

Investigation of ATG5, ATG12 and LC3 Genes Expression Related To Autophagy in Colorectal Cancer

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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 ATG12, ATG5, and LC3 genes.

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

Results: We reported that there was no significant difference in the expression level of ATG12 and LC3 genes in tumor samples compared to normal samples ($P > 0.05$), while the expression level of ATG5 gene with P Value=0.031 illustrated that this gene was reduced in colorectal tumor samples.

Keywords - Colorectal cancer, Autophagy, Real Time-PCR, ATG12, ATG5, 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 ATG12, ATG5 and LC3 genes (Cho, Jo, Kim, Park, & Kim, 2012; Guo et al., 2011; Koukourakis et al., 2010). One of the major functional groups in the formation of core machinery of autophagy is ubiquitin-like protein system which consists of two ATG12 and ATG8 (mammalian homologue LC3) proteins (Mari & Reggiori, 2007). The LC3 gene was the first marker involved in the autophagy mechanism that was suggested to interfere with colorectal cancer (Tanida, Ueno, & Kominami, 2004). The conjugation of ATG12 and ATG5 with cooperating ATG16L1, take part in the formation of the outer membrane of autophagosome (Geng & Klionsky, 2008; Tanida, Minematsu-Ikeguchi, Ueno, & Kominami, 2005). Various studies have reported that mutation in ATG5 and ATG12 genes are associated with colorectal and gastric cancers (Kang et al., 2009). There is also a correlation between mutations in the ATG5 gene and a decrease in the expression level of this gene in colorectal cancer (An, Kim, Yoo, Park, & Lee, 2011). Additionally, high level of ATG10 protein was found in metastatic colorectal cancer tissues (Jo et al., 2012). 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 = 30) 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 (3 Pmol/μl concentration). Real-time PCR amplifications were done as follows: for three 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 ($2^{-\Delta\Delta CT}$) method. The primers used for real-time PCR are listed in Table 1.

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

Target genes		Sequences (5' → 3')	Product length
ATG5	Forward	CGGGCAATCAATCGGAAACTC	127bp
	Reverse	CAGCCA CAGGACGAA ACA C	
ATG12	Forward	TGTATCAGTCCTTTGCTCCTTCC	131bp
	Reverse	GTTGCTTTTCTTTGTGGTTCATCC	
LC3	Forward	TACAGCAGATACGCGACCAG	193bp
	Reverse	TTCACCAGCAGGAAGAAGGC	
GAPDH	Forward	CATCAAGAAGGTGGTGAAGCA	120bp
	Reverse	GCGTCAAAGGTGGAGGAGTG	

2.3 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 ATG12 gene expression level in colorectal tumor tissues using Real Time-PCR method

According to figure 1 the outcomes obtained of ATG12 gene expression level showed that out of 30 colorectal

tumor samples, ATG12 expression level in 16 (53.3%) tumor samples were decreased compared with normal tissue and were increased in 14 (46.7%) tumor samples. But it should be noted that these changes in expression level of ATG12 gene indicate that there is no significant difference between the expression level of tumor samples and normal samples ($P>0.05$). (Figure. 4a shows the result of ATG12 gene expression level in tumor samples comparison with adjacent normal samples).

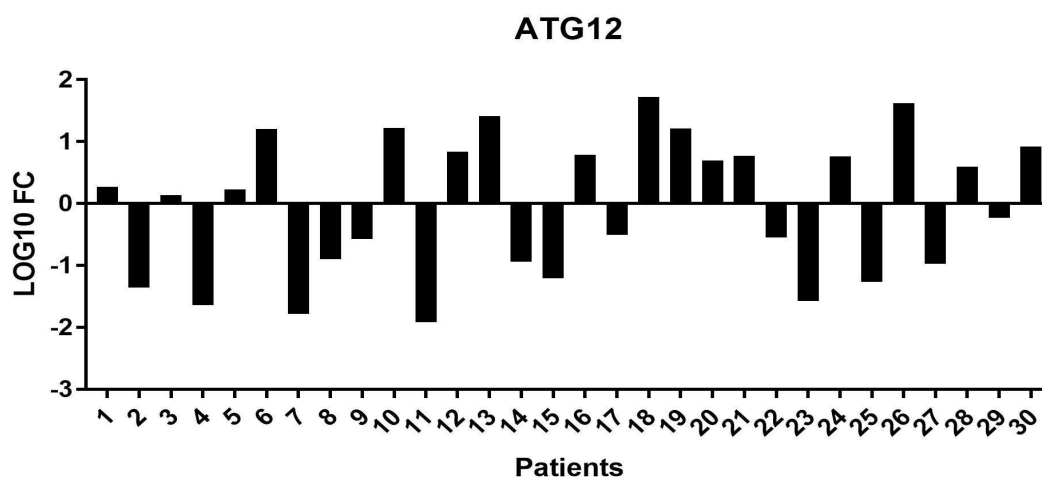


Figure.1. The graph related to ATG12 gene expression level

3.2 survey of changes on ATG5 gene expression level in colorectal tumor tissues using Real Time-PCR method

With us assess on ATG5, we showed that there is a significant difference between the expression levels of this gene in tumor samples and normal samples, so that was observed the down-regulation of ATG5 gene in colorectal

tumor tissues compared with normal tissues ($P=0.031$). Nevertheless, the high expression of this gene was observed in ten (33.3%) samples of thirty tumor samples, while there was a low expression in twenty (66.7%) tumor samples. (Figure. 2 and 4b show the result of ATG5 gene expression level in tumor samples comparison with adjacent normal samples).

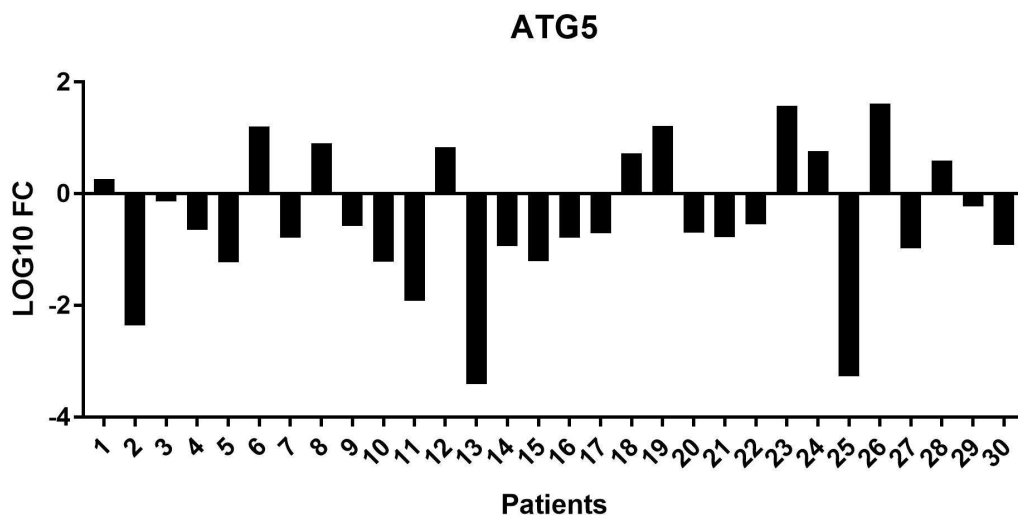


Figure.2. The graph related to ATG5 gene expression level

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

The results related to LC3 gene in 30 colorectal tumor samples demonstrated down-expression of this gene in 12 (40%) colorectal tumor samples and over-expression of LC3 gene in 17 (56.6%) colorectal tumor samples, while one sample (3.4%) did not show any changes in the expression

level of this gene, according to figure.3. Also, due to statistical analysis, was not observed significant difference between the expression levels of LC3 gene in tumor samples compared to normal samples ($P>0.05$). (Figure. 4c shows the result of LC3 gene expression level in tumor samples comparison with adjacent normal samples).

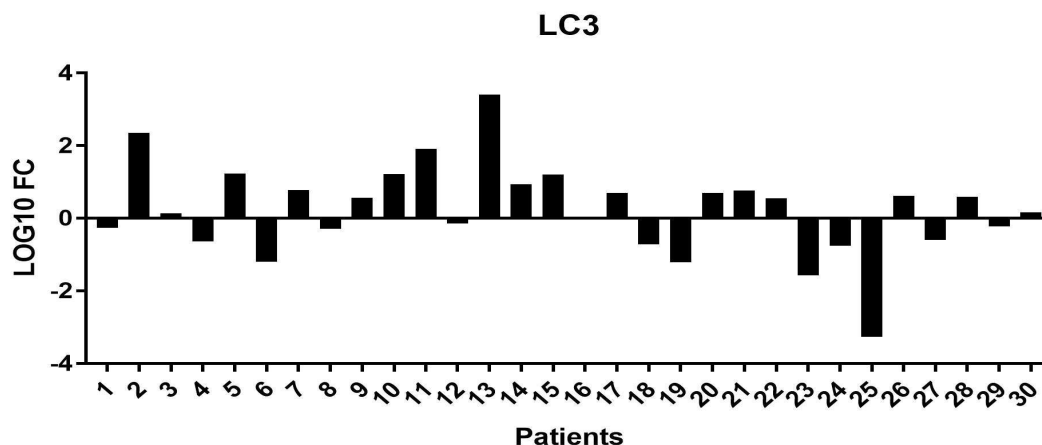


Figure.3. The graph related to LC3 gene expression level

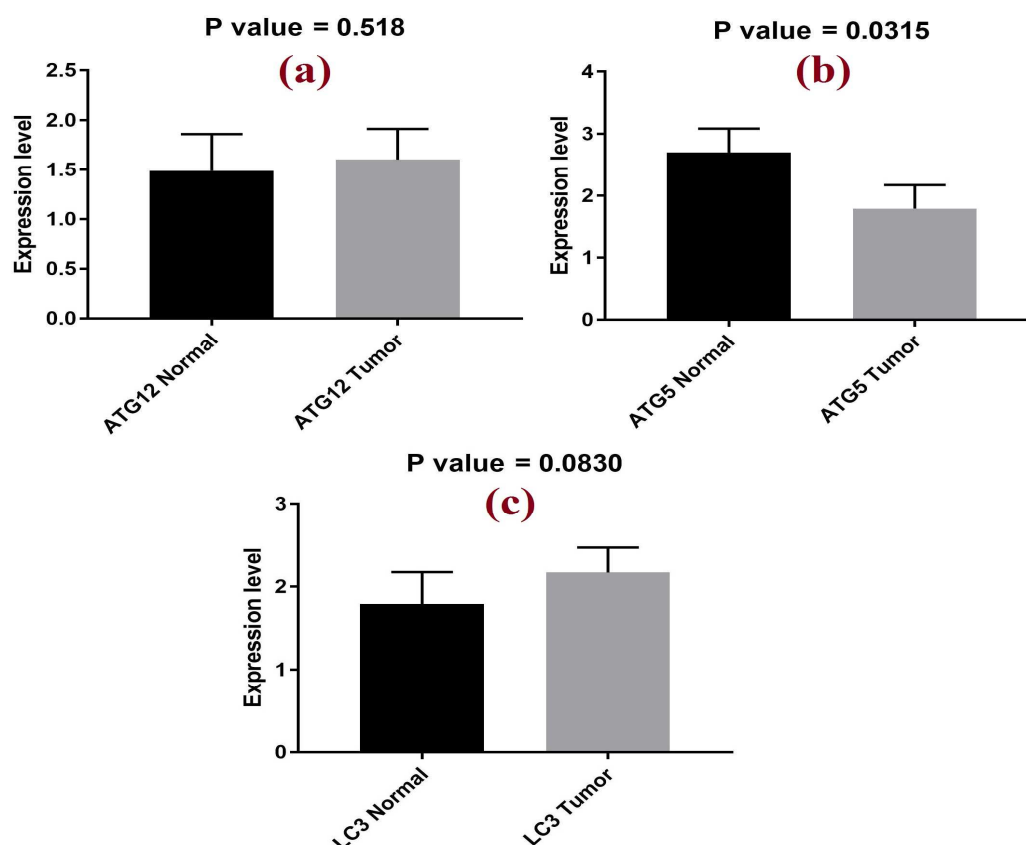


Figure.4. The graph (a), (b) and (c) related to ATG12, ATG5 and LC3 genes expression between colorectal tumor samples comparison with normal samples, respectively.

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 ATG5, ATG12 and LC3 genes in thirty colorectal cancer patients by real-time quantitative PCR.

Earlier research on these autophagy related genes in gastric cancer tissues, demonstrated that seven genes were highly expressed in the gastric cancer tissues, and lowly or moderately expressed in adjacent non-tumor tissues including ATG5, ATG12 and LC3. They also shown this over-expressions levels were correlated with advanced TNM stage and histological types for gastric cancer (Cao et al, 2016). The results of this study showed that ATG5 down-regulated in colorectal cancer and because of its important

role in autophagy pathway, this down-regulation can be very effective in progression of colorectal cancer. Also, this study didn't show any significant difference in expression level between tumor and adjacent non-tumor colorectal tissues for these LC3 and ATG12 genes. However, this study was designed as a pilot study, and further investigations are required to confirm our findings.

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