Multiple glycolytic enzyme target combination therapy is also worth exploring. The inhibition of glycolysis may not be enough to directly kill tumour cells, so adding inhibitors for other metabolic processes may prove more effective. Many clinical applications have revealed that a BRAF-targeted approach is crucial for identifying better methods to prevent, diagnose or treat ameloblastoma. Novel targeted therapies are required to treat or cure ameloblastoma effectively, and studies into BRAF-targeted therapies in ameloblastoma have yielded considerable advancements.

The correlation between BRAF mutations and enhanced glucose uptake, lactate generation and phosphoserine biosynthesis in colorectal cancer suggests a shift towards the glycolysis pathway. Overexpression of GLUT1, PKM2 and HIF-1 was also detected in papillary thyroid cancer, with BRAF mutations. These mutations are common in odontogenic tumours such as ameloblastomas and ameloblastic fibromas. In this study, however, no BRAF V600E mutation was detected in OKCs.

Previous research has implicated the chromosome 9q22.3-q31-mapped tumour suppressor gene PTCH1 in the development of OKCs. PTCH1 functions as a hedgehog receptor and inhibits the hedgehog signal transduction pathway by inhibiting the oncogene SMO. A study revealed that OKCs are susceptible to PTCH1 gene mutations. Inactivation of the PTCH1 gene causes its protein to lose its inhibitory effect on SMO, resulting in constitutive activation of the hedgehog pathway, which stimulates the odontogenic epithelium in the jaw to regain its proliferative potential. It has been demonstrated that hedgehog pathway activation can increase PKM2 mRNA levels, thus facilitating glycolytic reprogramming observed in medulloblastoma. Further research is required to determine whether PTCH1 gene mutations are associated with the metabolic abnormalities observed in OKCs.

Although the sample size of this study was small, the in-depth study of OKC-related gene profiles and metabolism-related targets is expected to explain their pathogenesis through the combined detection of these two aspects. This approach is an important target for the diagnosis and treatment of OKC.

**Conclusion**

In summary, the findings presented suggest that BRAF mutations do not play a significant pathogenic role in OKCs, and the role of BRAF-targeted therapy seems rather limited regarding OKCs. Metabolic reprogramming appears to play a key role in the occurrence and outcomes of OKCs. Further research is needed to establish the breadth of the mechanism and determine whether metabolic reprogramming studies for testing and therapy can support the precise medicine-based treatment of OKCs.

**Conflicts of interest**

The authors declare no conflicts of interest related to this study.

**Author contribution**

Drs Zhu YOU and Jing DU contributed to the data collection and drafted the manuscript; Dr Li Li XU contributed to the data collection; Drs He Yu ZHANG and Xue Fen LI contributed to the data analysis; Drs Zhi Peng SUN and Li Sha SUN contributed to the study design and revision of the manuscript. All authors approved the final version of the manuscript.

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