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Importance of autophagy in colorectal cancer: A cross-sectional study

Otofajinin kolorektal kanserde önemi: Kesitsel bir çalışma

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Abstract

Aim: Colon cancer is the third most common cancer in women and men all over the world. Colorectal cancer (CRC) is diagnosed in over 1.2 million people globally each year. The disease is responsible for approximately 609,000 deaths a year (10% of all cancer cases in women and men). Autophagy is the basic catabolic mechanism that involves cell degradation of unnecessary or dysfunctional cellular components through the actions of lysosomes. The development of autophagy plays a great role in the pathogenesis of many diseases. It was found that autophagy could influence on tumor progression and stimulation. The purpose of this study is to determine the relationship between autophagy and autophagy related ATG5, ATG12, Beclin-1 gene and protein expressions and clinicopathological features of colorectal cancer.

Methods: An observational study is planned. After approval of the ethical committee, the patients (n=45) operated for colorectal cancer was included to the study. There were totally 90 tissue samples taken and banked in liquid nitrogen: 1 tissue sample from tumor and 1 from normal from each patient. ATG5, ATG12, Beclin-1 gene expression levels in all samples were examined using SYBR- Green qPCR method, and, ATG5, Beclin-1, LC3 protein levels were analyzed using Western blotting technique. Expression levels were compared to clinicopathologic characteristics.

Results: Gene and protein expression in both tumor and normal tissue equivalents were studied in most of the examples. There was no significant correlation between gene expression levels and demographic or clinicopathological features. The TNM stage of cases significantly correlated with perineural invasion and lymphovascular invasion.

Conclusion: The results of this study suggest that autophagy may play a role in carcinogenesis of colorectal cancers. The further studies are required to determine the relationship between autophagy and clinicopathologic features associated with colorectal cancers.

Keywords: ATG, Autophagy, Colorectal cancer

Öz

Amaç: Kolon kanseri tüm dünyada kadınlar ve erkeklerde üçüncü en sık gözlenen kanserdir. Yılda yaklaşık 1.200.000 yeni vaka ve yaklaşık 609.000 ölüm tahmin edilmektedir. Erkek ve kadınlardaki kanserlerin yaklaşık %10'unu oluşturmaktadır. Otofaji, hücresel proteinlerin otofajik vakuoller aracılığı ile lizozomal degredasyonudur. Otofaji gelişimde, uzun yaşamda ve kanser gibi pek çok hastalığın patogenezinde büyük rol oynamaktadır. Tümör gelişimi ve uyarılması üzerine bazı etkiler gösterdiği saptanmıştır. Bu çalışmanın amacı, otofajinin, otofaji ilişkili ATG5, ATG12 ve Beclin-1genlerinin ve proteinlerinin ekspresyonu ve kolorektal kanserin klinikopatolojik özellikleri ile ilişkisinin belirlenmesidir.

Yöntemler: Çukurova Üniversitesi Tıp Fakültesi Hastanesi Genel Cerrahi Anabilim Dalında kolorektal kanser nedeniyle opere edilen 45 hasta dahil edildi. Hastaların hem tümör hem de eşlenik normal kolon dokularından alınan örnekler ameliyathaneden itibaren sıvı azotta bankalandı. Daha sonra SYBR Green qPCR yötemiyle ATG5, ATG12, Beclin-1 gen ekspresyonlarına ve Western Blot yöntemiyle ATG5, Beclin-1, LC3 protein ekspresyonlarına bakıldı. Ekspresyon düzeyleri ile klinikopatolojik özellikler karşılaştırıldı.

Bulgular: Gen ve protein ekspresyonları hem tümör hem de eşlenik normal doku örneklerinin çoğunda saptandı. Gen ekspresyon düzeyleriyle klinikopatolojik ve demografik veriler arasında anlamlı ilişki saptanamadı. Örneklerin TNM evreleriyle perinöral invazyon ve lenfovasküler invazyon arasında anlamlı ilişki saptandı.

Sonuç: Bu çalışmanın sonuçları otofajinin kolorektal karsinogenezisde işe karıştığını önermektedir. Genişletilmiş çalışmaların yapılması otofaji ve kolorektal kanser ile ilişkili klinikopatolojik özellikleri belirlemede faydalı olabilecektir.

Anahtar kelimeler: ATG, Otofaji, Kolorektal kanser

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Introduction

Colon cancer is the third most common cancer among men and women worldwide. Approximately 1,200,000 new cases and 609,000 deaths per year are observed. It accounts for about 10% of all cancers in men and women. In the United States, CRC is responsible for 10% of new cancer cases and 9% of cancer-related deaths, according to 2009 data. Despite the current treatment regimens, 5-year survival expectancy does not exceed 30-40% [1].

The incidence of colon cancer has decreased in western countries as a result of effective care and lifestyle changes. The interaction of genetic and environmental factors has an important role in colorectal carcinogenesis. Although the molecular mechanism behind colorectal cancer (CRC) is better understood in the last two decades compared to other solid tumors, especially in advanced stages, its prognosis was not significantly improved. According to the Survey Epidemiology and Results (SEER) program database analysis, 5-year survival rates increased from 56.5% for patients diagnosed in the early 1980s to 63.2% for patients diagnosed in the early 1990s and to 64.9% with early diagnosis and treatment lately [2]. The incidence of colon cancer has decreased in the last 30 years and mortality has decreased by 35% between 1990 and 2007. Possible reasons for this are the improvements about early diagnosis and treatment [3].

Primary treatment of early-stage CRC is surgery and cure can be achieved with high possibility. However, the prognosis of recurrence and metastatic patients is poor and the median survival time is 24 months for these patients [4]. Approximately half of the patients develop metastasis. The main treatment option in these patients is chemotherapy. Response rates and life expectancy are increased with targeted therapy agents and new chemotherapy agents [5].

Median survival of patients with metastatic CRC (mCRC) does not exceed 24 months despite these new agents. This may be due to the lack of enough biomarkers to select patients who will benefit from these agents [6].

Disorders of cell death signaling are one of the most important obstacles to the curative treatment of cancer [7]. Autophagy was first identified in yeasts in the 1970s, and then observed as an evolutionary conserved programmatic cell death mechanism in mammals. Several studies have been conducted in recent years on the role of autophagy in carcinogenesis. In these studies, there is evidence that autophagy can serve as a mechanism for both the survival of cancer cells, the initiation of carcinogenesis and, in contrast, the elimination of cancer cells escaping apoptosis. Thus, it has been suggested that treatment can be accomplished by the manipulation of autophagy (inhibition or activation). However, the available data are still insufficient to clarify these questions. Many studies focus on this subject lately.

Autophagy means literally self-eating. Autophagy is known as the process of disintegrating long-lasting proteins, damaged organelles, microorganisms and viruses in the cell by transporting them to the lysosome [8,9]. Autophagy genes (ATG); play a role in the regulation of autophagy [10]. Autophagy is defined by the presence of double-membrane vesicles in the cytoplasm called autophagosomes. Recent studies have shown that autophagy plays a role in metabolism, cancer, neurodegenerative diseases, infections, morphogenesis, aging, cell death and immune system [11].

Autophagy has two opposite roles; both cell survival and cell death [12]. Autophagy is called "programmed cell death II" because it causes cell death [13]. As a result of studies that demonstrate the relationship between autophagy and cancer, drugs are being developed to target autophagy-signaling pathways as new treatment strategies in cancer. For example, mTOR inhibitors that mediate the autophagy pathway are clinically used to treat renal carcinoma [15].

The mutation of UVRAG gene is defined in colorectal and gastric cancers [15]. It has been suggested that UVRAG, Bif1 genes can suppress tumorogenesis and these two genes may be tumor suppressor genes [15,16]. Somatic mutations have been described in the Atg5 gene in hepatocellular and gastric cancer and in Atg2B, Atg5, Atg9B genes in colorectal and gastric cancers [9,17]. As a result of these studies, it has been argued that the expression of autophagic genes decreases in cancer cells and autophagy plays an important role in carcinogenesis as a tumor suppressing mechanism [18]. It is a hypothesis that the role of autophagy in cancer can be regulated by tumor suppressors and oncogenes and autophagy genes may be tumor suppressors at the molecular level [19,20].

Disorders in apoptotic mechanisms cause both abnormal proliferation and resistance to cytotoxic therapy. Therefore, in the last decade studies on new agents that induce apoptosis have intensified [21]. Although autophagic response to starvation in cancer cells is less prominent, it appears as an important survival mechanism in many tumor types [22]. It is thought that suppression of carcinogenesis can also be achieved with autophagy. Another mechanism for the destruction of cancer cells, which evade apoptosis, may be autophagy [23]. In this study, the relationship between the clinical and pathological features of colorectal cancer and autophagy were investigated.

Materials and methods

Collection of samples

Between January 2015 and March 2015, 45 patients who underwent surgery for CRC were included in this study. Thirty of the patients were male and 15 were female. Before the study, the approval was obtained from the ethics committee of Çukurova University Faculty of Medicine. Written informed consent was obtained from all participants. Demographic (age, gender), pathological features (tumor localization, histological type, differentiation, vascular and perineural invasion, lymph node involvement), clinical stage and laboratory results of the patients included in the study were recorded.

Gene expression

We use chemicals such as TRIZOL (Guanidinium thiocyanate-phenol chloroform), Chloroform, %75 ethanol, İzopropanol and DEPC-water. For cDNA Synthesis we use PCR. Before starting the cDNA analysis, the total amount of RNA in each sample was determined using Nano drop to ensure the equalize reaction conditions of the samples. Accordingly, cDNA analysis is started by taking the determined volumes from the

tubes. The total amount of RNA from each tube should be 1000 ng/ul.

Protein Isolation

RIPA solution is placed according to the amounts in the tube and crushed on ice with homogenization bars. After dissolving well, it is centrifuged at maximum speed (14000 rpm) for 15 minutes. The supernatant between the top layer of fat and the bottom pellet is obtained. Proteins are in this supernatant. 1 ml of this supernatant and 29 ml of distilled water are added into the new tube to determine the total amount of protein in the spectrometer. 10 ml of this new heterogeneous mixture is taken and after adding 190 ml Bradfort solution the specimen is measured on the 595 nm wavelength spectrometer.

Statistical Analysis

In the statistical analysis of the data, SPSS package program was used. Descriptive statistics were expressed as number and percentage (%) for categorical measurements, mean and standard deviation for continuous measurements (median and minimum-maximum where necessary). In the comparison of continuous measurements between groups, distributions were checked and one-way Anova test was used for the parameters that are normally distributed according to the variables. Chi-square test statistics was used to compare categorical variables.

Results

In this study, 45 patients were included. Demographic and clinical data's of the study were given in Table 1. Thirty patients were male and fifteen were female. Before starting the study, approval was obtained from the ethics committee of Çukurova University Faculty of Medicine. Written informed consent was obtained from all patients.

Table 1: Demographic, clinical and pathological data

	n	%
Gender		
Female	15	33.33
Male	30	66.66
Localization		
Rectum	18	40.0
Cecum	5	11.1
Ascending colon	9	20.0
Sigmoid	10	22.2
Descending	1	2.2
Transvers	2	4.4
Histology		
Adenocarcinoma	41	91.1
Mucinous adenocarcinoma	1	2.2
Lymphovascular invasion		
Positive	37	82.2
Negative	5	11.1
Perineural invasion		
Positive	20	44.4
Negative	22	48.8
Histological Grade		
High	5	11.1
Low	36	80

RNA and protein expression were evaluated in twenty patients included in the study. Fifteen patients (75%) had low histologic grade and five patients (25%) had a higher histologic grade. The expression of ATG5, ATG12 and Becklin-1 was analyzed by SYBR Green qPCR in both tumor tissues and intact tissues of these patients. The quantification of the reference and target genes by using the cycle threshold (Ct) values obtained from the device was calculated and quantified. We referenced the GAPDH gene and the results were normalized to the intact tissue of patient number fifteen. Two patients were excluded on the basis of the inadequate RNA content of 1 (5%) low histological grade and 1 (5%) high histological grade of the gene expression before the cDNA synthesis phase.

There was no significant correlation between lymphovascular and perineural invasion and histological grade (p=0.104 and p=0.666, respectively). The relationship between TNM stages and perineural invasion and lymphovascular invasion was statistically significant (p=0.014). Becklin-1 gene expression with SYBR Green qPCR method in tumor tissue and intact tissue showed no significant correlation between TNM levels (p=0.093). Becklin-1 gene expression with SYBR Green qPCR method in tumor tissue and intact tissue showed no significant correlation with lymphovascular invasion (p=0.756). There was no significant relationship between ATG5 gene expression and TNM levels in tumor tissue and intact tissue by the SYBR Green qPCR method (p=0.055). There was no significant relationship between ATG12 gene expression in tumor tissue and intact tissue with TNPR Green qPCR method (p=0.292).

Discussion

CRC occurs as a result of the transformation of normal colon mucosa into invasive cancer with the accumulation of step by step genetic and epigenetic changes. Most of the CRC develops from adenomas that already exist and include the genetic characteristics of malignancy [24].

Autophagy has two opposite roles in cell death and life. Debates about the role of autophagy in cancer continue. Autophagy development suppresses the tumorigenic activity of cancer cells and inactivation of autophagy increases the tumor development as in the case of Beclin1 and UVRAG. However, blockade of autophagy makes resistant cells sensitive to radiotherapy [15].

Furthermore, the expression of Beclin1 and LC3 protein, which are autophagy-related proteins, up regulated in colon and gastric cancers indicates compensatory overexpression due to ATG mutations. These data indicate that autophagy genes have different roles in cell death and life depending on the content of the cell. In addition, autophagy is likely to have different roles in the progression of cancer and the treatment of cancer. Further functional studies are needed to investigate the contribution of mutations in ATG genes to the development, progress and treatment of cancer [22].

Especially in patients receiving metastatic CRC treatment, 5-year survival rates and treatment responses cannot exceed a certain percentage. The etiology, mechanisms of action and the role of autophagy in the progression of CRC are therefore important [5,6]. In this study, we tried to determine the role of autophagy in tumor samples of patients with CRC. The presence of autophagy in both normal and cancerous tissue may be due to changes in colorectal carcinogenesis in both tissues.

Limitation

The results we found in our study should be confirmed with larger patient series. However, the relationship between autophagy and CRC clinical characteristics can be better elucidated by planning the studies to eliminate the evaluation errors caused by the number of samples resulting from sub-groups by increasing the number of samples.

Conclusion

In addition to contributing to both tumor development and progression, autophagy has a negative effect on tumor development. In our study, ATG5, ATG12 and LC3 protein expression levels and ATG5, ATG12 and Beclin-1 RNA expression levels related to autophagy pathway, which is thought to have an important role in the early stages of cancer formation and in the progression of advanced cancers both in tumor tissue and conjugated normal colon.

RNA and protein expressions were detected in both tumor tissue and normal tissue. These results were compared with the demographic and histopathological features of the patients and there was no significant relationship between them.

As in all cancers, CRC requires more specific and less toxic treatment options. By identifying the characteristics of CRC and autophagy, it will be possible to reveal new markers and treatment options that include especially tumor development, prognosis, metastasis and treatment. In our study, optimal conditions were obtained in terms of study techniques and tissues were stored peroperatively and stored in liquid nitrogen until protein and RNA expression analyzes were performed. The tissues taken in the operating room were transferred to liquid nitrogen tanks where they were placed in Cryo tubes without being wasted at any time.

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