

Investigation of KLF11 and LC3 Genes Expression in Colorectal Cancer Patients

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Abstract

Background: Colorectal cancer is the fourth leading cause of cancer deaths around the world. This type of cancer, like other cancers, is caused by the influence of environmental and genetic factors. One of the important mechanisms involved in cancer, especially colorectal cancer, is the autophagy mechanism. In this mechanism, important genes have been identified, including KLF11 and LC3 genes.

Method: To evaluate the gene expression level, forty colorectal tumor samples and forty adjacent normal samples were used for carrying out Real-Time PCR method and western blotting analysis.

Results: We reported that there is a significant difference in the expression level of KLF11 and LC3 genes in tumor samples compared to normal samples ($P > 0.05$).

Key Words - Colorectal cancer, Autophagy, Real Time-PCR, Western blotting, KLF11, LC3.

I. INTRODUCTION

Colorectal cancer is one of the most common cancers in the gastrointestinal tract that is the second leading cause of death in women (9.4% of all cancer) after breast cancer and in men it is the third leading cause of death (10% of all cancers) after lung and prostate carcinoma, also it is the fourth cause of death of cancers in the world (Siegel et al., 2017). The annual incidence of this cancer in North America and Europe is reported to be between 30 and 50 cases per 100,000 people so that estimated at 3 to 7 per 100,000 in the Middle East (Bülow, 1980; Stewart & Kleihues, 2003). In 2016, 49,190 people were diagnosed with this cancer in the United States (Boyle & Leon, 2002).. The symptoms of colorectal cancer include intestinal problems such as diarrhea and constipation, dark stools, intestinal bleeding and weight loss (Astin, Griffin, Neal, Rose, & Hamilton, 2011). The cause of colorectal cancer, as with other cancers,

is unclear, but evidence and experience indicate that two important environmental and hereditary factors contribute to its formation and sometimes both factors with together can cause cancer (De Rosa et al., 2015). In addition, the risk factors for developing colorectal cancer include age, personal history of adenomatous polyps, personal history of inflammatory bowel disease, family history of colorectal cancer or adenomatous polyps, inherited genetic risk, also of the environmental factors can be mentioned to nutritional practices, physical activity and obesity, cigarette smoking, heavy alcohol consumption (Haggard & Boushey, 2009). One of the important mechanisms involved in cancer, especially colorectal cancer, is the autophagy mechanism (Burada et al., 2015). Autophagy is a process in which cell membranes undergo morphological changes and then destroy cellular proteins and cytoplasmic organs (Kondo, Kanzawa, Sawaya, & Kondo, 2005). According to recent studies, several genes are involved in the autophagy mechanism of colorectal

cancer which among them can point to LC3 gene (Cho, Jo, Kim, Park, & Kim, 2012; Guo et al., 2011; Koukourakis et al., 2010). The LC3 gene was the first marker involved in the autophagy mechanism that was suggested to interfere with colorectal cancer (Tanida, Ueno, & Kominami, 2004). Kruppel-like transcription factor 11 (KLF11), a member of the Sp1/Kruppel-like factor zinc finger transcription factor (Sp1/KLF) family (Kuroda et al., 2009), was first found in osteoblast cells. KLF11 shares a highly conserved C-terminal DNA-binding domain that contains three C2H2 zinc finger motifs. The zinc finger motif facilitates binding to GC-rich promoter elements which, in turn, regulate multiple cellular events, including suppressing cell cycle/proliferation, and promoting apoptosis. As an inhibitory transcription factor, KLF11 participates in the transforming growth factor- β (TGF- β) signaling pathway mainly through regulating the TGF- β induced expression of SMAD7 gene, which was thought to be regulated by a plethora of growth factors, cytokines, and hormones. Due to the high importance of these genes in the pathway of autophagy and colorectal cancer, we examined the expression levels of these genes in colorectal cancer tissues.

II. MATERIAL AND METHODS

2.1 Human specimens

Human colorectal cancer specimens ($n = 40$) and adjacent non-tumor tissues were obtained from patients at hospital with informed consent from each patient. The type

of the disease was diagnosed by the pathologists and the patients did not receive any type of treatment.

2.2 RNA extraction, cDNA preparation and Real-time quantitative PCR

Total RNA was isolated from each tumor tissue and adjacent non-tumor tissue by using RiboEx (GeneAll, Korea) according to the manufacturer's specifications. The concentration of total RNA in the final eluate was determined by spectrophotometry and the absorbance 260/280 ratio was controlled between 1.8 and 2.0. The synthesis of cDNA was performed using the Prime Script RT reagent kit (Takara, Japan) according to the manufacturer's specifications. The obtained cDNAs were stored in -70°C until use. Real-time PCR was performed using a StepOnePlus™ Real-Time PCR Systems (ABI Applied Bio-systems, Thermo Fisher Scientific, USA) in a 15- μl reaction containing 7.5- μl of RealQ Plus 2x Master Mix Green High ROX™ (Ampliqon, Denmark), 1- μl of cDNA, 5.5- μl of H₂O and 1- μl of mixed forward and reverse primers (6 Pmol/ μl concentration). Real-time PCR amplifications were done as follows: for two selected genes, PCR amplification was set to an initial 95°C for 15 min and then for all genes, a total of 40 cycles, 95°C for 15 seconds and 58°C for 1 min (step and hold). All samples were analyzed in duplicate. GAPDH was used as an internal control. Gene expression was calculated using the comparative threshold cycle ($2^{-\Delta\Delta\text{CT}}$) method. The primers used for real-time PCR are listed in Table 1.

Table 1. Primer sequences of two selected genes and GAPDH.

Target genes		Sequences (5' → 3')	Product length
KLF11	Forward	GCATGACAGCGAAAGGTCTAC	128bp
	Reverse	GGGGTCTTATCCGCAACAGG	
LC3	Forward	TACAGCAGATACGCGACCAG	193bp
	Reverse	TTCACCAGCAGGAAGAAGGC	
GAPDH	Forward	CATCAAGAAGGTGGTGAAGCA	120bp
	Reverse	GCGTCAAAGGTGGAGGAGTG	

2.3 Western blotting

Cell protein lysates were separated in 10% sodium dodecyl sulfate–polyacrylamide gels and transferred onto polyvinylidene fluoride membranes (Roche Diagnostics, Mannheim, Germany). All proteins were detected using antibodies purchased from Univ-bioInc (Shanghai, China).

2.4 Statistical Analysis

Statistical analysis was performed using the GraphPad Prism v7.03 (GraphPad Software Inc., USA) and T-test. For all tests, a P value <0.05 was considered statistically significant.

III. RESULTS

3.1 survey of changes on KLF11 gene expression level in colorectal tumor tissues using Real Time-PCR method

According to figure 1 the outcomes obtained of KLF11 gene expression level showed that out of 40 colorectal tumor samples, KLF11 expression level in 29 (72.5%) tumor samples were decreased compared with normal tissue and were increased in 11 (27.5%) tumor samples. But it

should be noted that these changes in expression level of KLF11 gene indicate that there is a significant difference between the expression level of tumor samples and normal samples ($P < 0.05$). Also, the results obtained from western blot analyzing showed a significant reduction in protein levels in this patients (Figure. 3a shows the result of KLF11 gene expression level in tumor samples comparison with adjacent normal samples).

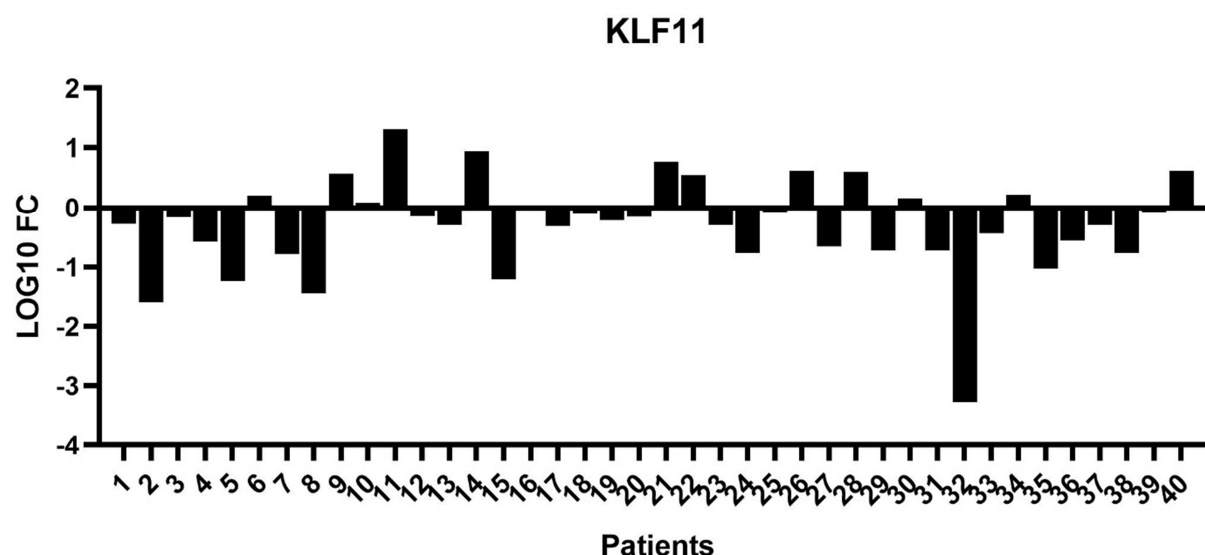


Figure.1. The graph related to KLF11 gene expression level.

3.2 survey of changes on LC3 gene expression level in colorectal tumor tissues using Real Time-PCR method

The results related to LC3 gene in 40 colorectal tumor samples demonstrated down-expression of this gene in 12 (30%) colorectal tumor samples and over-expression of LC3 gene in 28 (70%) colorectal tumor samples, according to figure.2. Also, due to statistical analysis, these changes in

expression levels of LC3 gene in tumor samples compared to normal samples was significant ($P > 0.05$). Also, the results obtained from western blot analyzing showed a significant increase in protein levels in this patients (Figure. 3b shows the result of LC3 gene expression level in tumor samples comparison with adjacent normal samples).

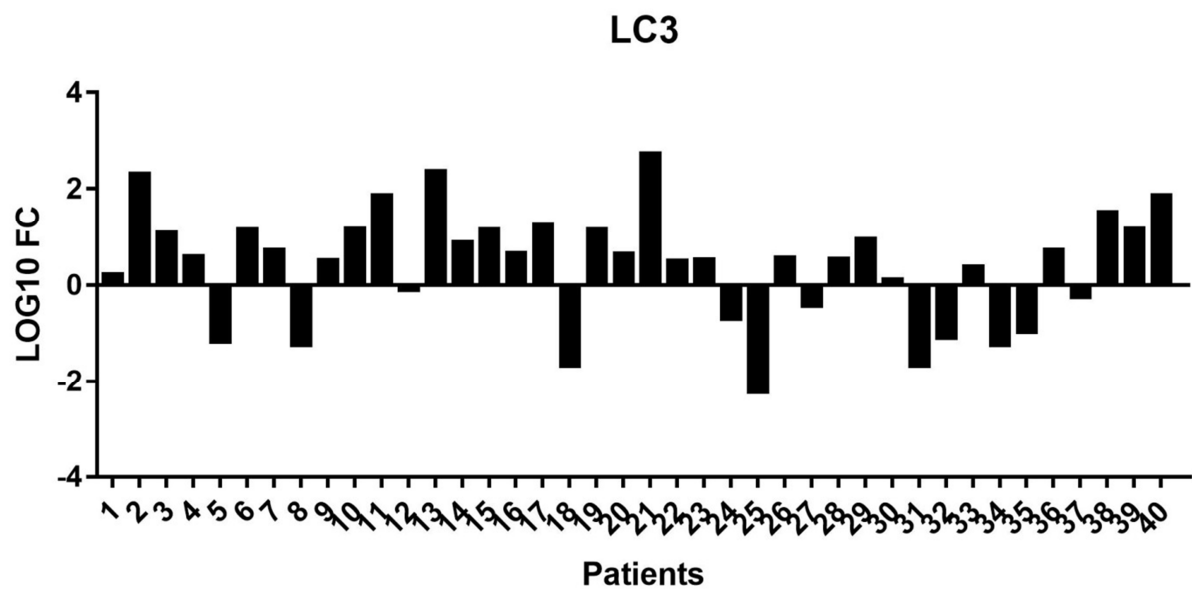


Figure.2. The graph related to LC3 gene expression level.

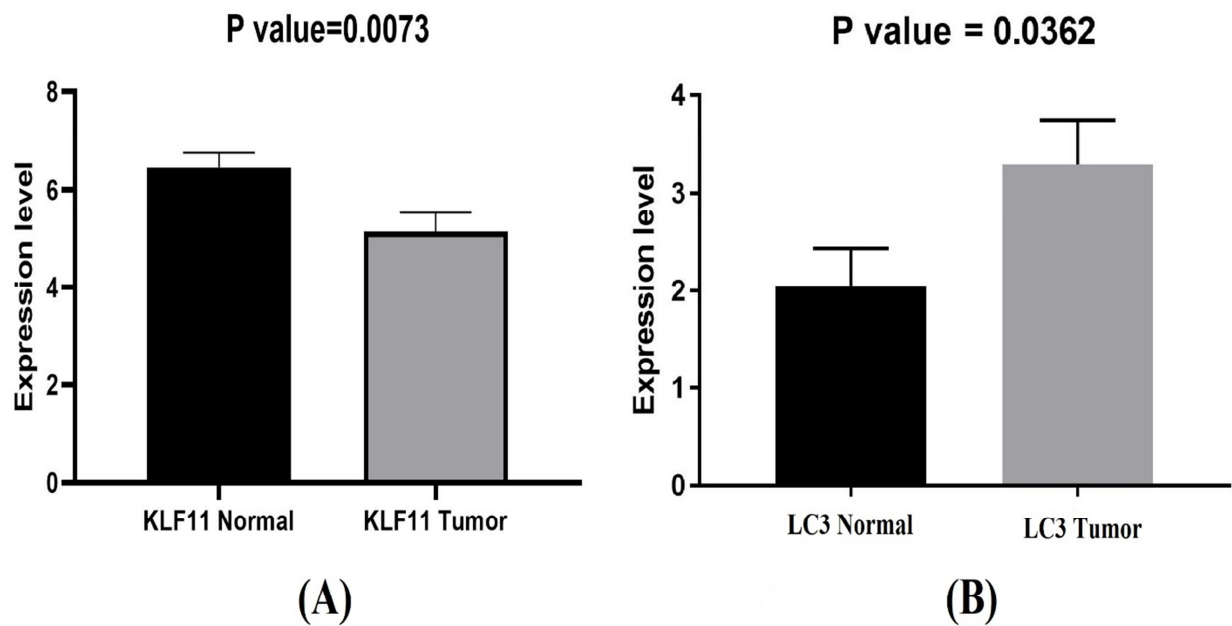


Figure.3. The graph (A) and (B) related to KLF11 and LC3 genes expression between colorectal tumor samples comparison with normal samples, respectively.

IV. DISCUSSION

Over the last few years, a great number of studies have reported aberrant patterns of gene expressions in various cancers including colorectal cancer. Despite the many advances in treating of colorectal cancer, the survival rate of colorectal cancer patients is still poor. Therefore, understanding of the different mechanisms involved in the onset and progression of this cancer can provide the basis for better treatment. In this study, we have analyzed the expression level of KLF11 and LC3 genes in 40 colorectal cancer patients by real-time quantitative PCR.

As mentioned, LC3 has been shown to be an autophagosomal marker in mammals and has been used to study autophagy in neurodegenerative and neuromuscular diseases, tumorigenesis, and bacterial and viral infections (Tanida, Ueno, & Kominami, 2004). Yoshioka et al. demonstrated that LC3 is upregulated in various gastrointestinal cancers and partly associated with Ki-67 index. Their results show that LC3 was highly expressed in 53% of esophageal, 58% of gastric and 63% of colorectal cancer tissues (Yoshioka et al, 2008).

Aberrant expression of the KLF11 gene has been reported in several human cancers. Wang et al. reported that the expression of KLF11 was significantly reduced in the tumor tissues compared to the normal ovarian tissues (Wang et al, 2015). This gene was also inactivated in myelodysplastic syndrome (Potapova et al, 2010). KLF11 along with TGF- β 2 involved in TGF- β signaling pathway and they accomplish a variety of activities including proliferation, differentiation, and apoptosis (Ellenrieder et al, 2008). The transforming growth factor TGF- β signaling, one of the most important signaling pathways that KLF11 plays in regulating it. This pathway controls a wide spectrum of cellular functions ranging from proliferation and differentiation to apoptosis. In carcinogenesis, TGF- β plays a dual role characterized by tumor suppression at early tumor stages and enhanced tumor progression at late stages of the disease. To date, Klf10 and Klf11 have been shown to be induced by several members of the TGF-beta superfamily and it's essential for normal cell functions. Therefore, down-regulation of this gene can beginning and development of CRC cancer.

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