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TRAF6 And LC3 Gene Expressions In Colorectal Cancer Patients And Predicting Metastasis Using Deep Machine Learning

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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. According to recent studies, several genes are involved in the autophagy mechanism of colorectal cancer which among them can point to TRAF6 and LC3 genes. we analyzed the expression levels of these genes in colorectal cancer tissues. Also, in this study, we predict metastatic CRC patients using the deep-learning.
Method: To evaluate the gene expression level, thirty-two colorectal tumor samples and adjacent normal samples were used for carrying out the Real-Time PCR method. Also, a model based on deep CNN was designed for the prediction of metastatic symptoms in CRC patients.
Results: We reported that there was a significant difference in the expression level of TRAF6 and LC3 genes in tumor samples compared to normal samples (P<0.05). However, this study was designed as a pilot study, and further investigations are required to confirm our findings.

Keywords – Colorectal cancer, Real Time-PCR, TRAF6, LC3, Deep learning.

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 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 (Haggar & 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 the LC3 gene. Two forms of LC3, called LC3-I and -II, were produced post-translationally in various cells, LC3-I is cytosolic, whereas LC3-II is membrane-bound (Kabeya et al., 2000). LC3 is an autophagosomal ortholog of yeast Atg8. A lapidated form of LC3, LC3-II, is an autophagosomal marker in mammals and

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has been used to study autophagy in neurodegenerative and neuromuscular diseases, tumorigenesis, and bacterial and viral infections (Tanida, Ueno, & Kominami, 2004). Various studies have also been conducted on this gene in various cancers that highlight the importance of this gene in tumorigenicity (Jiang, Shao, Wang, Yan, & Liu, 2012; Shen, Li, Wang, Deng, & Zhu, 2008). Recent studies have shown that TRAF6 is involved in some cancers. TRAF6 is a component of intracellular signal transduction proteins and is part of the TNFR family members (Chung, Park, Ye, & Wu, 2002) and this gene plays an important role in inherent immune responses (Lee & Lee, 2002). Differences in the expression of TRAF protein have been reported in human cancers and TRAF2 and TRAF6 have the highest levels of expression in human cancer cells (Rajandram, Bennett, Morais, Johnson, & Gobe, 2012; Zapata et al., 2000). 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 = 32) and adjacent non-tumor tissues were obtained from patients at the 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*.

Table 1. Clinicopathologic characteristics of colorectal cancer patients.

Variable	Value (n = 32)
Gender	
Male	23 (72%)
Female	9 (28%)
Smoking history	
Never-smoker	14 (44%)
Current smoker	18 (56%)
Tumor site	
Colon (L)	7 (22%)
Colon (R)	6 (19%)
Rectum	19 (59%)
Tumor grade	
I	8 (25%)
II	11 (35%)
III	9 (28%)
IV	4 (12%)

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 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 ($2^{-\triangle\triangle CT}$) method. The primers used for real-time PCR are listed in Table 2.

Table 2. Primer sequences of selected genes and GAPDH.

Target genes		Sequences (5`→ 3`)	Product length
TRAF6	Forward	TTGCCATGAAAAGATGCASAG	85bp
	Reverse	AGCCTGGGCCAACATTCTC	
LC3	Forward	TACAGCAGATACGCGACCAG	193bp
	Reverse	TTCACCAGCAGGAAGAAGGC	
GAPDH	Forward	CATCAAGAAGGTGGTGAAGCA	120bp
	Reverse	GCGTCAAAGGTGGAGGAGTG	

2.3 Statistical Analysis

Statistical analysis was performed using the GraphPad Prism v9.3.1 (GraphPad Software Inc., USA) and T-test. For all tests, a *P* value <0.05 was considered statistically significant.

2.4 Predict metastatic CRC patients using deep-machine learning

First, we created a symptoms-type classifier by CNN to enable the efficient recognition of cancer metastatic patients. These symptoms associated with colorectal cancer metastasis were extracted from approved articles in valid NCBI databases. Symptoms of metastasis to the most common organs of the body including metastasis to the lungs (shortness of breath, difficulty breathing, chest pain or a persistent cough), brain (headaches, confusion, memory loss or blurred vision) and other organs were defined by the CNN model. A deep-learning pre-trained model, was re-trained to recognize CRC metastatic symptoms in 7 different organs including lungs, brain, breast, liver, kidney, bowel and lymph nodes**.**

III. RESULTS

3.1 survey of changes on TRAF6 gene expression level in colorectal tumor tissues

According to figure 1 the outcomes obtained of TRAF6 gene expression level showed that out of 32 colorectal tumor samples, TRAF6 expression level in 5 (15.7%) tumor samples were decreased compared with normal tissue and were increased in 27 (84.3%) tumor samples. But it should be noted that these changes in expression level of TRAF6 gene indicate significant difference between the expression level of tumor samples and normal samples (P=0.02). (Figure. 3A shows the result of TRAF6 gene expression level in tumor samples comparison with adjacent normal samples).

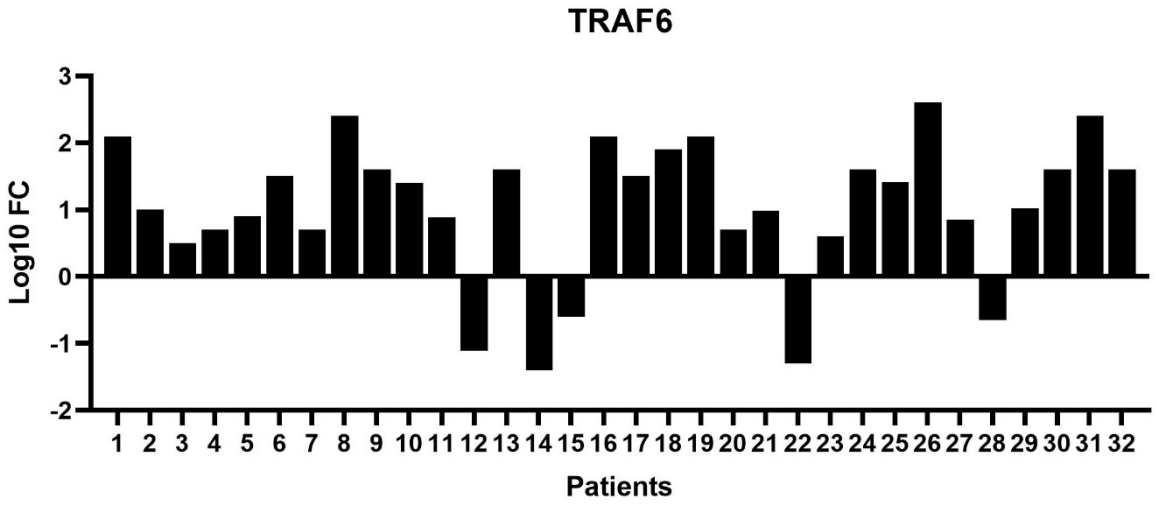

Figure.1. The graph related to TRAF6 gene expression level.

3.2 survey of changes on LC3 gene expression level in colorectal tumor tissues

The results related to LC3 gene in 32 colorectal tumor samples demonstrated down-expression of this gene in 6 (18.8%) colorectal tumor samples and over-expression of LC3 gene in 26 (81.2%) colorectal tumor samples, according to figure.2. Also, due to statistical analysis, we observed significant difference between the expression levels of LC3 gene in tumor samples compared to normal samples (P=0.001). (Figure. 3B shows the result of LC3 gene expression level in tumor samples comparison with adjacent normal samples).

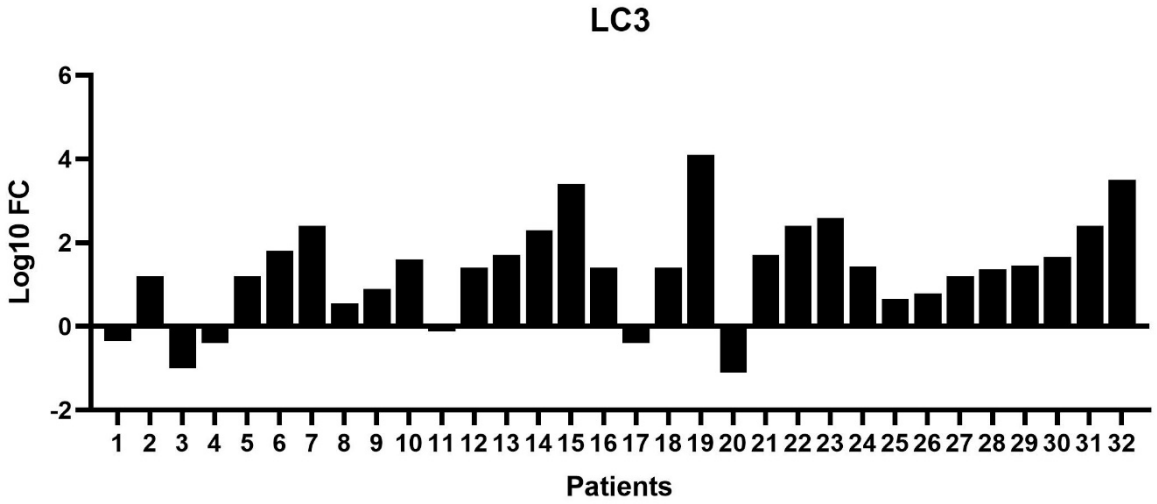

Figure.2. The graph related to LC3 gene expression level.

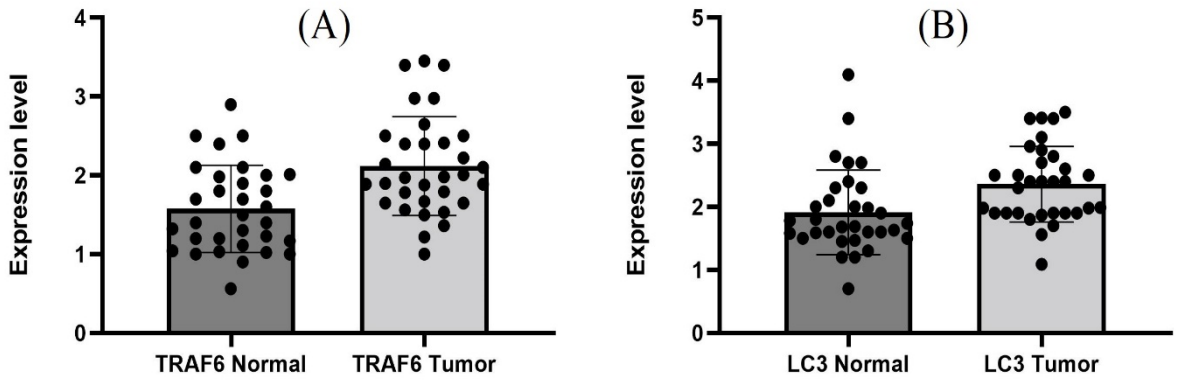

Figure.3. The graph (A) and (B) related to TRAF6 and LC3 genes expression between colorectal tumor samples comparison with normal samples, respectively.

3.3 predictive signs of metastasis of our model using deep learning

After examining the symptoms of the patients in this study, it was predicted that metastasis to other organs would occur in at least 12% of these patients. The highest predictors of metastasis were lung tissue for men and breast tissue for women. However, the number of patients studied in this study was small and the number of patients who were in advanced stages of the disease was very limited. Therefore, to better evaluate the accuracy of this study, 140 patients with a history of existing in the system were predicted and compared with the future history of the same patient. The results are summarized in the Table 3.

Table.3. analysis of predictive signs of metastasis of our model using deep learning.

CNN Model	Accuracy	Sensitivity	Specificity
Inceptionv3	51 %	59 %	48 %

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 TRAF6 and LC3 genes in 32 colorectal cancer patients by real-time quantitative PCR.

Aberrant gene expressions of TRAF6 have been reported in several human cancers. Meng et al. demonstrated that the expression rate of TRAF6 mRNA was significantly increased in osteosarcoma tissues rather than normal bone tissues (Meng et al. 2012). TRAF6 was also overexpressed in pancreatic cancer tissues (Rong et al. 2014). In another study, Sun et al. showed that the TRAF6 is upregulated in colon cancer which is associated with higher tumor grade (Sun et al. 2014). 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). The results of this study showed that TRAF6 and LC3 genes up-regulated in colorectal cancer and because of its important role in normal pathway, this up-regulation can be very effective in progression of colorectal cancer.

Also, in this study we predict metastatic CRC patients using deep-learning machine. The results did not go as we expected. In general, when patients are diagnosed with colorectal cancer in terms of symptoms, a variety of conditions, including history, how

the patient responds to the treatments used, and so on, depend on the treatment mechanisms. Therefore, the results obtained from predicting metastasis are very different from the results that actually occur. However, this study was designed as a pilot study, and further investigations are required to confirm our findings.

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