

RESEARCH ARTICLE

Editorial Process: Submission:05/02/2018 Acceptance:08/01/2018

Association of Autophagy Gene ATG16L1 Polymorphism with Human Prostate Cancer and Bladder Cancer in Turkish Population

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Abstract

Background: Urological cancers (prostate cancer and bladder cancers) are the most common cancers in Western population and its rate is increasing in the Eastern World. Autophagy has appeared as a fundamental repair mechanism for degrading damaged organelles and proteins. It was clear that autophagy gene polymorphisms are correlated with development of inflammatory bowel disease and it can also be related with prostate cancer (PCa) or bladder cancer (BCa). In this study, we aimed to determine if ATG16L1 (Thr300Ala) polymorphism is associated with an increased risk of developing PCa and BCa and to establish correlations between ATG16L1 genotypes and morphological parameters. **Methods:** This study included 269 healthy controls and 131 patients (62 PCa and 69 BCa) with PCa and BCa. The ATG16L1 (rs2241880) gene regions were amplified using polymerase chain reaction (PCR), detected by restriction fragment length polymorphism (RFLP). **Results:** At the end of our research, we found out that the genotype AG was prevalent on patients and controls (34% vs 42%), followed by genotypes AA (35% vs 27%) and GG (31% vs 31%) in PCa. The prevalence of genotypes of AA (wild-type), AG (heterozygous mutant) and GG (homozygous mutant) profiles for the ATG16L1 Thr300Ala polymorphism were 35%, 40% and 25% respectively in BCa patients, and 32%, 40% and 28% respectively in healthy control groups. The G allele frequency was 0.53 for in BCa patients and the control groups. **Conclusion:** No association was found between ATG16L1 (Thr300Ala) polymorphism and patients with PCa and BCa in Turkish population we studied.

Keywords: Autophagy- ATG16L1 gene polymorphism- prostate cancer- bladder cancer

Asian Pac J Cancer Prev, **19 (9)**, 2625-2630

Introduction

Prostate and bladder cancers are among the most widely seen occurring urological malignancies in today's developing world (Tong and Li., 2004; Ma et al., 2014; Amasyalı et al., 2015; Polat et al., 2015). Today prostate cancer (PCa) is one of the leading causes among cancer-related death in human males worldwide, being the second most current form of cancer in men. PCa grows locally and slowly, it usually takes many years; finally, it spreads outside the prostate into nearby tissues or invades distant tissues through blood (Ziparo et al., 2013). Bladder cancer (BCa) is the fourth most common cancer among cancers seen in men and it was estimated that 150,200 of the 386,300 new cases would end with death in 2008 worldwide (Jemal et al., 2011; Amasyalı et al., 2012). This cancer is a multi-factorial disease that is generated by both genetic and environmental factors (Polat et al., 2015).

Autophagy is an essential, homeostatic process involving the lysosomal degradation of cytoplasmic organelles or cytosolic components (Van Limbergen et al.,

2008; Choi, 2012; Chen et al., 2016). The term 'autophagy' was first coined by Christian de Duve in 1955 year, and was the first biochemist to describe the term "autophagy", which is derived from the Greek word meaning 'eating of itself', to distinguish lysosomal degradation, or cellular "eating" (phagy) of self (auto), from the breakdown of extracellular material (heterophagy). (Meijer and Codogno, 2004; Glick et al., 2010; Platini et al., 2010; Eng and Abraham, 2011; Jiang and Mizushima, 2014; Xu et al., 2015).

There are three major types of autophagic processes: macroautophagy, microautophagy, chaperone-mediated autophagy (Platini et al., 2010; Jiang and Mizushima, 2014). Autophagy is said to be tightly regulated by a definite number of highly conserved genes called autophagy regulators (ATGs) (for AuTophagy gene) (Chen and Debnath, 2010). Autophagy pathway was performed in Crohn's Disease by "Hampe et al., (2007)". These authors demonstrated association with a coding variant of the ATG16L1 gene (autophagy-related 16-like 1 gene, Ala197Thr polymorphism, rs2241880

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A/G) on chromosome 2q37.1. This polymorphism has mainly been studied for its association with Crohn's disease, ulcerative colitis and palmoplantar pustulosis (Palomino-Morales et al., 2009; Umeno et al., 2011; Douroudis et al., 2011). In addition, today, autophagy is a complex process, and changes in autophagic signalling pathways are often monitored in cancer. For this reason, mutations and polymorphisms of autophagic genes could possibly be related to cancer (Levine, 2007; Mathew et al., 2007; Yang et al., 2011; Karakaş and Gözüaçık, 2014; Nicoli et al., 2014).

Autophagy has two important roles in cancer. One of them is acting as a tumour suppressor by preventing the accumulation of damaged proteins and organelles and the other is being a mechanism of cell survival that can promote the growth of established tumours (Mathew et al., 2007; Karakaş and Gözüaçık, 2014). Tumour cells trigger autophagy in response to cellular stress and/or increased metabolic needs related to rapid cell proliferation (Karakaş and Gözüaçık, 2014). Autophagy has a controversial issue and its role in cancer hasn't been completely clarified yet (Ziparo et al., 2013). Moreover, autophagy defects are associated with increased tumorigenesis, but the mechanism behind this has not been determined. Recent studies demonstrate that autophagy provides a protective role to limit tumour necrosis and inflammation, and to mitigate genome defect in tumour cells in response to metabolic stress (Yang et al., 2015).

These roles of autophagy are protective and have an important role in countless pathological conditions, including neurodegeneration, Crohn's disease and pathogenic infections (Mathew et al., 2007; Eng and Abraham, 2011). But its role in cancer is not completely defined. The first genetic association-between autophagy and cancer occurred with the discovery that Beclin-1, a tumor suppressor, was an autophagy-related protein. The damages in Beclin1 are related in sporadic human breast, ovarian and prostate cancers (Eng and Abraham, 2011).

Autophagy-related (Atg) genes were first discovered in the 1990s. Since then there has been a proliferation of studies on the physiological and pathological roles of autophagy in a variety of autophagy knockout models (Jiang and Mizushima, 2014). The evolutionary highly conserved ATG proteins are essential components of the autophagy process. More than 30 of these proteins have been identified in yeasts (Nakatogawa et al., 2009; Glick et al., 2010; Plantinga et al., 2014). Nevertheless, accurate evidence of the connections between ATG gene dysfunction and human diseases has emerged only recently. There have been an increasing number of studies suggesting that mutations in the ATG genes were identified in various human diseases such as inflammatory bowel disease (Hampe et al., 2007; Rioux et al., 2007; Parkes et al., 2007; Henckaerts et al., 2011), neurodegenerative diseases (Saitsu et al., 2013), infectious diseases (Intemann et al., 2009; Raju et al., 2012), allergy (Martin et al., 2012), and cancers (Huijbers et al., 2012; Jiang and Mizushima, 2014; Plantinga et al., 2014).

BECN1, UVRAG, SH3GLB1 (Bif-1), Atg2B, Atg5, Atg9B, Atg12, and RAB7A are among the autophagy genes frequently stated as mutated in gastric and colorectal

human cancers (Kim et al., 2008; Kang et al., 2009; Liu and Ryan, 2012; Nicoli et al., 2014). The ATG16L1 gene polymorphism (Thr300Ala, rs2241880) has been reported to affect the autophagy pathway (Cooney et al., 2010). We tried to find out whether this ATG16L1 polymorphism is associated with prostate cancer (PCa) and bladder cancer (BCa). We compared the genotype frequencies detected in tree Turkish groups: patients diagnosed with PCa, BCa and healthy control subjects.

Materials and Methods

Subjects

A total number of 400 Turkish subjects (131 patients diagnosed with PCa and BC and 269 healthy controls) were included in this study. The patients were selected from urology clinic of Lüleburgaz and Niğde State Hospital, Turkey. The control group includes voluntary subjects who have no family history of cancer in same population. The DNA samples used in this study were obtained from the blood samples previously taken under 'the Investigation of Endothelial Nitric Oxide Synthase Gene Polymorphisms in Prostate and Bladder Cancer' titled project within the scope of ethical report (Çukurova University, Ethics Committee for Clinical Researches/KAEK 2013-29), in which both the patients and healthy volunteers were informed and their approvals were obtained and the DNA samples were used in accordance with the KAEK 2015-164 (Erciyes University, Human Ethical Committee for Clinical Researches) numbered ethical report.

DNA Extraction

Peripheral blood samples were collected from the participants in ethylenediaminetetraacetic acid tubes. Genomic DNA was extracted from 200 µL EDTA-anticoagulated peripheral blood leukocytes using the QIAamp DNA Blood Mini Kit (QIAGEN GmbH, Maryland, USA), according to the manufacturer's guidelines. The extracted DNA was stored at -20°C until analysis.

Genotyping of SNP T300A (rs2241880)

The polymorphism ATG16L1 (T300A) (rs2241880), an A to G polymorphism resulting in the amino acid exchange at position 300 of the protein (Thr300Ala), were genotyped using PCR and restriction fragment length polymorphism (RFLP) analysis according to the method of Scolaro et al., (2014) and Csöngöi et al., (2010).

A 282 bp fragment of the ATG16L1 gene was amplified by polymerase chain reaction with the following primers: forward 5'-CTCTGTCACCATATCAAGCGTGG-3' and reverse 5'-TCTAGAAGGACAGGCTATCAACAGATG-3'. The amplifications were conducted under the following reaction conditions: initial denaturation at 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing for 40 seconds at 55°C and extension at 72°C for 40 seconds completion with 72 °C extension for 5 minutes. All of the PCR products were electrophoresed on a 1.5% agarose gel with 1 × Tris-borate-EDTA buffer at 100 V for 30 minutes and

then observed under ultraviolet illumination.

After the PCR, amplicons were digested by endonuclease LweI (Fermentas, Burlington, USA) at 37°C during 1 hour, according to the manufacturer's instructions. Endonuclease LweI recognizes the polymorphic sequence GCATCN5 and cuts after six nucleotides in two fragments of 172 bp and 110 bp. The generated fragments were separated by 2.5% agarose gel electrophoresis containing ethidium bromide, followed by exposure to ultraviolet light. All samples were genotyped in duplicate and the genotyping was classified as follows: AA (282 bp), AG (282 bp, 172 bp, and 110 bp), and GG (172 bp and 110 bp).

Statistical analysis

The SPSS 15.0. package for Windows (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. The baseline characteristics were tested using the Student t test for equal variances. This data are expressed as the mean \pm SD. The comparisons of genotype and allelic frequencies among the different groups (prostate and bladder cancer patients and controls) were performed by means of chi-square test. For each polymorphism, unconditional logistic regression was used to calculate odds ratios (OR) and 95% confidence intervals (95% CI) for prostate and bladder cancer. Values of $p < 0.05$ were considered statistically significant. The Hardy-Weinberg equilibrium test was used to test the distributions of mutation genotype frequency. Deviations from Hardy-Weinberg equilibrium were analysed by using Michael H. Court's (2005-2008) online calculator.

(<http://www.tufts.edu/~mcourt01/Documents/Court%20lab%20-%20HW%20calculator.xls>).

Results

A total of 400 subjects (62 PCa patients and 113 healthy controls and 69 BCa patients and 156 healthy controls) were included in the study. The characteristics of the study groups are shown in Table 1. There were no differences in demographic characteristics such as age, sex, and smoking status between patients and controls (Table 1).

The genotype and allele frequencies of the ATG16L1, Thr300Ala polymorphism in the PCa patients and healthy controls are presented in Table 2. All analysed polymorphisms were in Hardy-Weinberg equilibrium both in PCa patients and healthy controls. Genotype distributions for ATG16L1 Thr300Ala polymorphism in control group ($p = 0.11$) were in agreement with Hardy-Weinberg equilibrium but not in patient group ($p = 0.01$).

The AA (wild-type) genotype for the ATG16L1 polymorphism in the PCa patients and in the control group were 22 patients (35%) and 30 healthy individuals (27%) respectively. The AG (heterozygous mutant) and GG (homozygous mutant) genotypes for the ATG16L1 polymorphism in the PCa patients and in the control group were 21 patients (34%) and 48 healthy individuals (42%) and 19 patients (31%) and 35 healthy individuals (31%) respectively. The G allele frequency was 0.40 for in PCa patients and the control groups (Table 2). We did

Table 1. Characteristics of Patients with Prostate Cancer (PCa), Bladder Cancer (BCa) and Healthy Controls

Parameters	Prostate cancer (PCa) (n = 62)	Control (n = 113)	p value
Age	72.83 \pm 7.60	71.60 \pm 7.09	0.28
Smoking status			
Yes	12 (69.66 \pm 6.40)	47 (71.46 \pm 6.20)	0.37
No	50 (73.60 \pm 7.72)	66 (71.69 \pm 7.71)	0.19
	Bladder cancer (BCa) (n = 69)	Control (n = 156)	p value
Age	62.07 \pm 10.48	61.81 \pm 14.25	0.89
Sex			
Male	61 (63.27 \pm 13.22)	126 (63.96 \pm 8.76)	0.72
Female	8 (52.87 \pm 17.32)	30 (52.80 \pm 15.10)	0.99
Smoking status			
Yes	36 (60.78 \pm 9.68)	53 (62.47 \pm 11.78)	0.47
No	33 (63.48 \pm 11.26)	103 (61.47 \pm 15.42)	0.91

$p < 0.05$; significantly different from control group; Yes, current smoker; No, never smoked.

not find any statistical significant difference between ATG16L1 (T300A) genotype and allele frequencies in our PCa patients and healthy control.

The genotype and allele frequencies of the ATG16L1 Thr300Ala polymorphism in the BCa patients and healthy controls are presented in Table 3. An analysis of Hardy-Weinberg equilibrium was used to test the deviation of genotype distribution. Genotype distributions for ATG16L1 Thr300Ala polymorphism in patient group ($p = 0.13$) were in agreement with Hardy-Weinberg equilibrium but not in control group ($p = 0.01$).

Table 2. Distribution of Genotype and Allele Frequencies of ATG16L1 Polymorphism in Patients with Prostate Cancer (PCa) and Healthy Controls

Gene/Genotypes	Patients n=62 n/%	Control n=113 n/%	P value	Odds ratio	95% (CI)	
ATG16L1						
AA	22 (35)	30 (27)	-	1	-	
AG	21 (34)	48 (42)	0.177	1.676	0.790-3.556	
GG	19 (31)	35 (31)	0.452	1.351	0.617-2.959	
A	65 (56)	108 (48)				
Alleles	G	59 (44)	118 (52)	0.407	1.204	0.776-1.866

Table 3. Distribution of Genotype and Allele Frequencies of ATG16L1 Polymorphism in Patients with Bladder Cancer (BCa) and Healthy Controls

Gene/Genotypes	Patients n=69 n/%	Control n=156 n/%	P value	Odds ratio	95% (CI)	
ATG16L1						
AA	24 (35)	50 (32)	-	1	-	
AG	28 (40)	62 (40)	0.856	0.941	0.486-1.821	
GG	17 (25)	44 (28)	0.566	0.805	0.383-1.690	
A	76 (55)	162 (52)				
Alleles	G	62 (45)	150 (48)	0.537	1.135	0.759-1.697

No significant differences were detected between the BCa patients and healthy controls groups for the ATG16L1 genotype and allele frequencies. The prevalence of genotypes of AA (wild-type), AG (heterozygous mutant) and GG (homozygous mutant) profiles for the ATG16L1 Thr300Ala polymorphism were 35%, 40% and 25% respectively in BCa patients, and 32%, 40% and 28% respectively in healthy control groups. The G allele frequency was 0.53 for in BCa patients and the control groups (Table 3).

Discussion

Autophagy is an evolutionarily protected lysosomal degradation pathway from yeast to mammals (Yang et al., 2011). This major pathway appears to be the key process that eliminates damaged macromolecules, including proteins, lipids, and dysfunctional organelles (Yang et al., 2011; Karakaş and Gözüaçık, 2014; Kumar et al., 2014; Yang et al., 2015).

Autophagy pathway has been identified approximately 30 Atg genes (Nakatogawa et al., 2009; Glick et al., 2010; Plantinga et al., 2014). These genes in autophagy pathway play an important role in innate and adaptive immunity and cancer (Brecha et al., 2009; Nakatogawa et al., 2009; Jiang and Mizushima, 2014; Plantinga et al., 2014; Kumar et al., 2014). The ATG gene BECN1 has been mapped to a locus on human chromosome 17q21 which is monoallelically loss in 40–75% of disease of ovarian cancers, breast cancers, and prostate cancers. BECN1 is probably important in tumorigenesis, on the other hand, some studies suggested that other ATG genes are oncogenically associated as well (Mizushima et al., 2008; Chen et al., 2009; Zhi and Zhong, 2015).

Recently, Huijbers et al., (2012) have described that a genetic variant in the autophagy gene ATG16L1 has an important impact on susceptibility to Epithelial cell derived non-medullary thyroid cancer (NMTC). The ATG16L1 protein is expressed in the colon, small intestine, intestinal epithelial cells, leukocytes, and spleen (Yang et al., 2011). The official name of the ATG16L1 gene is “autophagy-related 16-like 1 (*S. cerevisiae*)” located on chromosome 2 (Brecha et al., 2009; Kumar et al., 2014). This molecule encoded by ATG16L1 plays an important role within the pathways of autophagy through interactions, influencing several biological processes like LC3 synthesis by phosphatidylethanolamine (PE) or LC3-II formation (Glick et al., 2010).

Van Limbergen et al., (2008), in their work on adult and childhood CD, showed that, there is an association between rs2241880G-allele and adult-onset CD (60.7% versus controls 53.9%, $P=0.01$, OR 1.32, 95% confidence interval [CI] 1.07–1.63) in contrast to childhood-onset CD (54.1% versus controls, $P=0.95$, OR 1.01, 95% CI 0.80–1.26).

A Morocco study by Serbati et al., (2014) reported in a query of their dataset for IL23R and ATG16L1 variants in Moroccan patients with inflammatory bowel disease, 96 Moroccan IBD patients and 114 unrelated volunteers were genotyped for ATG16L1 (T300A) and IL23R (L310P) variants by PCR-restriction fragment length

polymorphism. They found that IL23R (L310P) variant conferred a protective effect for crohn’s disease (CD) but not for ulcerative colitis (UC) patients. The presence of ATG16L1 (T300A) mutated alleles was associated with CD type but not with UC disease. The findings of Sebati et al. showed that a possible role of ATG16L1 (T300A) on CD phenotype was suggested.

A study by Lauriola et al., (2011) in Italian population about IL23R, NOD2/CARD15, ATG16L1 and PHOX2B polymorphisms in a group of patients with Crohn’s disease and correlation with sub-phenotypes shows 19 Italian patients with CD and 20 healthy controls, that the analysis of correlation of genotype to sub-phenotypes showed an association of ATG16L1 rs2241879 with the lack of extra-intestinal manifestations (OR, 0.03; 95% CI, 0.002–0.45; $P=0.006$), and the patients defined as non-smokers displayed an increased frequency of the risk allele C ($P=0.03$).

Another study on evaluation of genotype frequency of ATG16L1 T300A polymorphism with susceptibility with Crohn’s disease in a study of New Zealand Caucasians with inflammatory bowel disease reported a healthy control group of 549 genotyped as having 23.67% (130) of the subjects with a GG genotype, 24.40% (134) with a AA genotype and 51.91% (285) healthy subjects with a AG genotype (Roberts et al., 2007).

ATG16L1 was found to be highly associated with CD by use of a haplotype and regression analysis (Hampe et al., 2007). Subsequent to the initial genome wide association study, many studies have consistently identified associations between the ATG16L1 (Thr300Ala) variant and CD (Rioux et al., 2007; Barrett et al., 2008; Van Limbergen et al., 2008; Serbati et al., 2014; Salem et al., 2015). It was found out that autophagy gene polymorphisms are correlated with development of chronic inflammatory lesions, which make up a risk factor for colorectal tumors (Boland, 2010; McAfee et al., 2012; Nicoli et al., 2014).

Our study aimed to examine the association of ATG16L1 (T300A) genetic variant with prostate and bladder cancers and controls in Turkish population. At the end of our research, we found out that the genotype AG was prevalent on patients and controls (34% vs 42%), followed by genotypes AA (35% vs 27%) and GG (31% vs 31%) in PCa. The prevalence of genotypes of AA (wild-type), AG (heterozygous mutant) and GG (homozygous mutant) profiles for the ATG16L1 Thr300Ala polymorphism were 35%, 40% and 25% respectively in BCa patients, and 32%, 40% and 28% respectively in healthy control groups. The G allele frequency was 0.53 for in BCa patients and the control groups. No significant differences were detected between the PCa and BCa patients and healthy controls groups for the ATG16L1 genotype and allele frequencies. We realized that the same results as the ones we have reached were gained in some studies on this gene polymorphism (Dema et al., 2009; Scolaro et al., 2014; Chatzikyriakidou et al., 2014). ATG16L1 was found to be highly associated with CD different population, for example Italian (Latiano et al., 2008; Lauriola et al., 2011). Hungarian patients (Lakatos et al., 2008), and Northern Europe (Van Limbergen et al., 2008).

A recent study has reported that ATG16L1 polymorphism (rs78835907) was associated with susceptibility of prostate cancers in Taiwan population (Huang et al., 2015). However, we found no association with prostate cancers and ATG16L1 polymorphism (rs2241880) in Turkish population. In conclusion, further molecular and functional studies in different ethnic groups will be required to ascertain the contribution of the ATG16L1 gene in patients with PCa and BCa.

Conflict of interest statement

None.

Acknowledgments

We would like to thank to Scientific Research Project Unit of Niğde Ömer Halisdemir University (Project No: FEB 2015-33, YÜLTEP)

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