

Evaluation of lncRNA FOXD2-AS1 Expression as a Diagnostic Biomarker in Colorectal Cancer

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Abstract

Background: Colorectal cancer (CRC) is still considered one of the prevalent cancers worldwide. Investigation of potential biomarkers for early detection of CRC is essential for the effective management of patients using therapeutic strategies. Considering that, this study was aimed to examine the changes in lncRNA FOXD2-AS1 expression through colorectal tumorigenesis.

Methods: Fifty CRC tumor tissues and fifty adjacent normal tissue samples were prepared and involved in the current study. Total RNA was extracted from the samples and then reverse transcribed to complementary DNA. Next, the expression levels of lncRNA FOXD2-AS1 were evaluated using real-time PCR in CRC samples compared to normal ones. Also, receiver operating characteristic curve analysis was used to evaluate the diagnostic value of FOXD2-AS1 for CRC.

Results: The obtained results showed that the expression level of FOXD2-AS1 gene was significantly ($p < 0.0001$) up-regulated in tumor tissues compared to normal marginal tissues. Also, a significant correlation was observed between higher the expression of FOXD2-AS1 and the differentiation of tumor cells. Furthermore, ROC curve analysis estimated an AUC value of 0.59 for FOXD2-AS1, suggesting its potential as a diagnostic target.

Conclusions: Taken together, the current study implied that tissue-specific upregulation of lncRNA FOXD2-AS1 might be appropriate diagnostic biomarkers for CRC. Nonetheless, more studies are needed to validate these results and further illustrate FOXD2-AS1 function through colorectal tumorigenesis.

Keywords: Biomarker, Colorectal cancer, FOXD2-AS1, lncRNA, qRT-PCR.

Introduction

Colorectal cancer (CRC) is the third most prevalent malignancy in both sexes, worldwide, with the 5-year survival rate near to 10-15% for patients suffering a metastatic form of CRC. Therefore, it is necessary to clarify the potential mechanism involved in CRC metastasis, which presents diagnostic and potential goals for better management of CRC patients using current therapeutic approaches (1, 2). Early detection of CRC has been shown to increase the 5-year survival

rates to 90% after removal of the local tumor, while detection in advanced and metastatic stages lowers it to 10% (3, 4). Therefore, it is necessary to explore new therapeutic and diagnostic targets with high sensitivity and specificity (5).

Long non-coding RNAs (lncRNAs) which are defined as non-protein-coding transcripts with more than 200 nucleotides in length, play imperative roles in the regulation of multiple biological and pathological processes,

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including cancer development and metastasis (6, 7). Several studies have shown that dysfunction/dysregulation of various lncRNAs is associated with abnormal cell proliferation, apoptosis, invasion, migration, and chemosensitivity (8-11).

In particular, recent studies have established the dysregulation of lncRNA FOXD2-AS1 through the development of multiple human cancers, including nasopharyngeal carcinoma (NC) (12), hepatocellular carcinoma (HCC) (13-16), gastric cancer (GC) (17), colorectal cancer (CRC) (1, 18), esophageal squamous cell carcinoma (ESCC) (19, 20), non-small cell lung cancer (NSCLC) (21, 22), breast cancer (23), glioma (24, 25) and so on. This lncRNA, with a transcript length of 2527 nucleotides, is located on chromosome 1p33 and functions as an oncogene through tumorigenesis (26). It was shown that FOXD2-AS1 upregulation could lead to provoked tumor cell proliferation, apoptosis inhibition, and induction of migration and invasion through regulating the Wnt signaling (20), Notch (1), and PI3K / Akt pathways (27, 28).

In addition, other studies have confirmed that increased expression of FOXD2-AS1 shows a significant correlation with poor

outcomes and clinicopathologic characteristics in cancer patients. Nevertheless, the prognostic potential of FOXD2-AS1 expression in various human cancers, including CRC has not been clearly defined due to limited sample sizes and/or methodology. Then, characterization of FOXD2-AS1 expression patterns in larger sample sizes is important to completely understand its value as a therapeutic and diagnostic target for CRC therapy.

Materials and Methods

Study population

In the present study, 40 samples of colorectal cancer tissues along with marginal normal tissue samples were prepared from patients diagnosed with a primary colorectal tumor in the Imam Reza Hospital (Tabriz University of Medical Sciences, Tabriz, Iran) without receiving any treatment during 2016 to 2018. The samples were immediately transferred into Qiagene RNAase inhibitor solution (Germany) and stored at -80 °C. Table 1 represents the pathological information of included patients. All patients had given written informed consent and the study was approved by the local ethical committee of Tabriz University of Medical Sciences (TBZMED.REC.1394.356).

Table1. General Characteristic of Study patients.

Parameters	Classification	Number of Patients
Age	<55	20
	>55	20
Sex	Female	18
	Male	22
Lymph node metastasis	positive	24
	negative	16
Tumor histology	Good	23
	Medium	13
	poor	4
Tumor depth	T2	3
	T3	9
	T4	28
Intravenous invasion	Positive	29
	Negative	11
Stage (AJCC)	I, III	14
	v, III	26
Hepatic metastasis	Positive	5
	negative	35
Tumor size	<3	3
	>3	37

RNA extraction and cDNA synthesis

Total RNA extraction from tissue samples was done using BioFACT (BioFACT TM, South Korea) following supplied instructions by the manufacture. After assessment of integrity, quality, and quantity of extracted RNA by gel electrophoresis and Nanodrop One/C Spectrophotometer (Thermo Scientific™, USA), respectively, the reverse transcription of RNA (1000 ng) to complementary DNA (cDNA) was performed by using BioFACT cDNA synthesis kit (Korea) in T100 thermal cycler system (BioRAD).

qRT-PCR

The quantitative real-time PCR (qRT-PCR) was used to assess the changes in FOXD2-AS1 gene expression in biological samples. The reactions

were performed in the Applied Biosystems Step One™ qRT-PCR system (Foster City, CA, USA) using 2X Real-Time PCR Master Mix (BioFACT TM, Seoul, South Korea). Each reaction was repeated three times in a total volume of 20 µl containing 10 µl master mix, 1 µl cDNA, 0.5 µl each of forward and reverse primers (4 pmol/µl) and 8 µl ddH₂O). To normalize FOXD2-AS1 expression levels, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as the internal control. The primer sequences and annealing temperatures are shown in Table 2. The condition for reactions was as follows: 15 min initial incubation at 95 °C, followed by 45 amplification cycles of denaturation at 94 °C for 10 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 20 sec.

Table 2. Characteristics of the primers used in the study.

Target	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon Size (bp)	Tm (°C)
FOXD2-AS1	TGGACCTAGCTGCAGCTCCA	AGTTGAAGGTGCACACTG	173	60

Statistical analysis

All data were expressed as the mean ± standard errors and statistical analysis was done using Graph Pad Prism 6 (Graph Pad Software Inc. CA, USA). To evaluate the statistical significance of differences in FOXD2-AS1 expression levels between malignant and normal samples, Kolmogorov-Smirnov's test was carried out. Also, the prognostic value of the target gene was evaluated using Receiver operating characteristic curve analysis. Only p values less than 0.05 were regarded as statistically significant through this study.

Results**Up-regulation of FOXD2-AS1 in tumor tissues**

The obtained results from qRT-PCR showed that the expression level of FOXD2-AS1 gene was significantly ($p < 0.0001$) up-regulated in colorectal tumor tissues compared to normal marginal tissues (Fig. 1). Furthermore, FOXD2-AS1 upregulation was shown to be significantly correlated with

tumor cell differentiation. The expression levels of FOXD2-AS1 were higher in well and moderately differentiated tumors compared to poorly differentiated ones ($p < 0.0001$) (Fig. 2). However, our results also showed that there was no significant correlation between FOXD2-AS1 expression levels and other clinicopathological characteristics of patients, including age, sex, lymph node metastasis, tumor histology and depth, tumor stage and size, intravenous invasion, and hepatic metastasis.

FOXD2-AS1 potential as a diagnostic biomarker

Considering the upregulation of FOXD2-AS1 in CRC samples compared to normal samples, its diagnostic potential was also evaluated. ROC curve analysis estimated the area under the curve (AUC) near 0.59 ($p < 0.089$), which suggested that FOXD2-AS1 could be considered as a diagnostic target for screening of CRC patients (Fig. 3).

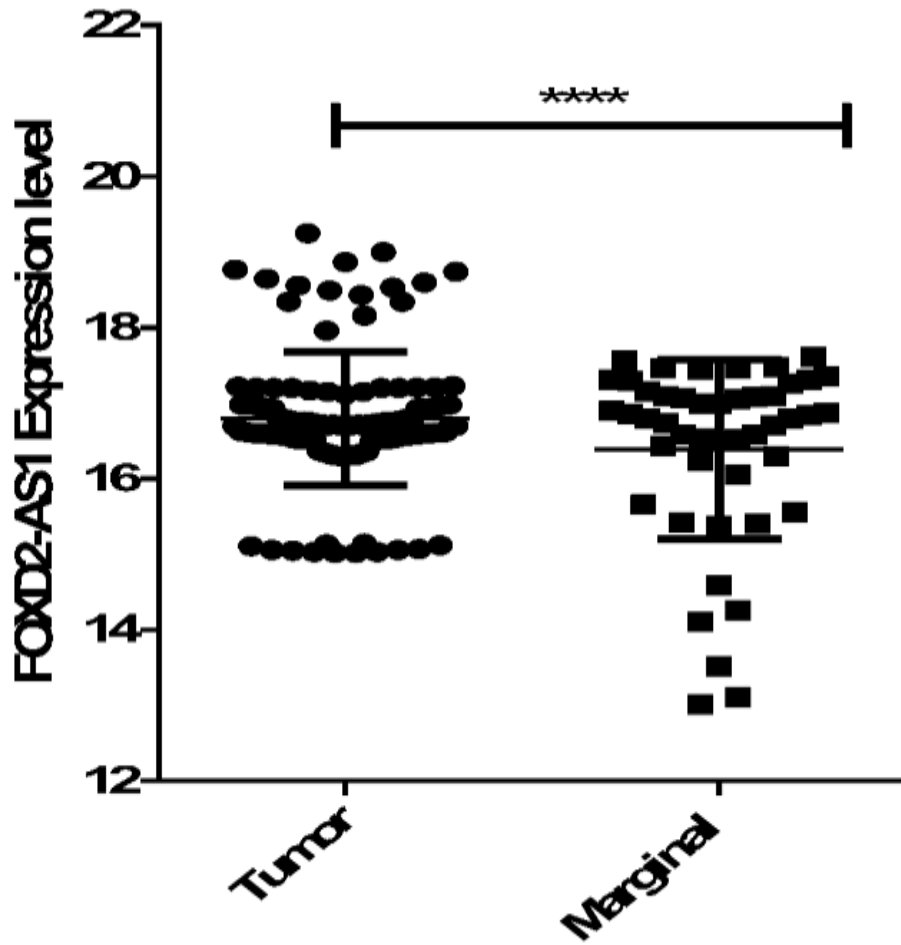


Fig. 1. qRT-PCR results showing Increased mRNA expression levels of FOXD2-AS1 in CRC tissue samples compared to normal tissue samples; **** $p < 0.0001$.

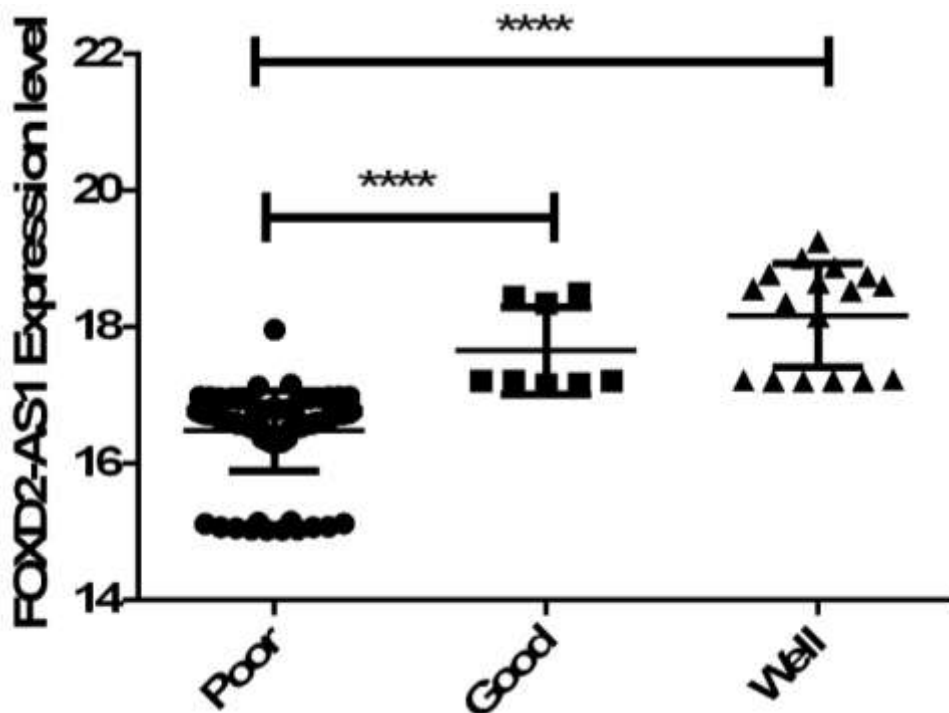


Fig. 2. The correlation between FOXD2-AS1 expression and tumor differentiation in CRC; **** $p < 0.0001$.

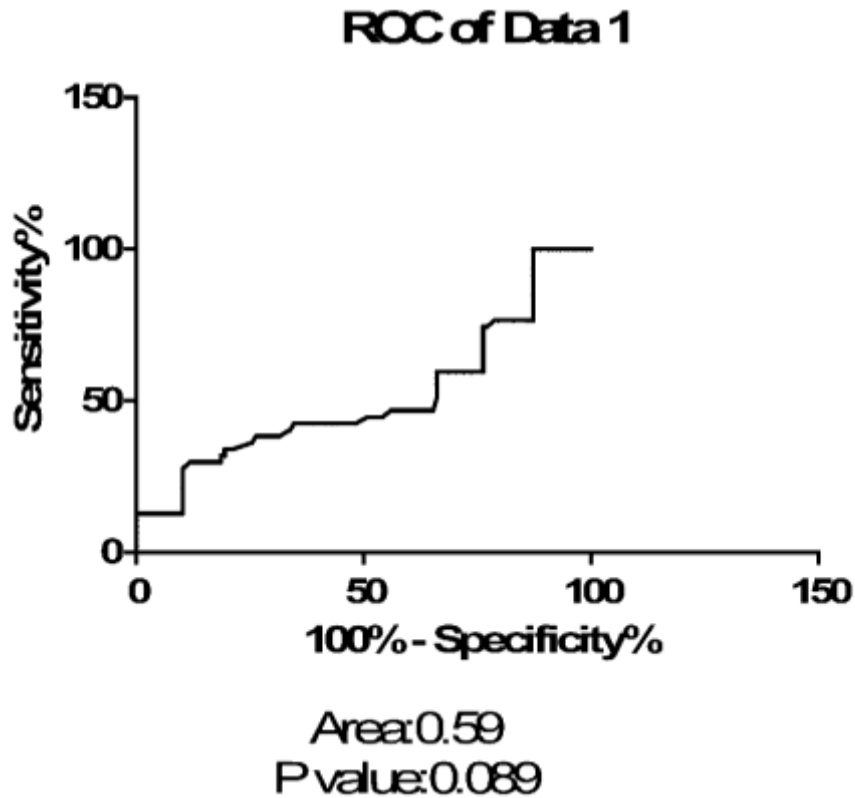


Fig. 3. ROC curve analysis; the accuracy of FOXD2-AS1 expression pattern as a diagnostic biomarker was evaluated.

Discussion

In the present study, the changes in expression levels of FOXD2-AS1 through colorectal tumorigenesis were investigated using qRT-PCR in Iranian patients, as a potential diagnostic target for CRC. The obtained results showed that CRC tumors have increased expression levels of FOXD2-AS1 in comparison with normal tissue samples. Moreover, upregulated levels of FOXD2-AS1 were observed to be significantly and positively correlated with tumor differentiation in CRC patients, the higher the expression of this gene, the greater the differentiation of tumor cells. Then, it was suggested that FOXD2-AS1 overexpression may be driver event through colorectal tumorigenesis, which is occurs in early stages of malignancy (29). These findings proposed that FOXD2-AS1 may act as a potential oncogene that induces CRC progression. Also, ROC curve analysis illustrated that FOXD2-AS1 expression patterns could be a considerable target for the differentiation of CRC patients from healthy cases.

Numerous findings have shown that lncRNAs acting as oncogenes or tumor suppressors through tumorigenesis of multiple human cancers possess great potential as the diagnostic and therapeutic targets (30). Particularly, lncRNA FOXD2-AS1 has been recently identified as an oncogene that is up-regulated in various types of solid tumors, showing a negative correlation with the survival of patients (24, 27, 31). More importantly, FOXD2-AS1 was previously reported to be upregulated in human CRC tissues and to be correlated with patients' poor survival and cancer progression (18). It was shown that FOXD2-AS1 exhibits high expression levels in CRC cells and its knockdown could significantly diminish *in vitro* cell invasion, migration and proliferation through modulating epithelial mesenchymal transition (EMT) and Notch signaling pathway (1). More interestingly, exosomal expression levels of FOXD2-AS1 has been recently illustrated to be significantly higher in the serum samples of

CRC patients compared to that of healthy individuals (32). Subsequently, our results further validated the therapeutic and diagnostic significance of FOXD2-AS1 for development of novel strategies to improve the outcomes in CRC patients.

As mentioned, the diagnostic and therapeutic value of FOXD2-AS1 has been identified in various human cancers as well. This lncRNA was also found to be upregulated in NSCLC tissue samples, showing a correlation with poor survival rate of patients. Besides, FOXD2-AS1 in NSCLC cells was indicated to provoke *in vitro* cell proliferation and *in vivo* tumor growth through upregulating Wnt/b-catenin signaling (20). In addition, FOXD2-AS1 has been reported to be overexpressed in gastric cancer patients and its high expression showed a significant relationship with poor prognosis of patients, larger tumor size and later pathologic stage. FOXD2-AS1 upregulation in gastric cancer cells increase tumorigenesis in part by reducing the activity of EphB3 through upregulation of EZH2 and LSD (17). Whereas silencing the expression of FOXD2-AS1 has been illustrated to inhibit cell cycle progression and cell proliferation and improve radiosensitivity in gastric cancer cells through miR-1913-mediated downregulation of SETD1A (33). Furthermore, Ziwei Yang and colleagues showed that FOXD2-AS1 level is also upregulated in plasma samples of gastric cancer patients and might be appropriate diagnostic biomarkers for this malignancy (5). Moreover, FOXD2-AS1 upregulation has been reported in papillary thyroid cancer tissue samples, showing a remarkable correlation with TNM stages, lymph node metastasis and poor prognosis of patients

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FOXD2-AS1 knockdown in thyroid cancer cells could suppress cell invasion and proliferation *in vitro* as well as diminish tumor growth *in vivo* through rescuing miR-185-5p expression (34). Besides, it has been reported that FOXD2-AS1 possesses good value as a prognostic target to predict the overall and disease-free survival of esophageal squamous cell carcinoma patients (35). Suppressing FOXD2-AS1 leads to inhibition of cell proliferation and invasion and induction of G1 phase cell cycle arrest by modulating miR-145-5p/CDK6 axis in esophagus cancer cells (22). Collectively, these studies further indicate the value of FOXD2-AS1 as a diagnostic and prognostic target for human cancers.

In conclusion, the current study illustrated that FOXD2-AS1 is upregulated in CRC patients and participates in colorectal tumorigenesis. Besides, alongside with previous studies, our study evidenced that FOXD2-AS1 expression levels may be considered as a predictive target for incidence and progression of various human cancers, including CRC. Nonetheless, more investigations are needed to validate these results and further clarify FOXD2-AS1 significance in human cancers, including CRC.

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