

SNHG6 203 and SNHG6 201 Transcripts Can be Used as Contributory Factors for a Well-Timed Prognosis and Diagnosis of Colorectal Cancer

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Abstract

Background: Long non-coding RNAs, as a big part of non-coding RNAs, are considered functionally more than past. These transcripts could be involved in carcinogenesis. SNHG6, as a long non-coding RNA, has been reported to be expressed more in colorectal cancer tissues than non-cancerous ones. Colorectal cancer as a malignancy needs fast prognostic and diagnostic methods for well-timed treatment. SNHG6 RNA and its relative variants can be considered as biomarkers for a well-timed treatment of colorectal cancer.

Methods: RNA extraction from 32 colorectal cancer tissues and their relative non-cancerous tissues were carried out and cDNA of the mentioned RNAs was synthesized and RT-qPCR was performed. Relative expression of SNHG6 201 and 203 were studied in colorectal cancer samples with different clinicopathological characteristics.

Results: The expression patterns of SNHG6 201 and 203 variants were different. SNHG6 203 was expressed significantly higher in colorectal tumor tissues than non-cancerous ones. In spite of SNHG6 203, SNHG6 201 was expressed significantly in colorectal non-cancerous tissues more than tumor ones. Additionally, expression of these variants in different colorectal cancer cell lines was different.

Conclusion: It seems that SNHG6 203 transcript might be considered as a prognostic and diagnostic biomarker in colorectal cancer case studies and treatments. Also, SNHG6 201 can distinguish precisely the tumor and non-tumor tissues in colorectal cancer.

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Introduction

Nowadays, long non-coding RNAs are considered as a big functional part of the transcriptome. These RNAs are proved as key regulators of cell behaviors, the role which was designated to the proteins before. LncRNAs are participated in

cell cycle progression, apoptosis, cell senescence, cell migration, cell viability and epithelial to mesenchymal transition (1-6). LncRNAs, as factors that affect cell cycle progression and apoptosis, could be involved in cancer progression. In fact, many of these transcripts express more in

tumor tissues than adjacent non-cancerous ones. For example, HOTAIR, as a well-known lncRNA, is more expressed in breast tumor tissues than non-cancerous ones (7). Higher expression of these RNAs in tumor tissues could be potentially applied as a prognostic and diagnostic factor for the well-timed treatment of cancers. Small nucleolar RNA host gene 6 (SNHG6), as a kind of long non-coding RNA, has been featured recently. SNHG6 RNA has a special sequence in its gene body. SNHG6 gene has u87 RNA (SNORD 87) sequence in its gene sequence, which is a small nucleolar RNA (snoRNA). Small nucleolar RNAs are able to alter rRNAs chemically for maturation (8-10). The process of rRNA alteration and maturation is critical for biogenesis of ribosomes and their maturation (11-14). Ribosomes as translation apparatus play crucial roles in carcinogenesis. SNHG6 RNA, as a long non-coding RNA that contains one snoRNA sequence in its gene body, might vary fate of the cancer cells express it. Up-regulation of SNHG6 RNA in a cancerous cell could increase ribosomes biogenesis and might be an advantage for proliferation of that cancerous cell (15-17). Splice variants of a long non-coding RNA might have important roles in carcinogenesis (19). Therefore, we were encouraged to study two splice variants of SNHG6 RNA. In this research, we studied the relative expression and expression pattern of SNHG6 201 and SNHG6 203 in 32 colorectal tumor tissues and their relative non-cancerous tissues. This study aimed to discover new molecular biomarkers, as contributory factors, for a precise prognosis and diagnosis of colorectal cancer.

Material and methods

Patients and specimens

The tumor and non-cancerous tissues of 32 colorectal cancer patients were obtained from Iran National Tumor Bank and they were stored at -185°C. The ethical committee of Kerman Graduate University of Advanced Technology approved the procedure of experiments. Also, written informed consent of patients was obtained from Iran National Tumor Bank.

RNA extraction

50-100 mg of the tissue was put in liquid nitrogen and then it was grinded. 1 ml of RnX plus solution was added to the grinded tissue and moved to a 1.5 ml micro tube. Then, 200 µl of chloroform was added to the tube and the contents were shaken vigorously for 15 seconds. The micro tube was placed on ice for 15 minutes and then centrifuged at 4°C, in 12000 rpm for 15 minutes. The above clear phase was removed and poured into a new micro tube. Next, an equal volume of cold isopropyl alcohol was added to the clear phase, and the contents were mixed gently by pipetting. The tube was kept in -20°C for an hour and then centrifuged at 4°C, in 12000 rpm for 15 minutes. After centrifugation, RNA pellet was placed at the bottom of the tube. The supernatant was poured out and the pellet was washed with ethyl alcohol 75% which was prepared with DEPC treated water. Final centrifugation was performed in 10000 rpm, at 4°C for 10 minutes. Then, the RNA pellet was dissolved in 30 µl of RNAase free water. The quality and quantity of extracted RNAs were assessed by agarose gel electrophoresis and 260/280 absorbance, respectively.

cDNA synthesis

1 µg of extracted RNAs was applied to synthesize cDNA. Briefly, in a 10 µl reaction, the extracted RNA was treated with DNAaseI to remove genomic co-purified DNA. Then, DNAase was inactivated by adding 1 µl of EDTA 50mM

solution. Next, 1 µl of random hexamer was added to the tube. At the 4th step, RT complex was added to the tube. Finally, RT enzyme was inactivated in 70°C for 10 minutes. The details are shown in Table 1.

Table 1. The steps and the conditions of cDNA synthesizing

DNAase treatment	1µl DNAase enzyme +1µl buffer + 1µg RNA + up to 10 µl nuclease free water	37°C	30 minutes
DNAase inactivation	1µl EDTA 50mM	65°C	10 minutes
Random hexamer addition	1µl random hexamer	65°C	5 minutes
RT complex addition	(1µl Reverse transcriptase enzyme, 4 µl Reverse transcriptase enzyme buffer, 2 µl dntp mix and 0.5 µl RNAase inhibitor)	25°C	10 minutes
		42°C	60 minutes
RT enzyme inactivation		72°C	10 minutes

RT-qPCR

Relative expression of SNHG6 201 and 203 variants was assessed by ABI real time instrument and the expression data was normalized using β actin as a housekeeping gene. Quantitative RT-PCR was performed in 10µl reaction on ABI step one plus qPCR system. 0.5 µl of cDNA as template, 0.5µl of primers (reverse and forward) and 5 µl of YTA qPCR master mix were mixed and up to 10 µl, nuclease free water was added. The primer sequences are shown in Table 2 and

thermal cycles of qPCR were carried out based on the information depicted in Table 3.

Polymerase chain reaction (PCR)

PCR was performed according to the amplicon PCR master mix protocol. The PCR conditions are shown in Table 4.

Table 2. Sequences of applied primers

	Forward primer	Reverse primer
β Actin	ACTCTCTTCCAGCCTTCCTTCCT	ACTGACAGCACTGTGTGGCGTA
SNHG6 203	GAGTGCCTAAGAGCTGTCTTCC	GCCGCGTGATCCTAGTAGTT
SNHG6 201	AAAACACTAGGATCACGC	CTAGTGAATGAGAATGGAG

Table 3. qPCR conditions of SNHG6 203 and 201

	Initial denaturation	Denaturation in cycles	Annealing and extension
SNHG6 203	95°C for 40 sec	95°C for 5 sec	62°C for 40 sec
SNHG6 201	95°C for 40 sec	95°C for 5 sec	65°C for 40 sec

Table 4. PCR conditions of SNHG6 203 and 201

	Initial denaturation	Denaturation	Annealing	Extension	Final extension
SNHG6 203 and 201	4 min	30 sec	30 sec	30 sec	5 min
	94°C	94°C	201 (65°C), 203 (62°C)		

Statistical analysis

The normalized relative expression of SNHG6 201 and 203 was analyzed by unpaired t test and significant level was considered at $P < 0.05$. To determine the sensitivity and specificity of SNHG6 203 and 201 as molecular biomarkers, ROC curve analysis was performed. P value < 0.05 was considered as significant. Graphpad prism 6 software was applied for Statistical analysis.

Cell culture

Sw1116 and sw480 cells were cultured in RPMI1640 medium with 10% of fetal bovine serum in a humidified incubator with 5% CO_2 atmosphere.

Results

SNHG6 203 was expressed in colorectal tumor tissues more than non-cancerous ones

SNHG6 203 expression assessment in colorectal tumor and non-cancerous tissues demonstrated that it was expressed in tumor tissues more than non-cancerous tissues and this expression difference was significant. ROC curve analysis of tumor and non-cancerous tissues showed high sensitivity and specificity of SNHG6 203 as a biomarker Figure 1 a and b.

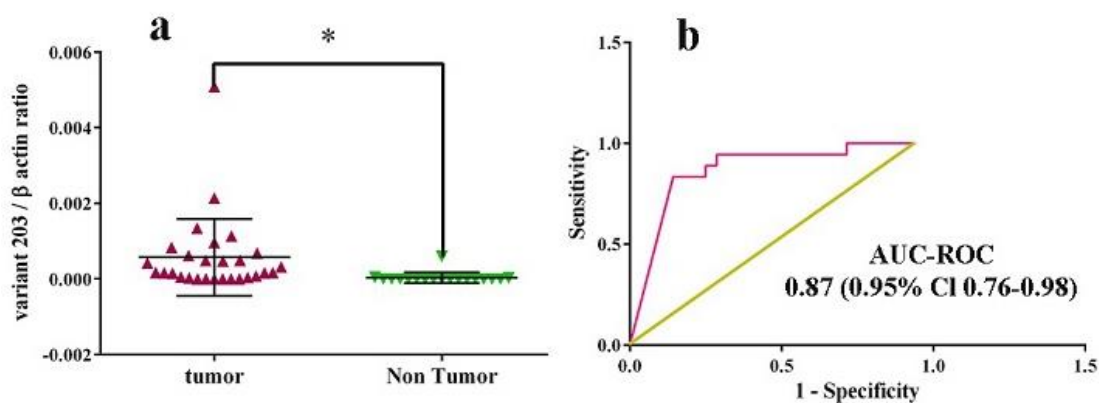


Figure 1. a: Colorectal tumor tissues expressed SNHG6 203 more than non-tumor ones. b: The ROC curve analysis result demonstrated SNHG6 203 could be as a suitable biomarker for discriminating of tumor and non-tumor tissues in colorectal malignancies.

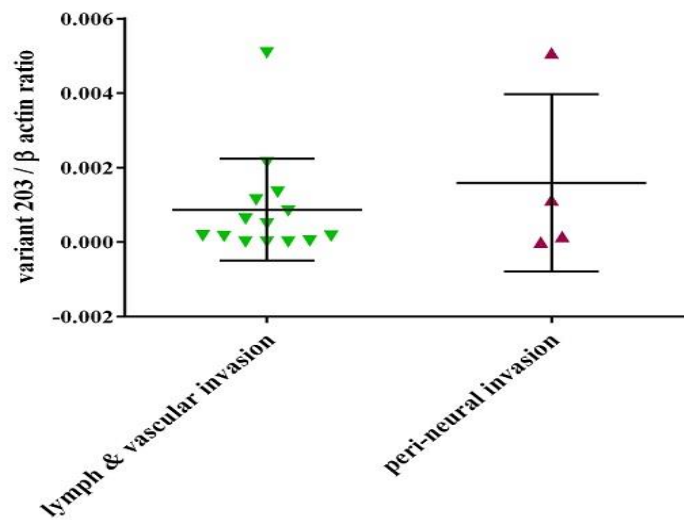


Figure 3. Non-significant expression difference of SNHG6 203 in colorectal tumors that invade their surroundings tissues was demonstrated

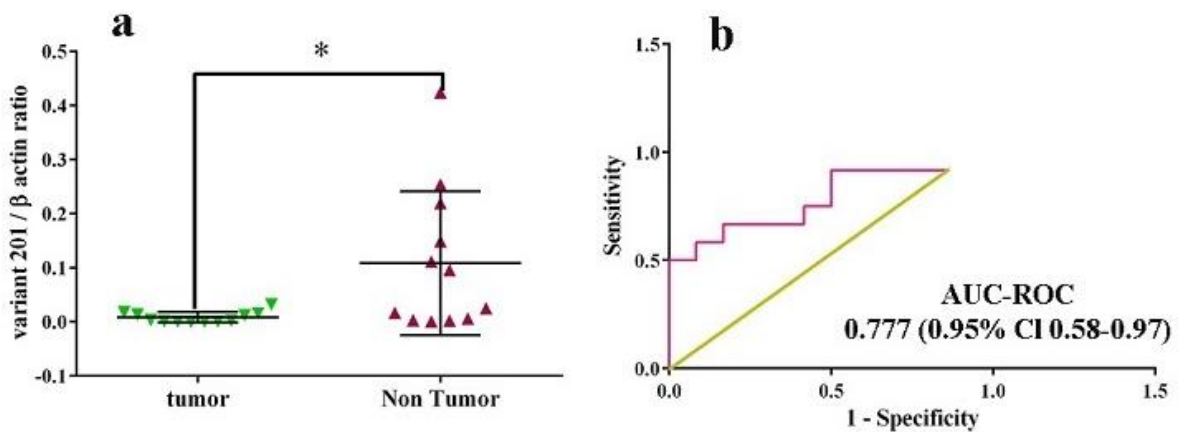


Figure 4. a: SNHG6 201 was expressed more in non-tumor tissues of colorectal cancer than tumor ones. b: based on ROC curve analysis of tumor and non-tumor tissues of colorectal cancer, SNHG6 201 can be used as a biomarker for identification of non-tumor tissues in colorectal malignancy

SNHG6 201 was expressed in low grade colorectal tumors more frequent than high grade ones

The expression patterns of SNHG6 201 in low grade colorectal tumors and high grade ones showed that this variant was expressed more frequently in low grade colorectal tumors Figure 5.

SNHG6 201 was expressed more frequently in tumors with lymph and vascular invasion

SNHG6 201 expression measurement in colorectal tumor samples with different invasion statuses indicated that this variant was expressed more frequently in tumors with lymph and vascular invasion than tumors with perineural invasion Figure 6.

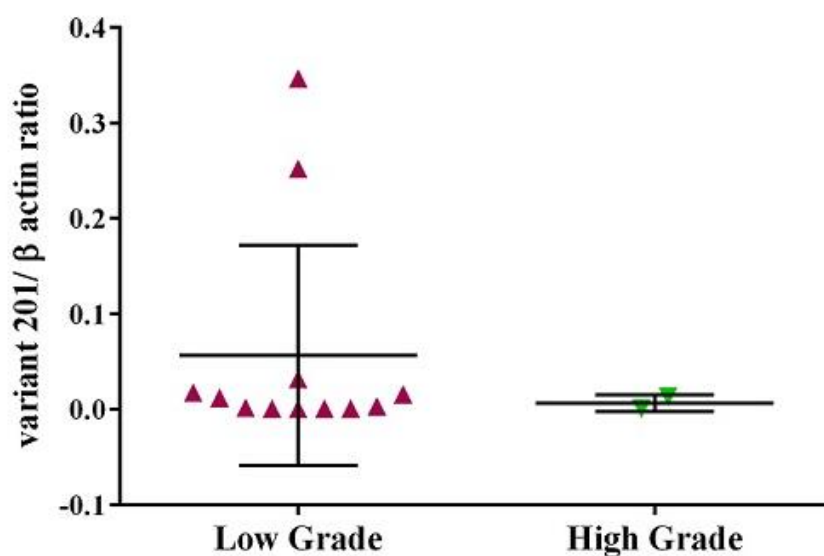


Figure 5. SNHG6 201 expression was happened in low grade colorectal tumors more frequent than high grade ones

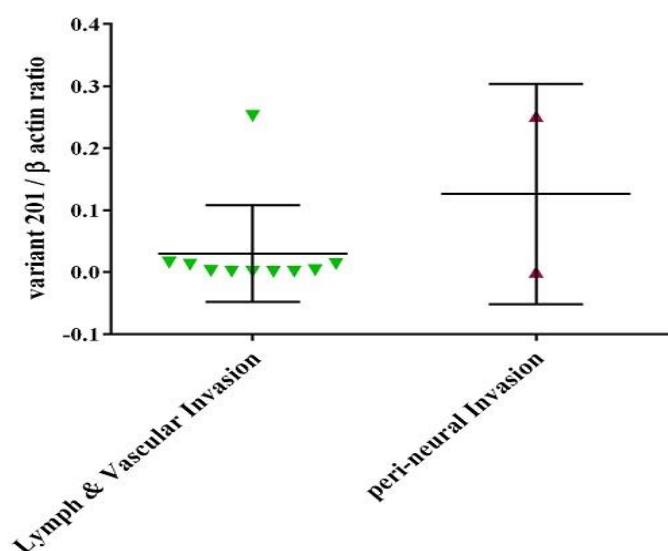


Figure 6. SNHG6 201 was expressed in lymph and vascular invading tumors more frequent than pre-neural invading tumors

SNHG6 201 and 203 were expressed differentially in colorectal cancer cell lines with different degrees of malignancy

SNHG6 expression was visualized in sw480 and sw1116 cell lines by agarose gel electrophoresis. A reverse expression pattern was indicated for SNHG6 201 and 203. SNHG6 203

was expressed in sw480 but it was not expressed in sw1116 cells. SNHG6 201 was expressed in sw1116 cells but it was not expressed in sw480 cells Figure 7.

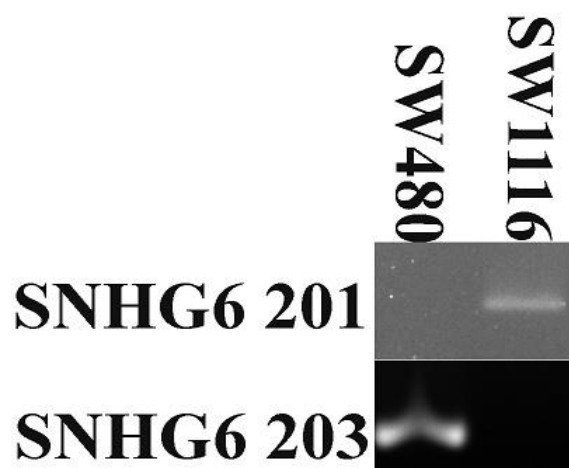


Figure 7. SNHG6 201 was expressed in SW1116 cells but not in SW480 cells. Reversely, SNHG6 203 was expressed in SW480 cells and was not being expressed in SW1116 cells

Discussion

RNA splicing is one of molecular mechanisms which causes diversity in proteins and also in long non-coding RNAs. As mRNA splicing results in protein diversity and protein structure evolution (20, 21), RNA splicing can be considered as an enhancer factor for diversity in other RNA molecules of the cells such as long RNAs.

Sometimes a special variant of a long non-coding RNA could extremely affect the cells express it (19,22,23), so its investigation is useful for better understanding of their carcinogenesis mechanism(s). As SNHG6 was proved as an oncogene long non-coding RNA (18,24,25), we were encouraged to study the two splice variants of it.

Colorectal cancer, as a widespread cancer among people, needs quick prognosis and diagnosis methods for a well-timed treatment. A quick diagnostic method will improve the patients' survival significantly. In this study, we analyzed the relative expression of SNHG6 201 and 203 variants in colorectal cancer cases to find an applied molecular biomarker for a well-timed prognosis and diagnosis of this cancer.

Relative expression analysis of SNHG6 203 & 201 in colorectal tumor and non-cancerous tissues indicated that the expression patterns of the variants were different. SNHG6 203 expression in colorectal tumor tissues was more than non-cancerous ones. In spite of SNHG6 203, the other studied variant (variant 201) demonstrated a reverse expression pattern than SNHG6 203 expression.

SNHG6 203, as a known splice variant of SNHG6, was proved as an oncogene transcript in hepatocellular carcinoma (23). This splice variant has not been studied in colorectal cancer yet. In this research, we realized that the expression pattern of SNHG6 203 varied significantly in tumor and non-cancerous tissues of colorectal cancer.

SNHG6 203 expression in colorectal tumor tissues was significantly more than non-cancerous ones Figure 1a and b. This difference in expression may position this transcript as a biomarker for distinguishing of colorectal tumor tissues and non-cancerous tissues. The significant expression difference of SNHG6 203 between these two groups of tissues might be useful as a contributory prognosis and diagnosis factor. Also,

the expression pattern of SNHG6 201 in colorectal tumor and non-cancerous tissues could be as an additional factor to distinguish colorectal tumors more precisely Figure 4 a and b.

Concordant with above, the ROC-curve analysis of SNHG6 203 & 201 expression proves these two variants can be used as two prognostic and diagnostic factors in relation to colorectal cancer. Additionally, we observed that these two variants were expressed differently in colorectal cancer cell lines. Expression of SNHG6 203 in sw480 (as an intermediate

malignant cell line) but not in sw1116 (as a less malignant cell line than sw480) could declare a probable role of this variant in colorectal malignancies induction.

Conclusion

SNHG6 203 and 201 can be applied as contributory factors for a well-timed prognosis and diagnosis of colorectal cancer.

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