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Research Article

HIF-1α: a potential biomarker in obstructive sleep apnea

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ABSTRACT

Obstructive sleep apnea (OSA) is a common breathing disorder characterized by repeated obstruction of the upper airway during sleep. Although polysomnography (PSG) is the widely used method for the diagnosis of OSA, it is a time-consuming and expensive method. Because it requires at least 1 night stay in the hospital and technical personnel are needed. Therefore, it has been focused on biomarkers that can be easily detected in blood for the diagnosis of OSA. Some studies have highlighted the relationship between OSA and cancer in humans, and HIF-1 is an important regulator in this process. KDM3A is a histone demethylase that increases hypoxic gene expression. We aimed to evaluate whether the expression levels of HIF-1α and KDM3A genes could be useful predictor in patients with OSA. Our study included 50 patients with OSA and 50 healthy controls. qRT-PCR was used to detect mRNA levels of HIF-1α, KDM3A, and epithelial-mesenchymal transition (EMT) genes in the leukocyte samples. The expression level of the HIF-1α gene was found significantly higher in the OSA group compared to the controls, and no statistically significant difference was detected in the expression levels of the KDM3A and EMT genes.

Keywords: Blood- based biomarker, HIF-1α, Obstructive sleep apnea, qRT-PCR.

1. INTRODUCTION

Obstructive sleep apnea (OSA), which affects 2-4% of middle-aged adults in the general population is a common respiratory disease.[1] OSA is stimulated by entire or partial obstructions of the pharynx during sleep repetitively. This obstruction in the pharynx leads to recurrent hypoxia cycles and re-oxygenation called intermittent hypoxia (IH).[2] IH is considered an important pathophysiological feature that causes most of the pathological phenotypes associated with OSA.[3] Polysomnography (PSG) is a gold standard method currently used for the diagnosis of OSA. Although PSG is the most used confirmation methods by clinicians, it is a time-consuming and high cost method.[4] Therefore, it is crucial to determine an easily detectable biomarker in blood for OSA diagnosis. Hypoxia-inducible factor-1α (HIF-1 α) is the first discovered signal molecule for IH. Its structure in the nucleus is oxygen-dependent. Under normoxia, HIF-1α is degraded in the proteasome, while under hypoxia with inadequate O_2, degradation pathway is interrupted, and HIF-1α accumulates in the nucleus.[5] There are some studies in the literature revealing the relationship between HIF-1α and OSA. In a study, HIF-1α was shown to be a useful diagnostic and prognostic biomarker for OSA.[4]

Lysine demethylase 3 A (KDM3A), also known as Jumonji domain-containing protein 1 A (JMJD1A), is an important histone demethylase that increases hypoxic gene expression. KDM3A triggers the process associated with tumor development by reducing H3K9 (Histone 3-Lysine 9) methylation of hypoxia-dependent genes). It was reported that KDM3A acted as a signal amplifier during hypoxia, and increased tumor growth in colon and renal carcinoma cell lines.[6]

OSA is an oxidative stress disorder and many in vivo experiments have shown the effects of hypoxia on tumor

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growth and metastasis. Therefore, we investigated the expression levels of several epithelial-mesenchymal transcription (EMT) factors such as E-cadherin, Claudine-1, Slug, and ZEB-1 to determine cancer-associated gene expression in OSA patients.

The purpose of this study is to examine whether the expression levels of HIF-1α, KDM3A and EMT genes could be useful biomarker in patients with OSA. The results of this study are important as it is the first expression study based on human peripheral leukocytes that investigated OSA and KDM3A relation. Future investigations handling whether these genes may use as prognostic markers are needed.

2. MATERIALS AND METHODS

2.1. Study groups

The study protocol was approved by the Ethics Committee of SANKO University (2018/05-03) and written informed consent was obtained from all participants. The study consisted of two groups; 50 healthy controls without sleep disorder, and 50 OSA patients diagnosed via conventional polysomnography. Blood samples were obtained from the OSA group at the time of diagnosis before treatment was started. Administrations of hormones, immune suppressors, drugs, or free radical scavengers were exclusion criteria for OSA and control groups. Also, individuals under the age of 18, patients who did not sign the informed consent form, and patients with psychiatric or neurological diseases were not included in the study. According to the severity of the apnea-hypopnea index (AHI), OSA patients were divided into three groups; mild (AHI 5-15), moderate (AHI 15-30), or severe (AHI>30).

2.2. Total RNA isolation and cDNA synthesis

PureLink® (Thermo Fisher) RNA Mini Kit was used to isolate the total RNA from peripheral blood samples according to the protocol suggested by the manufacturer. NanoDrop spectrophotometer was used to evaluate the RNA concentration and purities of each sample. Next, RNA samples (1µg) were reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher).

2.3. Quantitative real time-PCR

cDNA amplifications were performed StepOnePlus Q RT-PCR (Qiagen, Germany). The PCR mixture consisted of SYBR Green PCR Master Mix (Qiagen), 20 pmol of forward/reverse primers, RNase-free water, and the cDNAs in a total volume of 20 µL were constructed. The expression level of a housekeeping gene, β-actin, was used as an internal control. The PCR primers of each gene were summarized in Table 1. The cycling conditions were as follows: 95°C for 15 min, 40 cycles; at 95°C for 15 sec; at 60°C for 1 min, and at 72°C for 30 sec. Rotor-Gene Q Series software v2.1.0 (Qiagen) were used to examine data, and expression levels were calculated using the standard curve method. Each gene was performed in duplicate. $2^{-\Delta\Delta CT}$ method was used to determine the relative gene expression levels of HIF-1α, KDM3A, and epithelial-mesenchymal transition (EMT) genes.

Table 1. Primer sequences used for RT-PCR.

Gene	*Forward Primer*	*Reverse Primer*
HIF-1α	5′-TCCATGTGACCATGAGGAAA-3′	5′-CC AAGCAGGTCATAGGTGGT-3′
KDM3A	5′-GGCGGACTTTAGACGTTCCA-3′	5′-AGATGAGCCTTCCACTTGGC-3′
E-cadherin	5′-CGGGAATGCAGTTGAGGATC-3′	5′-AGGATGGTGTAAGCGATGGC-3′
CLDN1	5′-GATAGCAATCTTTGTGGCCACCGT-3′	5′-TTCGTACCTGGCATTGACTGGG-3′
Slug	5′-CATGCCTGTCATACCACAAC-3′	5′-GGTGTCAGATGGAGGAGGG-3′
ZEB-1	5′- GCCAATAAGCAAACGATTCTG-3′	5′- TTTGGCTGGATCACTTTCAAG-3′
ACTB	5′-GCCGGGACCTGACTGACTAC-3′	5′-TTCTCCTTAATGTCACGCACGAT-3′

2.4. Statistical analysis

SPSS software (standard version 22.0; SPSS) was used for all statistical analysis. The normal distributions of expressions were evaluated using the Shapiro-Wilk test. The results suggested expression levels did not conform to a normal distribution. Consequently, expression values were compared using the Mann-Whitney U non-parametric test. The measurement data were expressed as the mean±standard deviation, and the categorical data were presented as the median and interquartile range. The Kruskal-Wallis with Dunn's multiple comparison test was used to identify differences between patient subgroups. $p < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

Obstructive sleep apnea (OSA) is a common disease and is related with increased mortality and morbidity. There is growing evidence that patients with OSA have higher incidence of cardiovascular diseases, stroke, pulmonary

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diseases, and cancer. Intermittent hypoxia affects tumor growth and progression in many steps. Several clinical and epidemiological studies proposed that patients with OSA have higher incidences of cancer. The Spanish Sleep Network first designated a possible relationship between OSA and elevated cancer incidence.[7] In an Australian Brusselton Sleep Cohort, people with moderate to severe sleep apnea were shown to have elevated risks for mortality, cancer, and stroke compared to controls.[8] Also, mouse models of IH mimicking OSA revealed increased cancer growth, invasion, and metastasis.[9,10] PSG is a gold standard method for OSA diagnosis. However, it is a time-consuming and high-cost method as it requires an overnight stay in the hospital. Therefore, the detection of biomarkers in circulation blood could ensure the most useful test for OSA.

3.1. Participant characteristics

Total of 50 patients with OSA, 18 were women (36%), and the mean age was 50.14±13.65. The calculated percentages of severities in these cases were; 36.7% (mild), 12.2% (moderate), and 51% (severe). In the control group, the mean age was 52.82±12.10, and 35 were women (70%).

3.2. Comparison of gene expressions in OSA and control groups

HIF-1α, KDM3A, E-cadherin, Claudin-1, Slug and ZEB-1 genes were used in this study. The gene expression levels of these genes were studied by RT-PCR methods in the OSA and controls. The results comparing the OSA and controls were shown in Table 2.

Table 2. Comparison of gene expressions in OSA and control groups.

	Control	*OSA*	*p*
	Median [%25-%75]	*Median [%25-%75]*	
$Log_2^{(2-\Delta\Delta CT)}$ (HIF-1α)	-1.32[-5.18- 4.36]	3.03[-2.02-7.63]	**0.026**
$Log_2^{(2-\Delta\Delta CT)}$ (KDM3A)	0.06[3.45-3.05]	0.65 [2.92 -4.09]	0.625
$Log_2^{(2-\Delta\Delta CT)}$ (E-cadherin)	-1.19[-4.34-3.88]	0.69[-2.53 -4.38]	0.873
$Log_2^{(2-\Delta\Delta CT)}$ (CLDN1)	-0.39[-2.02-1.60]	3.06 [-3.60- 5.15]	0.143
$Log_2^{(2-\Delta\Delta CT)}$ (Slug)	0.56[-3.08-2.63]	0.54 [-3.66-2.83]	0.657
$Log_2^{(2-\Delta\Delta CT)}$ (ZEB-1)	0.65[-2.75-2.13]	1.42 [-2.98-3.33]	0.430

It has been demonstrated that HIF-1 is more accumulated in cells exposed to IH than those undergoing chronic hypoxia.[11,12] IH-stimulated increase in the protein levels of HIF-1α, has been proposed as an adaptive response to OSA.[13,14] Liu et al.[4] reported that HIF-1α mRNA was significantly up-regulated in the plasma of OSA patients compared to the control group. Some experimental in-vitro researches have demonstrated the relationship between HIF-1α and carcinogenesis and have shown consistent results regarding the effect of IH on colorectal cancer[15] and renal cell carcinoma.[16] In our study, HIF-1α expression level was significantly higher in the OSA group than in the control group (p=0.026) (Figure 1). This increased gene expression of HIF-1α could be a prognostic and diagnostic biomarker for OSA patients.

KDM3A is a histone demethylase that regulate hypoxic gene expression by reducing histone methylation in promoter regions. These studies define a regulatory mechanism in which the stimulation of KDM3A by HIF-1 acts as an epigenetic signal enhancer to improve cellular responses to hypoxia.[17] It was also demonstrated that KDM3A is increased in bladder[18], renal[17], liver[19], and colon[17,20] cancers in cell culture studies.

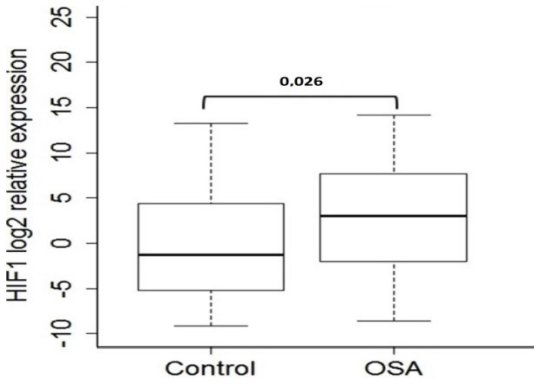

Figure 1. Box plot of RT-PCR results. The box border represents the interquartile range, the horizontal line in the box is the median, and circles represent outliers. Values are expressed as $log_2^{(2-\Delta\Delta CT)}$. Differences between the control and OSA of the HIF-1α gene were shown.

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In our study, KDM3A expression was increased in OSA patients due to hypoxia, but no statistically significant difference was found between the groups ($p>0.05$). This situation is thought to be due to the fact that our study is a circulation blood-based gene expression study, and it is not known which tissues are affected more by KDM3A activity caused by OSA yet. According to our literature search, this is the first study examining KDM3A expression in peripheral leukocytes of OSA patients.

EMT, a process in which epithelial cells loose their characteristic properties and transform into mesenchymal cells, was recognized in the 1980s.[21] Recent studies have provided evidence that EMT is associated with cancer progression and metastasis.[22] Hypoxic conditions can affect EMT directly or indirectly. Transcription factors, including Slug and ZEB1 are directly induced by HIF1.[23] On the other hand, HIF1 promotes EMT indirectly through signal transduction pathways. In EMT, mesenchymal-associated genes such as ZEB-1 and Slug were upregulated, whereas epithelial-associated genes such as Claudin and E-cadherin were downregulated.[24] In our study, no statistically significant difference was found in Slug, Claudin, and E-cadherin expressions between OSA and control groups ($p>0.05$). ZEB-1, a mesenchymal marker gene, was increased between groups with medians of 0.65, and 1.42, while no significant difference was observed. We could not find any relation between OSA and epithelial-mesenchymal markers.

In addition, HIF-1α, KDM3A, and EMT gene expression levels were investigated in OSA patients with different severities. There was no statistical significance between these subgroups ($p>0.05$) (data not shown).

4. CONCLUSIONS

Clinical *in-vivo* studies investigating OSA are are very limited and mostly based either on cell culture or animal studies. In our study, the HIF-1α gene expression was found to be associated with OSA and therefore could be a useful marker in circulating leukocytes. In this study, KDM3A and EMT genes were not found to be associated with OSA. Our study has a limitation in terms of the number of subjects and our findings need to be confirmed in larger clinical groups. Also, more biomarkers are needed to increase the reliability of the diagnosis. Despite limitations, this work reveals the first study to assess related gene expression levels on human peripheral leukocytes. Future studies are required to investigate the roles of identified genes and the genetic characteristics of OSA.

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Conflict of interests

I declare that there is no a conflict of interest with any person, institute, company, etc.

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