

Assessing Methylation of ATG5 and LC3 Related to Autophagy in Patients with Breast Cancer

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Abstract

Background: Breast Cancer is one of the leading causes of cancer deaths among women worldwide. The role of epigenetics as a distinct mechanism to alter gene expression in a tissue-specific manner has emerged as an important mechanism in the pathophysiology of cancer. One of the most important of epigenetic factor is DNA methylation which has been studied in various cancers.

Method: The methylation specific high resolution melting (MS-HRM) method was used for promoter methylation status investigation of ATG5 and LC3 genes in 30 breast tumor samples and 30 adjacent normal samples.

Results: For the ATG5 and LC3 genes, were methylated 2 (6.67%) of tumor samples and 1 (3.34%) of tumor samples out of max 30 tumor samples, respectively. Also, the rest of the samples showed no methylation change in their promoter region, and statistical results demonstrated that there is no significant difference between the promoter methylation status of these genes in tumor samples and normal samples ($P>0.05$).

Keywords - Breast Cancer, DNA Methylation, MS-HRM Method, Autophagy, ATG5, LC3

I. INTRODUCTION

Cancer can start almost anywhere in the human body, which is made up of trillions of cells. Cancer cells differ from normal cells in many ways that allow them to grow out of control and become invasive. Cancers are categorized according to the type of tissue involved and one of the most important of them is Breast cancer that is an increasing public health problem (Howell et al., 2014). The incidence is rising in most countries and is projected to rise further over the next 20 years despite current efforts to prevent the disease (Eccles et al., 2013; Rahib et al., 2014). Globally, breast cancer is the most frequently diagnosed cancer in women, with an estimated 1.38 million new cases per year.

Fifty thousand cases in women and 400 in men are recorded each year in the UK alone (Eccles et al., 2013). There are 458,000 deaths per year from breast cancer worldwide making it the most common cause of female cancer death in both the developed and developing world (Ferlay et al., 2010). Molecular classification of breast cancer has been proposed based on gene expression profiles of human tumors. Luminal, basal-like, normal-like, and *erbB2*+ subgroups were identified and were shown to have different prognoses (Rouzier et al., 2005). Breast cancer patients with tumors that are estrogen receptor (ER)-positive and/or progesterone receptor (PR)-positive have lower risks of mortality after their diagnosis compared to women with ER-

and/or PR-negative disease (Dent et al., 2007). Multiple factors affect breast cancer-related mortality rates, which reflect changes in incidence and survival (Coleman, 1999; Micheli et al., 2003), such as environmental factors, genetic factors and epigenetic factors, are involved in cancers. Epigenetic alterations, such as aberrant DNA methylation, are deeply involved in human cancer development (Ahmadi et al., 2018). The DNA hyper-methylation of 5'-CpG islands has been shown to be a major reason of silencing of tumor suppressor genes in human tumors (Nooshin, Karizi, Karimipour, & Nooshin, 2018), also autophagy is believed to have an important role in tumour development (Kisen et al., 1993). So far, many studies have been carried out on the methylation of genes involved in cancer mechanisms (Christensen et al., 2010; Widschwendter et al., 2004). In this study, the status of methylation of ATG5 and LC3 genes was also surveyed in breast cancer samples. The ATG5 gene is one of the genes involved in the autophagy pathway. The protein encoded by this gene, in combination with autophagy protein 12, functions as an E1-like activating enzyme in a ubiquitin-like conjugating system. The encoded protein is involved in several cellular processes, including autophagic vesicle formation, mitochondrial quality control after oxidative damage, negative regulation of the innate antiviral immune response, lymphocyte development and proliferation, MHC II antigen presentation, adipocyte differentiation, and apoptosis. Its expression is a relatively late event in the apoptotic process, occurring downstream of caspase activity (Karantza & White, 2007). Therefore, due to the high importance of this gene, various studies have been done on it (Cho, Jo, Kim, Park, & Kim, 2012; Kim, Song, Lee, Yoo, & Lee, 2011). LC3 gene is also involved in autophagy mechanism (Eskelinen & Saftig, 2009). 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 lipidated form of LC3, LC3-II, 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). Also, 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).

II. MATERIAL AND METHODS

2.1 Patients and Samples

Human breast tumor tissues (n = 30) and adjacent non-tumor tissues were obtained from patients at Masih daneshvari Hospital, Tehran, Iran, 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 DNA Extraction

DNA was extracted by using the Qiagen DNA Extraction Mini Kit (Qiagen, United States) from each tumor and normal tissues according to the manufacturer's specifications. The concentration of DNA was determined by spectrophotometry and Integrity of DNAs were assessed by 1.0% agarose gel electrophoresis.

2.3 Bisulfite treatment and Preparation of controls

Genomic DNA of each tumor and normal tissues were treated with Sodium bisulfite by using EZ DNA Methylation Kit (Zymo Research, Orange, CA), according to the manufacturer's recommendation. The entire converted DNA was stored at -70 °C until use. For unmethylated control, we used white blood cell DNA and then, fully methylated DNA was made by treatment a same white blood DNA extracted with *M. SssI* according to the manufacturer's recommendation (Thermo Scientific, United States). Finally, by diluting fully methylated DNA with unmethylated bisulfate-treated DNA, all controls with different ratios (5%, 10%, 25% and 50% methylated controls) were constructed.

2.4 MS- High resolution melting (MS-HRM)

MS-High Resolution Melting was performed in a 20-μl reaction containing 4-μl of 5x HOT FIREPol®EvaGreen® HRM Mix (ROX), 1-μl of bisulphite converted DNA, 14-μl of H₂O and 1μl of mixed forward and reverse primers (6 Pmol/μl concentration) by using an StepOnePlus™ Real-Time PCR Systems (ABI Applied Bio-systems, Thermo Fisher Scientific, USA). The thermocycler conditions were as follows: initial denaturation at 95°C for 15 min; followed by 40 cycles of 94°C for 15 seconds, 62°C for 30 seconds (annealing time), 72°C for 30 seconds (extension time); and a final extension at 72°C for 5 min. The melting curve stage was performed as follows: denaturation at 95°C for 15s, 62°C and 62°C for 1 min, followed by HRM step ramping from 60°C to 95°C, rising 0.3%. The primers used for amplification are listed in Table 1.

Table 1. Designed primer sequences for selected genes.

Target genes		Sequences (5' → 3')
ATG5	Forward	GGAGTTTTAGGTGAGGTAGAT
	Reverse	TCACCCTACCAACCTATCAC
LC3	Forward	CACGAACGCCTATCTCTACAA
	Reverse	GATGTCGGGGTAGTAAGTGAT

2.5 Statistical analysis

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

III. 3. RESULTS

3.1 Study of methylation status of ATG5 gene using HRM method in breast cancer specimens

The MS-HRM method was used to investigate the methylation status of ATG5 gene in 30 breast tumor samples and 30 normal samples. Also, controls for this study were considered 0% methylated (un-methylated), 25% and 100% methylated. So that cut off for methylation status in this study was selected 25% methyl control and it means that samples above this threshold line were considered methylated and lower samples of this threshold line un-methylated. Our assessing revealed that 2 (6.67%) of tumor samples out of max 30 tumor samples were methylated (were more than 25% methylated) and the remaining samples (93.33%) showed no methylation of this gene in tumor samples comparison with normal samples. Finally, the results illustrate that there is no significant difference between the methylation of this gene in tumor samples and normal samples ($P=0.15$). (Fig 1 and 3A illustrate the results obtained of ATG5 gene).

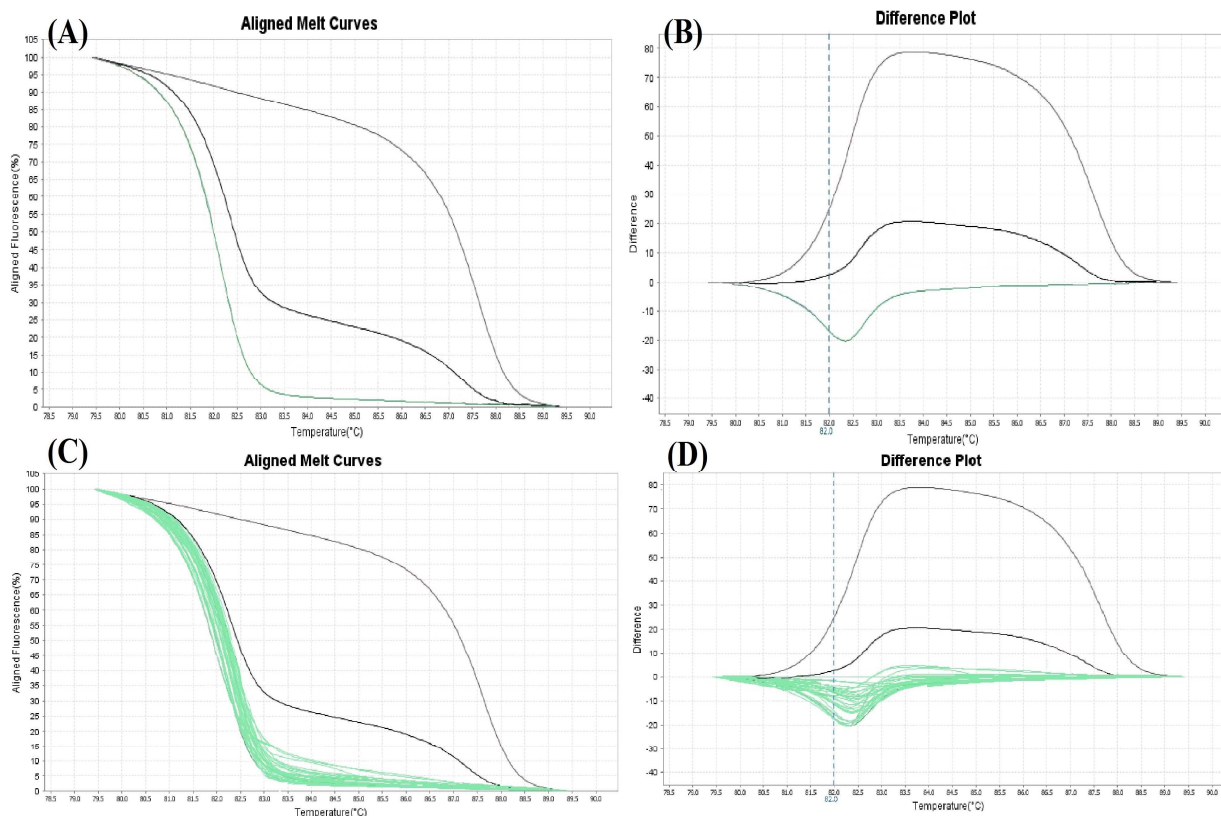


Figure 1. The graphs (A) and (B) related to methyl controls of ATG5 gene, So that controls are from down to up consists of 0%, 25% and 100% methylated. The graphs (C) and (D) related to ATG5 gene methylation status with controls and samples.

3.2 Study of methylation status of LC3 gene using HRM method in breast cancer specimens

In this paper, the methylation status of LC3 gene was examined in 30 tumor samples and 30 adjacent normal samples by MS-High resolution melting method. Contrary to the results of the ATG5 gene, controls for LC3 gene were selected 0% methylated (un-methylated), 10% and 100% methylated and cut off for this gene was considered 10%

methyl control. Our observations for methylation status of LC3 gene demonstrated that 1 (3.34%) of tumor samples were methylated compared to normal samples and 29 (96.66%) of tumor samples did not demonstrate any methylation of this gene. so that was observed no significant difference between tumor samples comparison with normal sample ($P=0.3$). (Fig 2 and 3B illustrate the results obtained of LC3 gene).

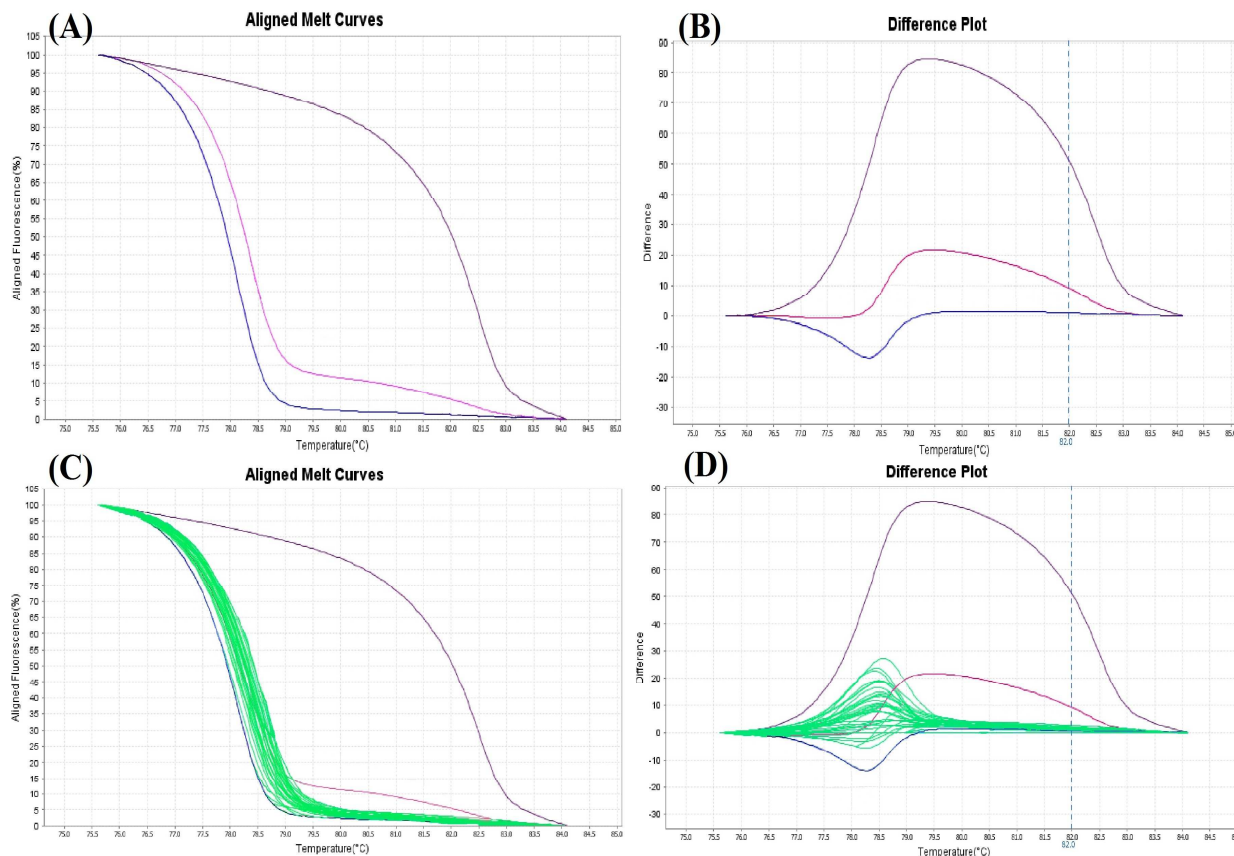


Figure 2. The graphs (A) and (B) related to methyl controls of LC3 gene, So that controls are from down to up consists of 0%, 10% and 100% methylated. The graphs (C) and (D) related to LC3 gene methylation status with controls and samples.

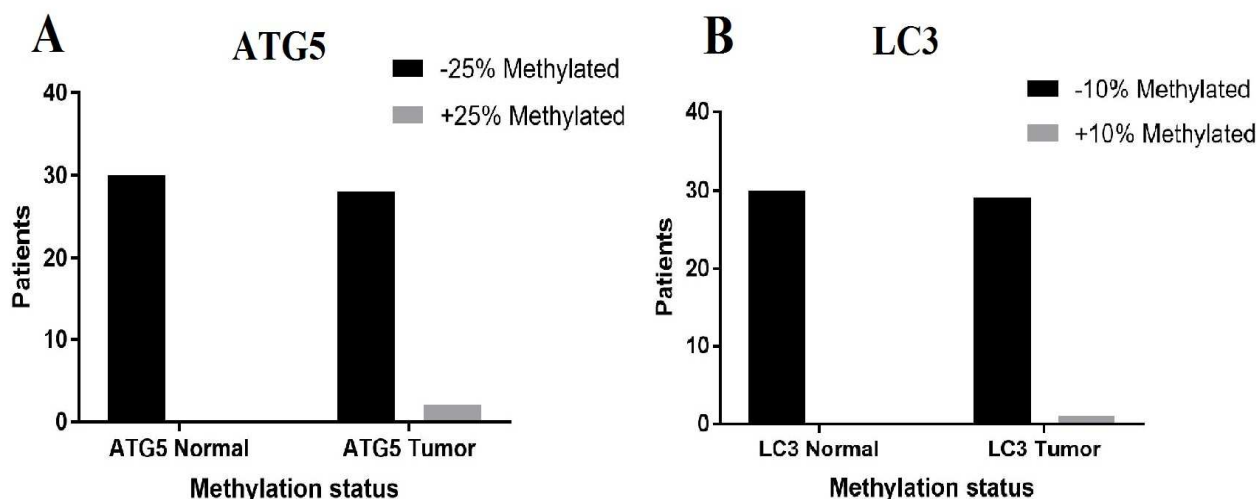


Figure 3. The graph (A) related to methylation of ATG5 gene in breast tumor samples compared to non-tumor adjacent samples and the graph (B) related to methylation of LC3 gene in breast tumor samples compared to non-tumor adjacent samples

IV. DISCUSSION

DNA hypermethylation of tumor suppressor genes was an important epigenetic event in the progression of early changes in the progression of breast cancer, the silencing of genes through promoter hypermethylation is now recognized as a major and causal epigenetic event. It is also closely associated with transcriptional silencing of genes. In this paper, we have analyzed ATG5 and LC3 genes promoter methylation status in 30 breast cancer patients by the MS-HRM method.

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).

Another gene that was studied in this paper was ATG5, and this protein is involved in several cellular processes, including autophagic vesicle formation, mitochondrial quality control after oxidative damage, negative regulation of the innate antiviral immune response, lymphocyte development and proliferation, MHC II antigen presentation, adipocyte differentiation, and apoptosis

(Karantza & White, 2007). On the Basis of research by Cho et al. ATG5 to be strongly down-regulated in colorectal cancer (Cho et al, 2012). Another study by Kim et al showed that this gene was altered in prostate cancer patients and they suggested that overexpression of this protein may be related to autophagy and might play a vital role in prostate tumorigenesis (Kim et al, 2011) but, in this study, we didn't see any significant difference in methylation level between tumor and adjacent normal breast tissues for LC3 and ATG5 genes. However, this study was designed as a pilot study, and further investigations are required to confirm our findings.

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