RESEARCH ARTICLE

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The Association of Ketolytic Enzymes Gene Expression Levels with Mitochondrial Activity and Content in Oral Squamous Cell Carcinoma

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Abstract

Background: Recent studies have pointed to the anti-tumour effects of a ketogenic diet (KD) in cancer. It is believed that patients with low ketolytic Enzymes gene expression levels are more sensitive and may respond better to the KD therapy. However, the ketolytic Enzymes gene expression levels and their association with mitochondrial activity and content in oral squamous cell carcinoma (OSCC) is not yet obvious. Therefore, the aim of this study was to explore the potential use of ketolytic enzymes as biomarkers for mitochondrial activity and content. **Materials and Methods:** Here we aimed to compare the mRNA expression levels of ketolytic enzymes (ACAT1, BDH1, BDH2 and OXCT1) between tumour and adjacent pre-tumor tissues of 16 OSCC patients. Additionally, we examined the association of the mitochondrial ketolytic enzymes, including ACAT1, OXCT1, and BDH1 gene expression with mitochondrial activity and content. **Results:** Our findings did not show any significant difference in ketolytic gene expression levels between tumour and pre-tumor tissues of OSCC patients. ACAT1 and BDH1 mRNA expression levels were significantly correlated with the mRNA level of ND2 in tumour of OSCC patients. The mRNA levels of ACAT1, BDH1 and BDH2 were not correlated with the mRNA expression of 16srRNA. **Conclusion:** Our data suggest that mRNA gene expression levels of BDH1 and ACAT1 correlate with the mitochondrial activity in tumour of OSCC patients. BDH2 mRNA level significantly anti-correlate with tumour grade. We offer clues on the potential of ACAT1 as a biomarker of mitochondrial activity, but future studies are needed to establish this concept.

Keywords: Oral Squamous Cell Carcinoma- Ketolytic enzymes- Mitochondria- mtDNA

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Introduction

OSCC is the sixth most common malignancy worldwide. Despite the progression of treatment methods, the five-year survival rate of patients is still less than 50% (Ferreira et al., 2021).Therefore, elucidating novel molecular markers and pathways in the assessment of OSCC carcinogenesis and treatment strategies is highly needed. Increasing evidence shows that fundamental differences exist between the metabolic pathways of normal and malignant cells (Meijer et al., 1998; Cognetti et al., 2008; Chen et al., 2017). One of the key hallmarks of cancer is metabolic reprogramming, which is crucial for metastasis. Hence, researchers became interested in studying the potential of metabolic enzymes as cancer therapeutic targets. A crucial factor for the carcinogenesis of OSCC is the metabolism of tumor (Grimm et al., 2014a), which has been shown to be associated with OSCC therapeutic strategies' resistance, such as radio- and chemotherapy treatments, as well as tumour recurrence (Grimm et al., 2013; Grimm et al., 2014b; PO et al., 2014; Zhang et al., 2014). Mitochondria are the centers of energy production and harbor important effects on cellular physiology, including ROS production, regulation of intracellular Ca^{2+} homeostasis and cell death pathways (Fontana and Limonta, 2021; Foo et al., 2021). The mitochondria have been shown to be involved in the processes of carcinogenesis(Fontana and Limonta, 2021). Although mtDNA comprises less than 1% of total cellular DNA, the proper function of normal cells relies on the gene products of mtDNA, and alteration of mtDNA has been reported in cancer progression and chemoresistance (Shoffner and Wallace, 1992; Chatterjee et al., 2006; Cullen et al., 2007; Mercer et al., 2011; Ju et al., 2014). Recent studies have shown deregulation of key metabolic enzymes in cancer cells in contrast to normal cells and their dependence on alternative metabolic pathways such as ketolysis. The deregulation of proteins in the ketone bodies metabolic pathway, including HMGCS2, acetyl-CoA acetyltransferase (ACAT1), D-hydroxybutyrate

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dehydrogenase (BDH1), 3-hydroxy-3-methylglutaryl-CoA lyase (HMGCL), 3-hydroxybutyrate dehydrogenase type 2 (BDH2), and 3-ketoacid-coenzyme A transferase 1 (OXCT1), has been shown in diverse cancers (Luo et al., 2017; Goudarzi, 2019; Li et al., 2020).

In the current study, we first analyzed mtDNA content and mitochondrial activity between tumour and adjacent pre-tumour tissues of OSCC patients. Next, we compared mitochondrial ketolytic enzymes gene expression including ACAT1, OXCT1 and BDH1 between tumour and adjacent pre-tumour tissues of OSCC patients and their association with mtDNA content and mitochondrial activity. Additionally, the correlation of mtDNA content, mitochondrial activity and ketolytic enzymes gene expression was evaluated with the grade and tumour site of OSCC patients. In summary, the main focus of this study was to address the potential of mitochondrial ketolytic enzymes as mitochondrial biomarkers of OSCC. Our results suggest that ACAT1 could be a novel biomarker of mitochondrial activity for OSCC.

Materials and Methods

Subjects

The tumour and adjacent pre-tumour tissues of 15 OSCC patients were collected from two hospitals (Bahman and Shariati hospitals) in Tehran, Iran for this case-control study. In each case, depending on the site of tumour, pre-tumour tissue was removed from the corresponding healthy part of it, which was used as a matched control for the tumour tissue. The clinicopathological features of patients are shown in Table 1. All patients had a histologically biopsy proven diagnosis of OSCC and belonged to an age group of 47–86 years. The anatomical locations of OSCC included tongue, lip/buccal mucosa, maxilla, mandibule and palate. Patients who were suffering from other diseases or were under treatment with medications that could affect their ketone body metabolism (diabetes, liver failure or thyroid disorders) were excluded. The tumours were graded histologically into grade I (well-differentiated), grade II (moderately differentiated) and grade III (poorly differentiated) based on WHO criteria. Clinical information, including age, gender and tumor location was obtained from the information files of patients. This study was performed under the ethical approval of Shahid Beheshti University of Medical Sciences IR.SBMU.MSP.REC.1400.609.

RNA extraction, cDNA synthesis and Real-Time qPCR

Total RNA was extracted from 15 paired samples of tumour and adjacent pre-tumour tissues of OSCC patients by the TRIzol reagent (RiboExTM,GeneAll) according to the manufacturer's instructions. The purity and integrity of RNA were investigated by the A260/280 ratios and agarose gel electrophoresis, respectively. Next, 500 ng of RNA was used for the cDNA synthesis by the PrimeScript 1st Strand cDNA Synthesis kit (Takara, Japan). PCR was accomplished using Ampliqon Real-Time PCR Master Mix (For SYBR Green I) in a Step-One-Plus TM real-time (ABI Applied Biosystems). The 2−ΔΔCT method was used to calculate the relative gene expression in tumour tissues of OSCC patients compared to their adjacent pre-tumours. The changes in mitochondrial activity were investigated by targeting ND2, ND3 and GAPDH genes. The primer sequences of all the studied genes are listed in Table 2.

DNA extraction

In order to extract DNA and RNA from the same tissue, after Trizol addition to the tissue and recovery of the upper clear inorganic phase for RNA extraction, the lower section was subjected to total cellular DNA extraction as follow: 250µl of extraction buffer (4M Guanidine hydrocholoride, 50mM Sodium citrate, 1M Tris, pH = 8) was added to the phenol phase and interphase of the microtubes (from which RNA had been separated) and incubated for 10 minutes in room temperature. Then microtubes were centrifuged at 12000 rpm 4˚C for 20 min. Next, the upper phase was removed and the equal volume of 100% isopropanol was added to it and incubated overnight at -70˚C. After centrifugation at 12000 rpm 4˚C for 20 min, the supernatant was removed and the pellet was washed with 70% ethanol three times. Finally, the pellet was dissolved in 20µl of elution buffer containing Tris EDTA (10 mM Tris, 0.1mM EDTA, pH = 8).

Relative quantification of mtDNA copy number (mtDNA-CN) was evaluated by qPCR targeting the mitochondrial 16srRNA and β2-microglobin genes. The PCR amplification process was performed using 100 ng of DNA as follows: one cycle at 95˚C for 10 min, 40 cycles at 95˚C for 15 sec, 60 ˚C for 20 sec, 72 ˚C for 25 sec. The qPCR results were calculated using the $2^{-\Delta\Delta C}$q method.

Statistical analysis

Data analysis was performed using GraphPad Prism version 8 and SPSS 22. Data normality was evaluated by the Shapiro–Wilk test. It should be noted that based on the normal and non-normal distributions, data were analyzed by parametric and non-parametric tests, respectively. The data is expressed as means and standard deviations (SDs). Statistical analysis was done using the paired students t-test. Correlation coefficients were calculated using the two-tailed Pearson's correlation and Spearman's correlation analysis. In all the above tests, a P-value < 0.05 was taken to be statistically significant.

Results

The mRNA expression level of ketolytic enzymes in tumour and adjacent pre-tumour tissues of OSCC patients

The gene expression of ketolytic enzymes including ACAT1, BDH1, BDH2 and OXCT1 in tumour and pre-tumour tissues of OSCC patients (n = 13 to 15) is shown in Fig. 1a to 1d. No significant difference was observed in mRNA levels of ketolytic enzymes in tumour and adjacent pre-tumour tissues of OSCC patients.

The mRNA expression of genes related to mtDNA and mitochondrial activity in tumour and adjacent pre-tumour tissues of OSCC patients

In order to compare the mtDNA content and mitochondrial activity between tumour and adjacent

pre-tumour tissues of OSCC patients, paired mRNA expression of 16srRNA, ND2 and ND3 genes was evaluated. No significant difference in the mRNA expression levels of ND2 (P = 0.3028), ND3 (P = 0.1677) and 16srRNA (P = 0.1205), between tumour and adjacent pre-tumour tissues of OSCC patients were observed (Figure 2a, b and c).

The association of ketolytic enzyme gene expression levels with mtDNA and mitochondrial activity

In order to determine if the mRNA level of ketolytic enzymes (ACAT1, BDH1, OXCT1) located in mitochondria could be a predictor of the DNA content (mtDNA) or mitochondria activity, the mRNA gene expression of the above-mentioned enzymes was correlated to that of ND2, ND3 and 16srRNA in tumour tissue of OSCC patients. Among them, the mRNA level of ACAT1 was significantly correlated with the mRNA levels of ND2 (P = 0.012, r = 0.674) and ND3 (P = 0.022, r = 0.679) in the tumour of OSCC patients Table. 3. BDH1 mRNA expression level showed a significant correlation with the mRNA level of ND2 (P = 0.018, r = 0.642) Table 3.

We then investigated the association of ketolytic enzymes, ND2, ND3 and 16srRNA gene expression with tumour grade and tumour site of OSCC patients. No significant correlation was observed with tumour site but BDH2 mRNA level was anti-correlated with tumour grade (P = 0.003, r = -0.824) Table S1.

Table 1. Clinicopathological Characteristics of of OSCC Patients

Clinicopathological Characteristic	Number of cases	%
Gender		
Female	7	46.7
Male	8	53.3
Age		
Mean	66.4	66.4
≤ 66	8	53.3
>66	7	46.7
Tumor location		
Tongue	5	33.3
Maxilla		26.7
Lip / buccal mucosa	3	20
Mandibule	2	13.3
Palate	1	6.7
Grade		
I	2	13.3
II	5	33.3
III	4	26.7

Discussion

Mitochondria have diverse and important functions in cellular physiology beyond ATP production(Carew and Huang, 2002). Alterations of mitochondrial metabolism have been shown in cancer, hence dietary therapeutic approaches could be considered (Chang et al., 2013). Chang et al. reported differential gene expression levels of ketolytic enzymes including ACAT1, BDH1, BDH2 and OXCT1 in malignant gliomas and suggested that patients with low or very low expression level of key ketolytic enzymes in their malignant gliomas may respond better to KD therapy than those patients with positive expression of these enzymes (Chang et al., 2013). The enzymes of mitochondria are encoded by both mtDNA and nuclear DNA (nDNA) (Goudarzi, 2019), thus the normal function of cells relies on the integrity and proper function of both mtDNA and nDNA as well as their content (Goudarzi, 2019). Additionally, mtDNA alterations have been reported in a variety of cancers and these alterations lead to cancer progression (Aminuddin et al., 2020). MtDNA-CN serves as a biomarker of mitochondrial function and levels of mtDNA-CN are associated with diseases, including cardiovascular disease, chronic kidney disease, and cancer (Castellani et al., 2020). Among the above-mentioned ketolytic enzymes, ACAT1 has recently been reported to play a role in cancer proliferation and tumour growth (Fan et al., 2016). Several studies have shown that cancer cells depend on aerobic glycolysis and that carbohydrate limitation decreases the blood glucose level in cancer cells while increasing the fatty acid and ketone body

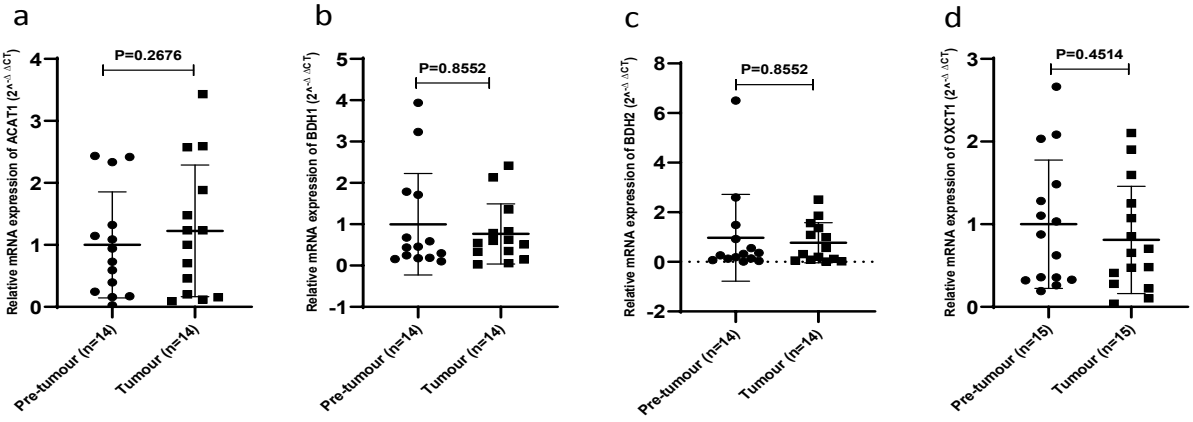

Figure 1. Expression of ACAT1 (a), BDH1 (b) BDH2 (c) and OXCT1 (d) Genes in the Tumour and Adjacent Pre-tumour Tissues of OSCC Patients (n = 13 to 15). Results are normalized to the corresponding value of the housekeeping gene (GAPDH).

Table 2. Forward and Reverse Primers used for Real-time qPCR

Primer	Sequence
BDH2 F	GCTTCCAGCGTCAAAGGAGTT
BDH2 R	CAGTTGCGAATCTTCCCGTC
BDH1 F	GAAAGTGGTGGAGATTGTCCGC
BDH1 R	TGTAGGTCTCCAGGCTGGTGAA
OXCT1 F	GCTTTGGTGAAAGCCTGGAAGG
OXCT1R	CTCTACCACTGTGGTTTCTGCAG
ACAT1 F	AAGGCAGGCAGTATTGGGTG
ACAT1 R	ACATCAGTTAGCCCGTCTTTTAC
GAPDH F	GCTCAGACACCATGGGGAAG
GAPDH R	TGTAGTTGAGGTCAATGAAGGGG
B2M F	TGCTGTCTCCATGTTTGATGTATCT
B2M R	TCTCTGCTCCCCACCTCTAAGT
ND2 F	CTTAAACTCCAGCACCACGAC
ND2 R	AGCTTGTTTCAGGTGCGAGA
ND3 F	CCGCGTCCCTTTCTCCATAA
ND3 R	AGGGGCTCATGGTAGGGGTAA
16srRNA F	GCCTTCCCCCGTAAATGATA
16srRNA R	TTATGCGATTACCGGGCTCT

utilization in normal cells. In some cancer patients, the altered expression of certain enzymes involved in ketone body metabolism deprives the tumour cells from obtaining energy from ketolysis, suggesting that KD therapy and carbohydrate limitation could selectively starve and kill cancer cells but not normal cells. It is of note that KD therapy in combination with other therapeutic strategies, such as chemotherapy and radiotherapy, could achieve satisfactory results, whereas the positive effects of KD therapy alone are still controversial. In the current study, first we investigated the mRNA gene expression levels of ketolytic enzymes, ND2, ND3 and 16srRNA by RT-qPCR on a series of 13 to 15 OSCC patients and compared the results between tumour and adjacent pre-tumour tissues of OSCC patients. Next, the potential of ACAT1, BDH1 and OXCT1 as mithochondrial biomarkers was evaluated. Our findings did not show any statistical gene expression difference of ketolytic enzymes, ND2, ND3 and 16srRNA between tumour and pre-tumour tissues of OSCC patients (Fig. 1 and Fig. 2). Taking into account the non-significant gene expression difference of ketolytic enzymes between tumour and pre-tumour tissues of OSCC patients, it seems to us that carbohydrate restriction and KD therapy might not be an effective therapeutic therapy for OSCC patients. Larsen et al. found that the activity of mitochondrial enzymes such as citrate synthase, complex I and complex IV, protein content and activity of complex II, and protein content of complex V were associated with mitochondrial content(Larsen et al., 2012). Levin et al. examined mitochondrial biogenesis in the fibroblasts of rats which were defective in cytochrome c oxidase (COX), they revealed that decreased activity of COX in rats

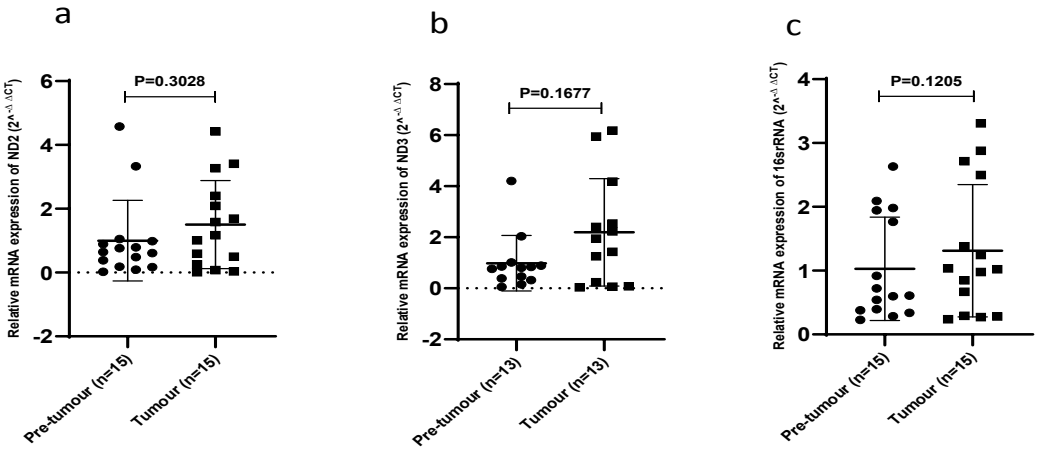

Figure 2. Expression of ND2 (a), ND3 (b) and 16srRNA (c) Genes in the Tumour and Adjacent Pre-tumour Tissues of OSCC Patients (n = 13 to 15). The results of 16srRNA, ND2 and ND3 were normalized to the corresponding value of the housekeeping gene (β2-Microglubolin) and GAPDH, respectively.

Table 3. Correlation of ACAT1, OXCT1, and BDH1 Gene Expression Levels with Mitochondrial Activity (ND2 and ND3) and content (16srRNA).

	ND2 mRNA expression		ND3 mRNA expression		16srRNA mRNA expression	
	Correlation coefficient (r)	P-Value	Correlation coefficient (r)	P-Value	Correlation coefficient (r)	P-Value
ACAT1 mRNA expression	Pearson's corr. coef.=0.674	**0.012**	Spearman's corr. coef.=0.679	**0.022**	Spearman's corr. coef.=-0.173	0.571
BDH1 mRNA expression	Spearman's corr. coef.=0.642	**0.018**	Spearman's corr. coef.=0.545	0.083	Spearman's corr. coef.=-0.044	0.887
OXCT1 mRNA expression	Pearson's corr. coef.=0.461	0.098	Pearson's corr. coef.=0.472	0.121	Spearman's corr. coef.=0.262	0.366

Bold values represent significant correlations as evaluated by Pearson's and Spearman's correlation analysis.

was related to increased mitochondrial content and ROS production (Kogot-Levin et al., 2016). From the fact that ACAT1, BDH1 and OXCT1 are encoded by nDNA but located in mitochondria, we expected to find a relationship between their gene expression levels (ACAT1, BDH1 and OXCT1 mRNA) and the mtDNA content/activity. To test this hypothesis, we evaluated the correlation of ACAT1, BDH1 and OXCT1 mRNA levels with the gene expression levels of ND2 and ND3 (as the marker of mitochondria activity) and 16srRNA mRNA (as the marker of mtDNA content). We found that among the evaluated ketolytic enzymes, ACAT1 and BDH1 are significantly correlated with mitochondrial activity Table 3.

To the best of our knowledge, this is the first study evaluating the association between ketolytic enzymes with mitochondrial activity and content. We revealed that ACAT1 could be a novel biomarker and indicator of mitochondrial activity in OSCC patients. The main limitation of this study was the number of OSCC patients, future works would be needed to measure and compare the enzymatic activities of ACAT1, BDH1 and OXCT1 between tumour and adjacent pre-tumour tissues of OSCC patients.

Author contribution statement

AG designed and supervised the study. MY was involved in the collection of samples and performed the experiments. AG and MY performed statistical analyses. AK provided samples and supervised the clinical part of the experiments. AG and MY wrote the manuscript. All authors reviewed and approved the final manuscript for submission.

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Availability of data

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

The authors declare no competing interest.

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