Correlation Between Expression levels of lncRNA FER1L4 and RB1 in Patients with Colorectal Cancer

Marjan Ostovarpour
University of Tabriz
Mohammad Khalaj-Kondori (khalaj@tabrizu.ac.ir)
University of Tabriz
Tayyebeh Ghasemi
University of Tabriz

Research Article

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Abstract

Colorectal cancer is one of the most common cancers in the world. Studies demonstrated that lncRNA FER1L4 was downregulated in different cancers and its expression was positively correlated with RB1 in a competing endogenous RNAs network. We investigated expression levels of FER1L4 lncRNA and RB1 in patients with colorectal cancer. 50 paired colorectal tumor and non-tumor marginal tissues, as well as 30 paired adenomatous colorectal polyps and matched adjacent normal tissues were obtained from the patients. Total RNA was extracted from the samples and cDNAs were synthesized. Their expression were quantified by qRT-PCR. Correlation between FER1L4 and RB1 expression levels was analyzed by Pearson correlation test. Finally, ROC curve analysis was used to evaluate their biomarker potency. We observed significant downregulation of FER1L4, but upregulation of RB1 in the colorectal tumors compared with non-tumor as well as with the adenomatous colorectal polyp tissues. However, RB1 expression was positively correlated with the FER1L4 expression both in the tumor and polyp samples. ROC curve analysis showed that both FER1L4 and RB1 expression levels could discriminate tumor from non-tumor and tumor from polyp samples. None of clinicopathological characteristics of patients were associated with FER1L4 or RB1 expression levels. Despite downregulation of FER1L4 and upregulation of RB1 in tumors compared with non-tumor tissues, the expression of RB1 was positively correlated with the expression of FER1L4 in the colorectal tumor as well as in the adenomatous colorectal polyp tissues. FER1L4 expression level might be considered as a potential biomarker for colorectal cancer development.

Introduction

Colorectal cancer (CRC) as a gastrointestinal malignancy is the third most common cancer and also the second leading cause of cancer-related deaths in the world [1, 2]. In recent decades, CRC incidence rate is increasing due to lifestyle changes including dietary habit, obesity, smoking, inflammatory bowel disease, diabetes mellitus and lack of physical activity [2–4]. Distant metastasis and recurrence of disease are the cause of most CRC-related deaths [5]. Therefore, it is essential to identify novel biomarkers for early stages and to understand the molecular mechanisms of CRC to discover new therapeutic targets [5, 6].

Long noncoding RNAs (lncRNAs) are a class of RNAs with a length longer than 200 nt and less than 1 Mb, but they have little ability to code proteins [7, 8]. LncRNAs participate in many biological functions, including cell proliferation, apoptosis, metastasis, and gene expression regulation; thus, they could play crucial roles in cancer biology [8–10]. Different cancer types have been reported to be associated with aberrant expression of lncRNAs and the related dysregulation of mRNAs [5, 8, 11]. In colorectal cancer, some lncRNAs have been demonstrated to play a role as tumor suppressor or oncogene, implying their potential as a diagnostic marker or therapy target for CRC [12, 13].

Fer-1-like protein 4 (FER1L4) is a long noncoding RNA which is located on the chromosome 20 [14]. This lncRNA has been confirmed to be involved in tumorigenesis and tumor development [15]. LncRNA FER1L4 expression has been reported to decrease in various cancers, including gastric cancer [14],

colorectal cancer [16], osteosarcoma [17], endometrial carcinoma [18] and hepatocellular carcinoma [19]. However, there is little published data about involvement of FER1L4 in colorectal cancer [16]. On the other hand, the retinoblastoma gene, RB1, acts as a tumor suppressor gene in different tumor types, but its expression has been reported to increase in colorectal cancer [20–27]. The lncRNA FER1L4 and RB1 cooperate in a competing endogenous RNAs (ceRNAs) network involving in cancer biology, where, the expression of FER1L4 positively correlates with the RB1 expression. This study aimed to evaluate FER1L4 and RB1expression levels in colorectal tumor vs. paired marginal non-tumor tissues as well as in adenomatous colorectal polyps (ACPs) vs. their matched adjacent normal tissues. Finally, correlation between FER1L4 and RB1 expression levels in colorectal cancer was further explored.

Materials And Methods

Study subjects

A total of 160 specimens, including 50 colorectal tumors, 50 corresponding adjacent non-tumorous tissues, 30 adenomatous colorectal polyps, and 30 polyps' matched adjacent normal tissues were obtained from the Shahid Mahallati Hospital (Tabriz, Iran). The study was approved by the Research Ethics Committee of University of Tabriz (Tabriz, Iran) and written consents were collected from all patients. No patient received clinical treatment (radiotherapy, chemotherapy, or immunotherapy) before surgery or colonoscopy. The diagnosis of every specimen was confirmed by a pathologist.

RNA extraction, cDNA synthesisand expression analysis

RNX-plus Reagent (SinaClone, Iran) was used to isolate total RNA from tissues according to the manufacturer's instructions. The quality and quantity of isolated RNAs were measured using agarose gel electrophoresis and NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher). A total of 1000 ng RNA was reverse transcribed into cDNA in a volume of 10 µl using PrimeScript RT Reagent kit (TaKaRa) following the manufacturer's protocol. Primers were; RNU6 Forward: 5′-CTCGCTTCGGCAGCACAT-3′ and Reverse: 5′-GGAACGCTTCACGAATTTGC-3′, FER1L4 Forward: 5′-CCGTGTTGAGGTGCTGTTC-3 and Reverse: 5′-GGCAAGTCCACTGTCAGATG-3′, RB1 Forward: 5′-GCGTGCGCTCTTGAGGTT-3′ and Reverse: 5′-AGCCATGCAAGGGATTCCA-3′. The genes expression levels were quantified by qRT-PCR using Master Mix Green (RealQ plus 2x, AMPLIQON) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems). All reactions were performed in a total volume of 20 µL in duplicate format. The reaction conditions were: Initial denaturation at 95˚C for 10 min, followed by 40 repeats of a cycling stage consisting of 20 sec at 95˚C (denaturation), 30 sec at 58˚C (RB1), at 60˚C (FER1L4), at 65 ˚C (RNU6) (primer annealing) and 20 sec at 72˚C (extension). Data were normalized to RNU6 and the relative expression of FER1L4 and RB1 were calculated by the $2^{-\Delta Ct}$ method.

Statistical analysis

Data were analyzed using GenEx 7, SPSS v25, and GraphPad Prism v8 software programs. Kolmogorov–Smirnov, one-way analysis of variance test, Kruskal-Wallis, independent samples T-test, and Mann-

Whitney U test were used as appropriate. The Kolmogorov-Smirnov test was used to determine the normality of the data. Expression levels in tumor and polyp tissues and their margins were assessed by ANOVA test. The clinicopathological features in the two groups by independent samples T-test and and Mann-Whitney U test were evaluated in normal and abnormal data, respectively. Non normal data in more than two groups were analyzed first by Kruskal-Wallis test and then ANOVA test. A receiver operating characteristic (ROC) curve was established to evaluate the diagnostic value. Also, the Pearson test was used to investigate the correlation between FER1L4 and RB1. The data were presented as the mean ± SD and $P < 0.05$ was considered statistically significant.

Results

Study subjects

Clinicopathological characteristics of the study subjects are shown in Tables 1 and 2. Briefly, 80 patients with colorectal cancer or polyp were assessed. 78% of patients with the tumor were male while 22% were female. 62% of the patients with tumor were recognized as stage I or II, and 38% as stage III. Of the patients with polyp, 53.33% were men and 46.66% women.

LncRNA FER1L4 is downregulated in colorectal cancer

We analyzed the expression level of FER1L4 in 50 paired CRC tumor and non-tumor tissues as well as in 30 paired adenomatous colorectal polyps and their adjacent normal tissues by qRT-PCR. The results indicated that the expression level of lncRNA FER1L4 was significantly reduced in the colorectal cancer tissues compared with their matched adjacent non-tumor tissues ($p = 0.0027$, Fold change: 0.268), however, there was no significant difference between its expression in the adenomatous colorectal polyps and their matched adjacent normal tissues ($p = 0.99$, Fold change: 0.930). Comparing its expression between the tumor and polyp tissues revealed a significant decreased expression in tumor tissues ($p = 0.00029$, Fold change: 0.159) (Fig. 1a).

Additionally, the receiver operating characteristic (ROC) curve suggested a significant diagnostic ability for FER1L4 in CRC patients. The results demonstrated that the area under the ROC curve (AUC) was 0.764 (99% confidence interval = 0.5993 to 0.9310; $p = 0.0003$) for the paired tumor vs. marginal tissues, and 0.907 (99% confidence interval = 0.7716 to 1.0000; $p < 0.0001$) for the tumor vs. polyp tissues (Fig. 2).

The association of FER1L4 expression level in CRC, as well as in polyp tissues, with the clinicopathological features of the patients including gender, age, smoking status, size, location, histological grade, and TNM stage, was further analyzed. The results showed no association between its expression level neither in the CRC nor in the polyp tissues with the clinicopathological features (Tables 1 and 2).

RB1 is upregulated in colorectal cancer

RB1 expression level was also compared between tumor and non-tumor as well as between polyp and their adjacent normal tissues. As shown in Fig. 1b, the expression level of RB1 was significantly higher in the tumor tissues compared with the corresponding adjacent non-tumor tissues ($p = 0.00065$, Fold change: 3.678), however, there was no significant difference between polyps and matched normal tissues ($p = 0.90$, Fold change: 1.317). Comparing RB1 expression levels between tumor and polyp tissues revealed that its expression was significantly increased in tumor tissues ($p = 0.01343$, Fold change: 3.067).

ROC curve analysis identified an area under the curve (AUC) of 0.766 (99% confidence interval = 0.6202 to 0.9117; $p < 0.0001$) for tumor vs. non-tumor and an AUC of 0.708 for tumor vs. polyp analyzes (99% confidence interval = 0.5388 to 0.8779; $p = 0.0040$) (Fig. 2).

Furthermore, the association between clinicopathological characteristics of the patients and the RB1 expression level was analyzed. As shown in Tables 1 and 2, none of the clinicopathological characteristics showed a significant association with its expression in the tumor or polyp groups.

RB1 expression positively correlates with FER1L4 expression

Correlation between FER1L4 and RB1 expression levels both in tumor samples as well as in polyp samples were investigated. We observed a statistically significant positive correlation between FER1L4 and RB1 expression levels in tumor ($R = 0.407$, $P = 0.023$) and in polyp samples ($R= 0.518$, $P = 0.033$) (Fig. 3).

Discussion

Over 1.8 million patients are diagnosed annually with colorectal cancer [1]. So, identification of the pathogenic molecular pathways and determination of therapeutic targets are needed. It has been recognized that mutations within the noncoding genome and deregulation of noncoding RNAs play critical roles in human diseases [13]. LncRNA, as a novel molecular target impacts on cancer pathogenesis [28]. As an example, LncRNA FER1L4 has been confirmed to retard tumor progression [6]. It is downregulated in several cancers, including gastric cancer [14], osteosarcoma [17], endometrial carcinoma [18], hepatocellular carcinoma [29], esophageal squamous cell carcinoma [30], lung cancer [31]. However, its expression in glioma is upregulated [15]. Furthermore, expression level of FER1L4 significantly associates with lymph node metastasis, distant metastasis, and TNM stage of diverse cancers [6, 14, 17, 18]. Previous experimental studies determined that FER1L4 could suppress proliferation, migration and invasion but induce apoptosis [6, 19, 30–33]. FER1L4 functions as a ceRNA (competing endogenous RNA) to regulate the expression of PTEN through miR-106a-5p and miR-18a-5p in gastric cancer and Osteosarcoma [10, 34]. It also interacts with RB1 which is mediated by miR-106a-5p. Both RB1 and FER1L4 are targets of miR-106a-5p. It was reported that knockdown of FER1L4 by siRNA could decrease both FER1L4 and RB1 levels in gastric cancer. FER1L4 knockdown elevates miR-106a-5p level that provides more chance to bind to other targets, such as RB1 and PTEN mRNAs [35].

To reveal if the lncRNA FER1L4 deregulates in CRC, here we analyzed its expression level in 50 pairs of colorectal tumors and their non-tumor marginal tissues as well as in 30 pairs of adenomatous colorectal polyps and their adjacent normal tissues. Furthermore, the expression level of RB1 as a ceRNA of FER1L4 was also investigated. The results showed that FER1L4 was significantly downregulated in the colorectal tumor tissues, but, there was no significant difference between the polyps and their adjacent normal tissues (Fig. 1a). Consistent with this findings, Yue et al. observed that FER1L4 significantly downregulated in colorectal tumor compared with the marginal non-tumor tissues [16]. Interestingly, as Fig. 1a shows, we observed a significant difference between FER1L4 expression in the colorectal tumor and polyp tissues. This observation implies that deregulation of FER1L4 might be occurred after transformation of adenomatous polyps to colorectal tumors. Furthermore, ROC curves analysis confirmed that FER1L4 expression level can significantly discriminate colorectal tumors from non-tumor tissues, as well as from adenomatous polyps, which implies its potential as a biomarker for colorectal cancer development. Although Yue et al. reported association of FER1L4 expression with cilinicoapathological features including depth of tumor invasion, lymph node metastasis, vascular invasion and clinical stage [16] we did not observe any significant association with the cilinicoapathological features.

RB1 is one of the first described tumor suppressor genes in a wide variety of human cancers, but its involvement and function in colorectal cancer is largely controversial. Some researchers reported its downregulation [36, 37], but, others observed its upregulation in CRC [20–23, 25–27]. Here we also observed significant upregulation of RB1 in the colorectal tumor compared with their matched marginal non-tumor tissues. However, its expression was not significantly different between polyps and their matched normal tissues. As stated above, miR-106a-5p targets the RB1 mRNA and regulates its function in a ceRNA network. Sponging miR-106a-5p by the lncRNA FER1L4 improves RB1 level and its appropriate tumor suppressor function [35]. As a general role, this ceRNA interaction network suggests that downregulation of FER1L4 should result in the downregulation of RB1 mRNA, implying a positive correlation between their expression levels. Despite downregulation of FER1L4 and upregulation of RB1 that we and some other researchers [20–27, 36] observed in colorectal tumors compared with their corresponding non-tumor tissues, a positive correlation between their expressions levels was identified when their expressions were analyzed just in tumor or polyp tissues in our study. This observation was consistent with their molecular relationship in the ceRNA network. Probably, the RB1 upregulation which was observed by us and some other researchers might be explained by its increased gene copy number and infrequent allelic loss at the RB1 locus in colorectal cancer [20, 38–40]. Lai et al. observed allelic imbalance (AI) of RB1 in colorectal carcinomas and suggested that AI was indicative of allelic loss [25]. So in this situation, remaining allele could increase RB1 expression by dosage compensation or homeostatic mechanisms, because mRNA and protein of RB1were observed in all samples with AI [25]. Furthermore, RB1 mRNA, Rb protein and Rb Phosphorylation were reported to be increased in colorectal carcinoma compared with paired normal colonic tissue [22–24, 26, 27]. RB1 commonly performs its tumor suppressor role by restraining E2F transcription factors, but this inhibition is abolished by

phosphorylation of the Rb protein. Rb phosphorylation as an oncogenic driver leads to increased proliferation and decreased apoptosis in CRC [21, 22].

Conclusions

In summary, we observed significant downregulation of lncRNA FER1L4 but overexpression of RB1 in colorectal cancer. However, their expression was positively correlated both in the tumor and polyp samples. Neither FER1L4 nor RB1 expression levels were associated with clinicopathological characteristics of the patients. However, both FER1L4 and RB1 expression levels could discriminate colorectal tumor from non-tumor tissues, as well as from the adenomatous polyps, highlighting their potential as biomarker for colorectal cancer development.

Declarations

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Conflicts of interest/Competing interests

The authors declare they have no conflict of interest.

Availability of data and material

Not applicable

Code availability

Not applicable

Authors' contributions

Marjan Ostovarpour: Conceptualization, Methodology, Writing- Original draft preparation, Data analysis, Investigation. Mohammad Khalaj-Kondori: Conceptualization, Methodology, Writing - Review & Editing, Resources, Validation, Project administration. Tayyebeh Ghasemi: Writing - Review & Editing, Methodology.

Ethics approval

This study was approved by the Ethics Committee of University of Tabriz (approved number: IR.TABRIZU.REC.1398.013) and informed written consent was obtained from patients

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent for publication

Author Declaration and Consent to Publish agreement

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Tables

Table 1

Association between FER1L4 or RB1 expression ($2^{-\Delta Ct}$) and different clinicopathological features in CRC patients

Clinicopathological features	No. of cases (Percent)	FER1L4 relative expression Mean ± SD	P value	RB1 relative expression Mean ± SD	P value
Gender			0.21		0.52
Male	39 (78)	0.21 ± 0.41		0.90 ± 1.48	
Female	11 (22)	0.03 ± 0.03		0.58 ± 0.45	
Age (years)			0.173		0.175
< 65	19 (38)	0.09 ± 0.24		0.82 ± 1.74	
≥ 65	31 (62)	0.22 ± 0.42		0.84 ± 1.07	
Smoking status			0.753		0.935
Ever and current	35 (70)	0.15 ± 0.28		0.95 ± 1.50	
Never	15 (30)	0.22 ± 0.55		0.43 ± 0.34	
Histological grade			0.53		0.084
Well	40 (80)	0.19 ± 0.40		0.97 ± 1.46	
Moderately, Poorly	10 (20)	0.10 ± 0.14		0.30 ± .037	
Tumor stage			0.734		0.859
I/II	31 (62)	0.16 ± 0.42		0.85 ± 1.17	
III	19 (38)	0.18 ± 0.32		0.81 ± 1.58	
Lymph node metastasis			0.777		0.989
Negative	27 (54)	0.17 ± 0.43		0.81 ± 1.17	
Positive	23 (46)	0.17 ± 0.31		0.86 ± 1.54	
Vascular invasion			0.961		0.569
Absent	11 (22)	0.04 ± 0.04		1.24 ± 2.45	
Present	39 (78)	0.20 ± 0.40		0.75 ± 1.01	
Tumor size (cm)			0.44		0.095
< 5	18 (36)	0.06 ± 0.11		0.65 ± 0.98	
≥ 5	32 (64)	0.22 ± 0.43		0.91 ± 1.48	
Location			0.873		0.565
Rectum	15 (30)	0.04 ± 0.04		0.95 ± 1.38	
Colon	35 (70)	0.20 ± 0.41		0.79 ± 1.35	

Table 2

Association between FER1L4 or RB1 expression ($2^{-\Delta Ct}$) and different clinicopathological features in patients with adenomatous colorectal polyps

Clinicopathological features	No. of cases (Percent)	FER1L4 relative expression Mean ± SD	P value	RB1 relative expression Mean ± SD	P value
Gender			0.434		0.159
Male	16 (53.33)	0.32 ± 0.32		0.36 ± 0.44	
Female	14 (46.66)	0.35 ± 0.21		0.30 ± 0.74	
Age (years)			0.071		0.942
< 58	16 (53.33)	0.29 ± 0.40		0.47 ± 0.77	
≥ 58	14 (46.66)	0.32 ± 0.35		0.16 ± 0.11	
Smoking status			0.177		0.767
Ever and current	12 (40)	0.27 ± 0.30		0.33 ± 0.44	
Never	18 (60)	0.40 ± 0.24		0.34 ± 0.69	
Size of ACP			0.202		0.150
1–5 mm	9 (30)	0.53 ± 0.30		0.69 ± 1.04	
6–9 mm	18 (60)	0.29 ± 0.26		0.17 ± 0.22	
10 mm or more	3 (10)	0.16 ± 0.02		0.42 ± 0.55	
Location			0.933		0.896
Anal, Rectum	8 (26.66)	0.29 ± 0.31		0.27 ± 0.38	
Sigmoid,	15 (50)	0.36 ± 0.26		0.39 ± 0.72	
Descending colon	2 (6.66)	0.22 ± 0.01		0.05 ± 0.07	
Transverse colon	5 (16.66)	0.34 ± 0.38		0.36 ± 0.59	
Ascending colon, cecum					
Polyp type			0.344		0.685
Tubular	24 (80)	0.37 ± 0.29		0.38 ± 0.65	
Tubuvillus	6 (20)	0.15 ± 0.02		0.14 ± 0.13	
Indications for			0.164		0.339
referral	10 (33.33)	0.40 ± 0.29		0.49 ± 0.90	
Abdominal pain	9 (30)	0.23 ± 0.20		0.16 ± 0.11	
Constipation	6 (20)	0.12 ± 0.14		0.07 ± 0.07	
Bleeding per rectum	3 (10)	0.77 ± 0.11		0.86 ± 0.66	
Anemia	2 (6.66)	0.57 ± 0.13		0.19 ± 0.12	
Diarrhea					