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ORIGINAL ARTICLE

KDM3A, A NOVEL BLOOD-BASED BIOMARKER IN COLORECTAL CARCINOGENESIS

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ABSTRACT

Colorectal cancer (CRC) is one of the leading causes of cancer-linked deaths globally. The determination of biomarkers is important in the prognosis and treatment of CRC. Previous studies emphasized the relationship between hypoxia and CRC in humans, and there is strong evidence that this process is strongly related to HIF-1. KDM3A is a histone demethylase that could directly bind to HIF-1α, a subunit of HIF-1. This study aimed to reveal whether the expression level of the *KDM3A* gene could be used as a predictor of CRC. The expression levels of *HIF-1α, KDM3A*, and Epithelial-Mesenchymal Transition (EMT) genes were evaluated by qRT-PCR in leukocyte samples of 50 CRC patients in different stages and 50 healthy controls. *HIF-1α* and *KDM3A* expression levels were significantly higher in the CRC group, compared to the controls. *Slug* and *ZEB-1* genes, the mesenchymal markers, showed the same significance pattern between groups. We acquired 0.664 AUC with 54% sensitivity and 85.4% specificity for separating controls from CRC patients by using the *KDM3A* expression levels in ROC analysis. This data support that *KDM3A* could be a novel supplementary biomarker in diagnosis of CRC, which could be noninvasively detected in circulation.

Keywords: biomarker, colorectal cancer; *KDM3A*; qRT-PCR

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INTRODUCTION

Colorectal cancer (CRC) is a leading cause of morbidity and mortality throughout the world. It is the third most frequent cancer among worldwide and the second most common cause of death (1). Although the high incidence and mortality have increased interest to better understand the pathogenesis of CRC, the molecular mechanism which triggers CRC progression is not clearly identified (2). Also, determination of blood-based biomarkers for diagnosis and treatment in CRC is one of the most studied subjects.

The *HIF-1* gene is the key regulator of hypoxic cell response and development of many cancer types. Hypoxia-inducible factor-1 (HIF-1) consists of two subunits: HIF-1α and HIF-1β. Its construction is O_2-dependent in the nucleus. Under normoxia with adequate O_2 conditions, the HIF-1α subunit is degraded in the proteasome (3). But under hypoxic conditions, this degradation pathway is disrupted, and HIF-1α accumulates in the nucleus (4). The HIF-1α function has been shown to influence particular Jumonji C-domain-containing histone demethylases (JHDM) (5, 6). These histone demethylases form a broad family of enzymes, each of which has a specific ability to influence transcriptional repression on specific histone residues (7). *KDM3A* regulates the expressions of some pro-angiogenic hypoxia dependent genes by reducing histone methylation in promoters (8-10). Uemura et al. demonstrated the association between *KDM3A* and CRC in human colon cancer cell lines and tissue samples and this gene was indicated to be a useful biomarker for hypoxic tumor cells and a prognostic marker that could be a therapeutic target against CRC (11). These data suggest a link between tumorigenesis in the colon and *KDM3A* gene expression.

Early detection of CRC influences the survival rate of patients (12). Colonoscopy is the gold standard for the diagnosis of CRC; however, it is an invasive, time-

consuming and expensive method (13). Therefore, it is important to find an easily detectable, and non-invasive blood-based biomarker for the early detection of CRC. We aimed to evaluate whether the expression levels of the *KDM3A* gene could be useful predictors of colorectal carcinogenesis in circulation.

Epithelial-Mesenchymal Transition (EMT) ensures a mechanism for cancer cells to obtain a more aggressive phenotype which is controlled by several transcription regulators, including *E-cadherin, Claudin-1, Slug,* and *ZEB-1*. In the process of EMT, expression levels of epithelial markers such as *E-cadherin* and *Claudin-1* are decreased, whereas those of mesenchymal markers such as *Slug,* and *ZEB-1* are increased (14). We investigated the expression levels *E-cadherin, Claudin-1, Slug,* and *ZEB-1* to determine this association.

In this study, it was determined that *KDM3A* is related to CRC, and in our opinion, these results are very valuable as this is the first expression study based on human peripheral leukocyte samples that showed this association. Based on this data, *KDM3A* is an important target in the diagnosis in CRC.

MATERIALS AND METHODS

Study design

The study consisted of two groups; 50 healthy controls without CRC, inflammatory bowel disease, and positive family history, and 50 pathologically confirmed CRC patients in different stages. All blood samples collected from the patient and control groups were taken early in the morning. Furthermore, blood samples were collected from the patient groups at the time of diagnosis before treatment was started. Usage of drugs, hormones, immune suppressors, cytotoxins, or free radical scavengers were exclusion criteria for all groups. Depending on the severity of the CRC, patients were divided into four groups (stage I-IV). The study was approved by the Ethics Committee of SANKO University (2018/05-03). Written informed consent was acquired from all individuals who agreed to participate in the study.

Total RNA Isolation and cDNA Synthesis

PureLink® (Thermo Fisher) RNA Mini Kit was used to extract the total RNA from peripheral blood leukocytes, according to the protocol recommended by the manufacturer. The RNA concentration of each sample was measured, and purities were evaluated by the NanoDrop spectrophotometer. Then, cDNA transcriptions from RNA samples (1µg) were done using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher).

Quantitative Real Time-PCR

The gene expression levels of *HIF-1α, KDM3A, E-cadherin, Claudin-1, Slug,* and *ZEB-1* were determined using quantitative real-time polymerase chain reaction (qRT-PCR) from total leukocyte RNA of peripheral blood samples. StepOnePlus QRT-PCR (Qiagen, Germany) was used for cDNA amplifications. The PCR mixture was composed of SYBR Green PCR Master Mix (Qiagen), 20 pmol of forward/reverse primers, RNase-free water. The cDNAs were constructed in a total volume of 20 µL. *β-actin* (*ACTB*), the housekeeping gene, expression level was used as an internal control to evaluate the integrity of each sample. The PCR primers of each gene are described in table 1. Cycling conditions were 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. Data were examined by Rotor-Gene Q Series software v2.1.0 (Qiagen), and expression levels were calculated by using the standard curve method. Each gene was analyzed separately and ran by duplicate. The mean CT threshold values for each sample were used to calculate the relative gene expression levels using the $2^{-\Delta\Delta CT}$ method expressed as Fold-Change (FC) (15).

Statistical analysis

Statistical analysis was performed using SPSS software (standard version 22.0; SPSS). The normal distribu-

Table 1. The detailed PCR primers of each gene

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′
Claudin-1	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′

tions of expressions in groups were analyzed using the Shapiro-Wilk method. As descriptive statistics, median values were used when expression levels were not normally distributed, and mean values were used when expression levels were normally distributed. A Non-parametric Mann-Whitney U test was used for non-normally distributed genes and the student-t test was used for normally distributed *Claudin-1* gene. Differences between patient subgroups were tested using Kruskal-Wallis with Dunn's multiple comparison test. The *KDM3A* diagnostic value was assessed by the ROC curve. P <0.05 was considered statistically significant.

RESULTS

Characteristics of study subjects

Of 50 CRC patients, 22 (44%) were women, and the mean age was 59.98±15.58. In all, 12.2% of patients were diagnosed as stage I, 28.6% were stage II, 20.4% were stage III, and 38.8% were stage IV. And the mean age of the control group was 52.82±12.1, and 35 of them (70%) were women.

Analysis of HIF-1α, KDM3A and EMT marker gene expressions in CRC and control groups

The gene expression levels comparing the groups were shown in table 2. *HIF-1α, KDM3A, Slug*, and *ZEB-1* expression levels were detected as statistically significant between the CRC and the control groups. *HIF-1α* expression tended to increase among groups (median -1.32, and 4.25), and the difference was significant (p=0.0045) (Fig. 1A). The expression level of the *KDM3A* gene was significantly higher in the CRC group compared to the controls (p=0.0049) (Fig. 1B). Furthermore, the expression levels of the *Slug* and *ZEB-1* mesenchymal marker genes was significantly higher in the CRC group compared to the controls (p=0.008 and p=0.029) (Fig. 1C-1D) There was no statistically significant difference between the groups in terms of *E-cadherin* and *Claudin-1* gene expressions (p>0.05).

The areas under the receiver operating characteristic (ROC) Curve (AUC), as well as the specificities and sensitivities for the optimal cut-points were computed to determine whether the *KDM3A* might be a prognostic factor in CRC. ROC analysis produced an AUC of 0.664

Table 2. Comparing the control and CRC groups gene expression levels

	Control	**CRC**	
$Log_2^{(2-\Delta\Delta CT)}$	***Median [%25-%75]***	***Median [%25-%75]***	***p***
HIF-1α	-1.32 [-5.19- 4.59]	4.25 [0.72 -7.4]	**0.0045**
KDM3A	0.06 [-3.56-3.12]	4.29 [-0.74-5.97]	**0.0049**
E-cadherin	-1.19 [-4.40-4.03]	0.46 [-2.89-4.01]	0.189
Slug	0.56 [-3.18-2.64]	2.74 [0.75-3.51]	**0.008**
ZEB-1	0.65 [-2.75-2.18]	2.49 [-1.09-3.58]	**0.029**
$Log_2^{(2-\Delta\Delta CT)}$	***Mean±SD***	***Mean±SD***	***p***
Claudin-1	0.00058±3.45	0.74±4.03	0.42

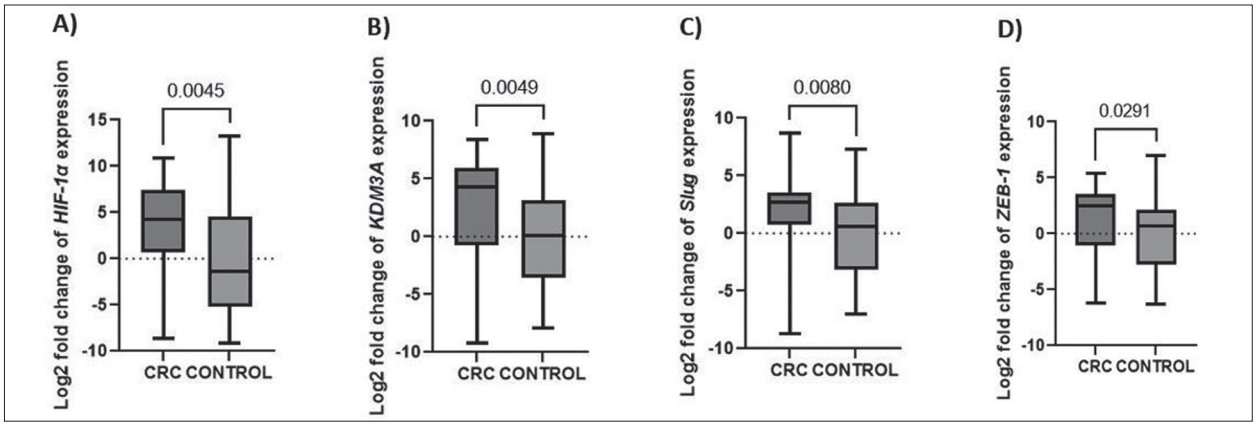

Figure 1. A-C. Box plot of RT-PCR results. The box border represents the interquartile range, the horizontal line in the box is the median, and circles represent outliers. Values are expressed as $log_2^{(2-\Delta\Delta CT)}$. Significant differences between control and CRC of *HIF-1α* **(A)**, *KDM3A* **(B),** *Slug* **(C),** *ZEB-1* **(D)** genes were shown.

for separating CRC patients from controls by using the *KDM3A* expression level with 54% sensitivity and 85.4% specificity (Fig. 2).

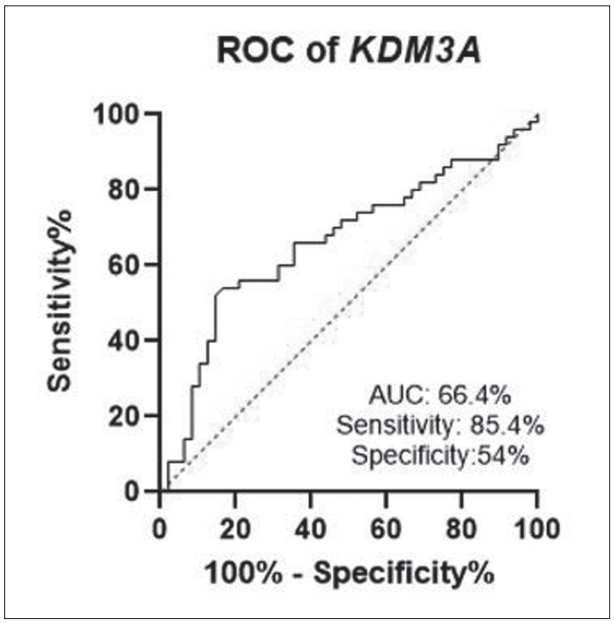

Figure 2. ROC curve, comparing control and CRC cases using *KDM3A* expression levels

Gene expressions correlated with clinical features

We next examined the *HIF-1α, KDM3A, E-cadherin, Claudin-1, Slug, and ZEB-1* gene expression levels in different stages of CRC patients. No significant difference was observed between these subgroups ($p>0.05$).

DISCUSSION

Hypoxia is a very common characteristic in solid tumors. While normal cells cannot resist prolonged hypoxia and undergo either apoptosis or necrosis, cancer cells can adapt to hypoxia by changing the expression of genes involved in many cellular processes like proliferation, metabolic reprogramming, and angiogenesis (16). Chronic tumor hypoxia is linked to poor cancer outcomes and activates the transcription HIF-1, which regulates the expression of many cancer-related genes, particularly those involved in angiogenesis (17).

HIF-1α is shown to be related to CRC (18), and experimental *in-vitro* studies have revealed consistent findings regarding the effect of hypoxia on colorectal cancer. Martinez et al. found that HIF-1α protein increased in HCT116 colorectal cancer cells exposed to O_2 changes. This changed gene and protein expressions in pathways regulating hypoxia, glycolysis, and extracellular matrix remodeling (17). In addition, several studies proposed that the expression level of *HIF-1α* is associated with the prognosis, recurrence, and metastasis of CRC (19). Also, it was reported that high expression levels of *HIF-1α* in patients with CRC might be a potential biomarker for the progression of CRC. In our study, we demonstrated that *HIF-1α* expression was significantly increased in leukocytes samples of CRC patients. To the best of our knowledge, this is the first study to show expression changes in peripheral leukocytes.

KDM3A is also one of the hypoxic response genes, and HIF-1α up-regulates its expression. It was shown that loss of *KDM3A* reduced tumor growth *in-vivo*, consistent with its role in regulating histone methylation during hypoxia (9). In another study, it was emphasized that *KDM3A* was mainly overexpressed in human CRC specimens, and knock-down of *KDM3A* significantly suppressed CRC cell proliferation, colony formation, invasion, migration, and metastasis (2). These studies identify a regulatory mechanism in which the induction of KDM3A by HIF-1 acts as an epigenetic signal enhancer (9). Several types of research have shown that abnormal expression of *KDM3A* exists in numerous types of cancers (9, 11, 20, 21). In this study, this gene has demonstrated to be a novel prognostic marker and a therapeutic target for CRC. It was detected to be statically significant in CRC groups, and the AUC was calculated as 66.4%. We could not find a study investigating KDM3A in CRC patients from human blood samples in our literature searches.

EMT is a dynamic process in which epithelial cells obtain a mesenchymal phenotype with decreased intercellular adhesion and increased cell mobility. Many transcription factors, including Slug and ZEB-1 have been reported to induce EMT by downregulating E-cadherin and Claudin (22, 23). In our study, *E-cadherin* and *Claudin-1* were not statistically significant between groups. However, the significance determined between the groups at *Slug* and *ZEB-1* expression levels was found to be compatible with *KDM3A*. These results indicate that mesenchymal markers are more important in CRC.

In conclusion, clinical *in-vivo* studies have been very limited investigating *KDM3A* and colorectal carcinogenesis relation. Furthermore, the existing ones are either animal or cell culture studies. Concerning all our data, *KDM3A* is a novel biomarker in the development of CRC, which deserves more detailed research.

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article**.**

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