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Selected ALKBH dioxygenases are overexpressed in salivary gland tumours

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Salivary gland tumours (SGTs) are a heterogeneous group of benign tumours of various origins and pathologies, showing a number of DNA modifications. Previously, in malignant head and neck cancer (HNSCC), we found overexpression of ALKBH proteins, the homologs of *Escherichia coli* AlkB 2-oxoglutarate and Fe(II) dependent dioxygenase. Moreover, we proved the connection of some of these dioxygenases with cancer development. Here, we studied the expression of five of these ALKBH dioxygenases: 1, 3, 4, 5, and FTO in benign SGTs. Using Western blot analysis, we found overexpression of three proteins: ALKBH1, 4, and FTO in SGT as compared to the surrounding, unaffected tissue. ALKBH4 was overexpressed in 76% of patient samples, whereas ALKBH1 and FTO in 65% of the samples. These results differ from those obtained in HNSCC, where FTO overexpression has been observed in 90% of patient samples. We also investigated the relationships between ALKBHs' expression levels in normal and SGT tissues and identified two correlated pairs: ALKBH1-ALKBH3 and ALKBH1-ALKBH5. Additionally, in tumour tissue ALKBHs: ALKBH1, ALKBH3, ALKBH4, and ALKBH5 levels were correlated with each other. Together, these findings show that the ALKBH proteins exhibit pro cancerogenic action in SGT, even though the levels ALKBHs are generally lower in benign SGT than in malignant HNSCC. We suggest that the overexpression of the ALKBHs, especially FTO, may be used as a cancer marker and for its grading.

Keywords: salivary gland tumours, ALKBH proteins, Western blot

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Abbreviations: 2OG, 2-oxoglutarate; ALKBH, AlkB homologs; EDTA, Ethylenediaminetetraacetic acid; FPS, First progression survival; FTO, Fat Mass and Obesity-Associated; HCC, Hepatocellular carcinoma; HNSCC, Head and neck cancer; LUAD, Lung adenocarcinoma; m6Am, N62'-O-dimethyladenosine; N1 meA, N1 methyladenosine; N6meA, N6methyladenine; NSCLC, Non-small cell lung cancer; OS, Overall survival; PBST, phosphate buffer saline with 0.1% TWEEN-20; PMSF, Phenylmethylsulfonyl fluoride; PPS, Post-progression survival; RIPA, Radioimmunoprecipitation assay; SDS-PAGE, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SGT, Salivary gland tumours; WB, Western blot; WT, Warthin's tumour

INTRODUCTION

Salivary gland tumours (SGTs) are relatively rare (1-6% of neoplasms of the head and neck) and diverse in reference to origin and pathology. This heterogeneous group of tumours include as much as 24 histological subtypes (Thompson, 2006) but generally, they are divided into two groups: arising from major and minor salivary glands.

Among such tumours, Warthin's tumour (WT), also known as papillary cystadenoma or adenolymphoma, is a benign neoplasm that arises almost exclusively in the parotid (major) gland and is the second most common parotid tumour of the parotid gland that accounts for 15% of all parotid cancers. A retrospective comparative study of 96 cases of Warthin tumour indicate that it is a benign neoplasm occurring in the salivary gland area (McGurk, 2004; Teymoortash *et al*., 2006; Faur *et al*., 2008). It originates from two types of cells: myoepithelial and epithelial cells present in the salivary ducts. This tumour is the most common around the submandibular and parotid glands, but occasionally occurs around the minor salivary glands of the palate, lips and cheeks (Samolczyk-Wanyura *et al*., 2013).

A characteristic feature of neoplastic tissues is the intensification of metabolic processes resulting in an increased number of DNA modifications. This state leads to the induction of DNA repair systems that remove lesions blocking replication, which facilitates neoplastic transformation.

AlkB dioxygenase plays a role of one-protein DNA/RNA repair system in *Escherichia coli*. When induced within so called adaptive response, AlkB removes alkylation lesions from nucleic acids in the presence of the 2-oxoglutarate (2OG) and Fe (II) (Nieminuszczy & Grzesiuk, 2007). The homologs of AlkB protein have been found in almost all organisms, with nine human homologs (ALKBH1-8 and FTO) that perform various biological functions including DNA repair and RNA metabolism, histone demethylation, adipogenesis, cell cycle progression, fatty acid metabolism, carcinogenesis, etc.

Overexpression of individual proteins from the ALKBH family has been observed in various cancer types (Konishi *et al*., 2005; Hotta *et al*., 2015; Shimada *et al*., 2009, 2012; Choi *et al*., 2011; Tasaki *et al*., 2011; Gao *et al*., 2011; Xu *et al*., 2017; Yamato *et al*., 2012; Zhang *et al*., 2012, 2016; Kaklamani *et al*., 2011; Johannessen *et al*., 2013; Zhang *et al*., 2017; Zhou *et al*., 2018) indicating pro-carcinogenic function of at least some of these proteins. We have found as much as seven out of nine ALKBH proteins were overexpressed in head and neck cancer (HNSCC), with FTO protein overexpressed in 90% of HNSCC patient samples (Pilžys *et al*., 2019). Moreover, the expression level of this protein has been proved to correlate with cancer size and stage of development (Pilžys *et al*., 2019).

The aim of this study was to determine if the levels of selected ALKBH proteins were also elevated in SGT tumours as observed in malignant HNSCC.

With the use of Western blot analysis, we examined expression of five ALKBH proteins: 1, 3, 4, 5, and FTO in SGTs and their healthy surrounding tissue.

ALKBH1 acts mainly as a tRNA demethylase removing N^1 methyladenosine (N^1 meA) from various tRNAs, preferentially from tRNA on a stem loop structure (Liu *et al.*, 2016). It regulates translation initiation and elongation in response to glucose deprivation. ALKBH1 also shows DNA lyase activity that does not require 2OG and Fe (II) (Müller *et al.*, 2010). One of the roles of ALKBH1 in DNA repair is introduction of double strand breaks (DSB) at apurinic/apyrimidinic (AP) sites (Müller *et al.*, 2017). ALKBH4 localizes in the cytoplasm and nucleus and is involved in regulation of gene expression and chromatin maintenance (Tsujikawa *et al.*, 2007; Bjørnstad *et al.*, 2012). Its functions seem to be dependent on cellular localization. Like other ALKBH proteins it is a demethylase of monomethylated lysine-84 on actin, and N^6-methyladenine in DNA (Li *et al.*, 2013). FTO (fat mass and obesity associated) protein eliminates N^6methyladenine (N^6meA) adducts, the most abundant modification in eukaryotic mRNA (Jia *et al.*, 2011) in wide spectrum of substrates. Not only does it bind several RNA species: mRNA, snRNA and tRNA, but also several N^6meA adducts, including internal N^6meA in mRNA and snRNA, $N^6$2'-O-dimethyladenosine (m6Am) adjacent to the mRNA cap, and N^1 methyladenosine (N^1 meA) in tRNA (Wei *et al.*, 2018). Several studies indicate a regulatory role of FTO in transcript stability, gene expression, and multiple levels of biological processes in normal and cancerous cells.

Here, we show ALKBH 1, 4, and FTO were overexpressed in SGT.

MATERIALS AND METHODS

Clinical samples

Twenty-three tissue samples (blinded) of salivary gland tumours (originating from 11 males and 12 females) were collected at the Department of Otorhinolaryngology, Faculty of Medicine and Dentistry at Medical University of Warsaw between 2011 and 2014. Nine of the patients suffered from Warthin's tumour, further six suffered from adenoma multiforme. The research was performed in accordance with the relevant guidelines and regulations. Patients were not treated with chemo- or radiotherapy. Samples after histopathological examination have been made available in the archives. Patients agreed to the use the samples in accordance with the current procedure. The biochemical tests on the samples and their subsequent utilization according to routine procedures of biohazard were accepted by Ethical Committee. Samples were anonymized with no access to patient personal data. The clinicopathological data are presented in Table 1.

Tissue samples and Western blot analysis

Samples were prepared and analysed as described in Pilzys and coworkers (Pilžys *et al.*, 2019). Briefly, frozen samples were homogenized in liquid nitrogen and extracted with RIPA buffer (Sigma-Aldrich) supplemented with 50 mM EDTA and 4 mM PMSF in the presence of protease inhibitor cocktail (Sigma-Aldrich). Cellular de-

Table 1. Clinicopathological features of the patients suffering from salivary gland carcinomas taking a part in this study (n = 23).

Characteristic N (%) or mean [IQR]			
Age	54.5 [50.0-61.0]	Weight	66.8 [59.2-75.8]
BMI	25.0 [20.9-29.3]		
Sex			
Male	11 (48%)	Female	12 (52%)
Tumour Examination (%)			
Acinic cell carcinoma	1 (4%)	Adenoid cystic carcinoma	1 (4%)
Adenoma multiforme	6 (26%)	Ca planoepitheliale partim keratodes G1	1 (4%)
Ca planoepitheliale partim keratodes G2	1 (4%)	Lymphoma	1 (4%)
Mucoepidermoid carcinoma G3	1 (4%)	Warthin's tumour	9 (39%)
Unknown	2 (8%)		
Grading (%)			
1	1 (4%)	2	1 (4%)
3	1 (4%)	Unknown	20 (88%)
Other diseases N (%)			
Jaundice		Asthma	
Yes	1 (4%)	Yes	0 (0%)
No	16 (70%)	No	17 (74%)
Unknown	6 (26%)	Unknown	6 (26%)
Hypertension		Problems with blood coagulation	
Yes	4 (17%)	Yes	2 (8%)
No	13 (57%)	No	15 (66%)
Unknown	6 (26%)	Unknown	6 (26%)

bris was spun down and supernatant protein content was measured using the Bradford assay (Bio-Rad). Samples were diluted with SDS-PAGE loading buffer to a protein concentration of 3 µg/µl and 10 µl was loaded onto Mini-PROTEAN TGX 4-15% gradient gels (Bio-Rad). The Western blot analysis was performed with specific primary monoclonal and polyclonal antibodies used at dilutions: 1:200-500 against ALKBH1, ALKBH3, FTO (#sc-374301, #sc-376520, # sc-271713; Santa Cruz Biotechnology), ALKBH5, (#HPA007196; Sigma-Aldrich) and ALKBH4 (#19882-1-AP; Proteintech) with appropriate 1:2000 secondary anti-mouse IgG antibody (Sigma-Aldrich) or anti-rabbit IgG antibody (Santa Cruz Biotechnology) conjugated with horseradish peroxidase. All incubations were performed in 5% milk/PBST (PBST – phosphate buffer saline with 0.1% TWEEN-20). Chemiluminescence was measured using the ChemiDoc MP Imaging System (Bio-Rad). Total protein was standardized in four steps: (i) equal masses of the tissue were taken for extraction in RIPA buffer (50 mg); (ii) extract was then assayed by Bradford assay for protein content; (iii) equal amounts were loaded on the gel and verified by Coomassie blue staining or Stein Free gels imaging; (iv) protein transferred to the nitrocellulose membrane was visualized by Ponceau-S reversible staining prior to the final Western blot.

Statistical analyses

All analyses were performed using R software (version 3.3.0, www.r-project.org) with *outliers* and *gplots* packages. The significance level α of 0.05 was assumed in all statistical tests. The Shapiro-Wilk test was used to assess the agreement of ALKBHs content with the Gaussian distribution (Bonnini *et al.*, 2014). Because even after filtering of extreme values with the Grubbs' test for putative outliers (Grubbs, 1969) the vast majority of the distributions were found to be non-Gaussian, further analyses were based on the non-parametric methods. The statistical significance of the differences observed in protein levels were tested using Wilcoxon tests; the paired (signed-rank) version for individuals or non-paired version (rank-sum, Mann-Whitney) for the whole group. Correlation matrixes were calculated for the levels of seven proteins using Spearman's rank correlation coefficients. Hierarchical cluster analysis of these matrixes was performed according to the Ward criterion.

Ethics approval and consent to participate

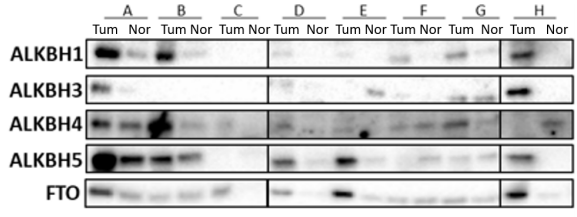
All participants were informed about the purpose of the study and gave their written informed consent. The study was approved by Warsaw Medical University Bioethical Committee permission for working with human tissues/tumours no. KBO/17/11 April 12th, 2011. All research was performed in accordance with the relevant guidelines and regulations.

RESULTS AND DISCUSSION

This study included 23 SGT patients.. The clinicopathological characteristics, including main data concerning patients, tumour grading, and accompanying diseases, are shown in Table 1.

Using Western blot (WB) analysis, we detected overexpression of three out of the five examined ALKBH proteins (ALKBH1, 3, 4, 5, and FTO) in SGT tu-

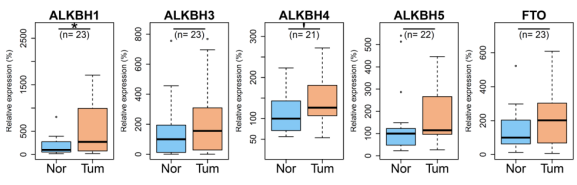
A)



B)

Protein	Protein level: Tumour vs. normal			N	p
	Higher	Lower	Undetectable		
ALKBH1	15 (65%)	3 (13%)	5 (22%)	23	**5.52E-04***
ALKBH3	10 (43%)	8 (35%)	5 (22%)	23	2.40E-01
ALKBH4	16 (76%)	5 (24%)	0 (0%)	21	**1.86E-03***
ALKBH5	13 (59%)	9 (41%)	0 (0%)	22	5.42E-02'
FTO	15 (65%)	7 (30%)	1 (4%)	23	**1.49E-02***

C)



D)

	ALKBH1	ALKBH3	ALKBH4	ALKBH5	FTO
1	3.1	1.5	1.1	2.0	2.0
2	0.5	0.1	0.9	0.6	0.4
3	3.9	ND	3.2	1.5	0.9
4	20.2	89.1	1.5	8.3	3.8
5	ND	ND	1.1	0.8	4.8
6	3.9	9.0	1.2	5.9	8.6
7	15.2	18.4	NA	2.7	7.9
8	2.1	0.4	1.6	0.5	0.6
9	0.7	0.3	0.9	0.7	0.6
10	4.6	2.0	1.0	4.6	5.8
11	1.5	0.6	0.9	0.6	0.8
12	2.3	0.6	1.4	0.9	1.4
13	1.9	ND	1.9	1.3	2.2
14	5.8	2.4	2.1	4.6	3.6
15	1.3	0.7	1.2	0.7	ND
16	4.4	0.7	1.2	2.9	3.4
17	0.3	0.3	0.8	0.3	0.4
18	5.2	3.2	1.6	2.6	2.5
19	6.2	5.0	1.9	2.6	1.4
20	ND	ND	0.8	0.9	1.1
21	ND	16.4	NA	NA	2.2
22	ND	ND	1.8	4.5	0.2
23	ND	1.9	1.4	1.4	1.8

Protein not detected in patient (ND) | Protein level decreased in tumour | Protein level increased in tumour | Data not available (NA)

Figure 1. ALKBH expression in salivary gland tumours.
(**A**) WB analysis of ALKBH expression in SGT samples; Nor, normal periphery; Tum, tumour; A–H, tumour samples. (**B**) ALKBHs expression in tumour and normal tissues from SGT patients. Samples were classified into three groups according to the expression level of each protein: (i) stronger signal from tumour than normal surrounding; (ii) weaker signal from tumour than normal surrounding; (iii) no detectable expression of the proteins in the normal and tumour tissue. N, number of patients; *p*, *p*-value obtained from the Wilcoxon signed-rank test for paired samples. (**C**) Nonparametric Wilcoxon rank-sum test (for groups) was performed. n- number of samples from each group; *p*-values with Benjamini-Hochberg adjustment: '$p < 0.1$; *$p < 0.05$; **$p < 0.005$ (**D**) Heat map of changes of individual protein expression in SGT. Fold changes were calculated for tumour *vs.* adjacent normal tissue and colour coded in each rectangle. White, no detectable expression of the particular protein in cancer and adjacent tissue. Blue, decreased relative protein level in the tumour. Orange, increased relative protein level in the tumour. Grey, no data gathered.

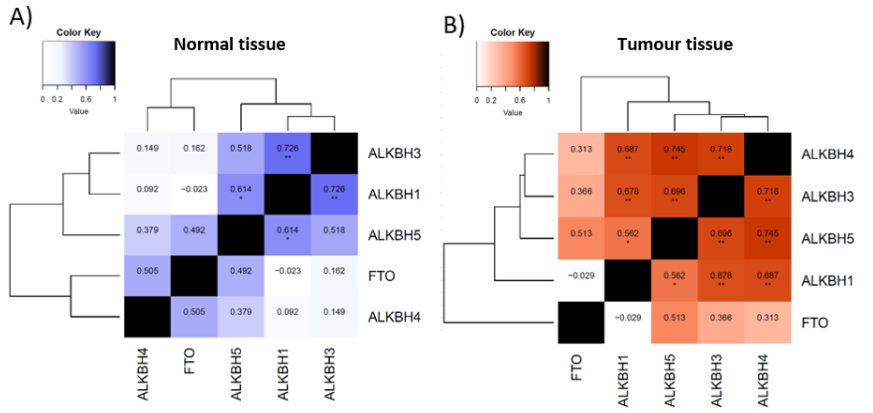


Figure 2. Relationships between protein levels within ALKBH family members.
(**A**) Relationship between the levels of ALKBH proteins in healthy surrounding tissue and (**B**) in salivary gland tumour. Each tabulated entry presents the Spearman's rank correlation coefficient and indicates the p-value of correlation. Hierarchical cluster analysis of matrices presents similarities between examined ALKBHs. *p*-values with Benjamini-Hochberg adjustment: '$p < 0.005$; *$p < 0.0025$; **$p < 0.0005$.

mour, as compared to the surrounding, unaffected tissue (Fig. 1A). The significant increase in expression were observed for ALKBH1, 4, and FTO (Fig. 1B). Further analysis indicated that among overexpressed ALKBH proteins, the greatest difference between tumour and surrounding tissue was observed for ALKBH1 (3-fold) (Fig. 1C). Thus, simultaneous overexpression of the indicated dioxygenases in cancer tissue may be used in cancer diagnosis as a meta-marker (Patent no 235564).

Elevated levels of the above ALKBH family members have been observed in other types of cancer, including HNSCC (Pilžys *et al.*, 2019). Interestingly, in SGT, ALKBH4 is overexpressed the most often (in 76% of samples), whereas in HNSCC the first place is taken by FTO protein (overexpressed in 90% of the samples). However, in general, the tested ALKBHs had higher expression in HNSCC than in SGT. Later studies also showed the involvement of ALKBH1, 4 and FTO in cancer homeostasis and development. ALKBH1 and 4 mRNA levels were significantly elevated in patients with non-small cell lung cancer (NSCLC). In addition, higher ALKBH 4 and 6 expression correlated with poor overall survival (OS), first progression survival (FPS) and post-progression survival (PPS) (Wang *et al.*, 2022). Subsequent studies on this cancer have also indicated elevated levels of ALKBH4 protein (Dworakowska, 2005).

The role of ALKBH1 in lung cancer development has been intensively studied. Another group found that ALKBH1 expression levels in lung cancer tissues and cells were upregulated. The invasive and migratory abilities of lung cancer cells were significantly suppressed *in vitro* when ALKBH1 was silenced, while they were significantly promoted when it was overexpressed (Li *et al.*, 2021).

Analysing the levels of ALKBHs in SGT, we observed the simultaneous increase in expression of at least two ALKBH proteins in approx. 78% of patients and at least four ALKBHs in over 52% of patients. More than 30% of samples tested showed high expression of all five ALKBH proteins (Fig. 1D).

Further, we assessed the relationship between the expression levels of individual ALKBH proteins in normal surrounding tissue and in SGT (Fig. 2A, B). In the healthy periphery, we identified two correlated pairs,

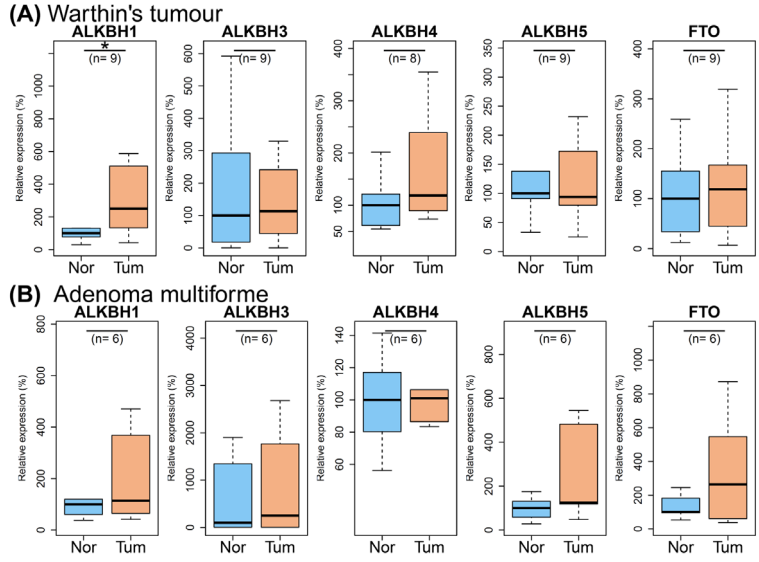


Figure 3. ALKBHs expression in cancer and normal tissues from patients with Warthin's tumour (A) and Adenoma multiforme (B).
Nonparametric Wilcoxon rank-sum test (for pairs) was performed. n, number of samples from each group; *p*-values with Benjamini-Hochberg adjustment: *$p < 0.05$.

with the highest Spearman's rank correlation coefficients ($\varrho > 0.55$, $p < 10^{-3}$) calculated for ALKBH1-ALKBH3 and ALKBH1-ALKBH5 pairs. In the cancer tissue, we observed a similar correlation involving ALKBH1-ALKBH3 and ALKBH1-ALKBH5, but also noted that all ALKBH1, ALKBH3, ALKBH4, and ALKBH5 were correlated with each other.

Other research groups have also indicated that changes in ALKBH protein levels are related e.g. in the case of hepatocellular carcinoma (HCC), where the expression levels of ALKBH1, 2, 3, 4 and 7 were significantly elevated compared to normal tissues (Peng *et al.*, 2021). Another tumour with changes in the expression levels of multiple ALKBHs is lung adenocarcinoma (LUAD). In LUAD ALKBH1/2/4/5/7/8 expression levels were significantly increased, while on the contrary, ALKBH3/6 and FTO expression decreased (Wu *et al.*, 2021).

In the patients studied here, two tumour types predominated: Warthin's tumour and Adenoma multiforme (Fig. 3). Only in the case of the Warthin's tumour we observed statistically significant difference in ALKBH1 levels between the healthy periphery and the tumour. For the other ALKBH proteins and Adenoma multiforme, no changes were observed; however, this may be a result of a small number of samples examined.

We also assessed whether tumour virulence could be related to ALKBH protein levels. To do so, we compared previously published results from HNSCC tumours with the ones obtained from SGT samples in this study. We observed that there was a statistically significant increase in the level of FTO in HNSCC compared to SGT (Fig. S7 at https://ojs.ptbioch.edu.pl/index.php/abp/).

The results obtained in this study may be further developed with ample modern molecular technics to identify mutations and polymorphisms. Additional Western blots detecting proteins belonging to important regulatory pathways can be included for a broader perspective.

CONCLUSIONS

Overexpression of individual protein members of the ALKBH family has been detected in various type of cancers and in many cases connected with cancer progression. Considering the results obtained here, we suggest that some of the ALKBH proteins, especially FTO, show pro cancerogenic action in SGT. This most probably happens through repairing alkylation lesions in DNA, RNA, and proteins enabling cancer cells to proliferate. Moreover, the level of the one of the ALKBHs proteins, namely FTO, is not as high in benign SGT as it is in high malignancy HNSCC. For instance, the FTO overexpression has been observed in 65% of SGT patients samples in comparison to 90% of HNSCC (Pilžys *et al.*, 2019). Independently on the ALKBHs overexpression level, this feature can be used for cancer diagnosis and estimation of its malignancy (grading) (Patent no 235564).

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