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ORIGINAL ARTICLE

ATG16L1 T300A Polymorphism is Correlated with Gastric Cancer Susceptibility

Florin Burada[1] · Marius Eugen Ciurea[1] · Raluca Nicoli[1] · Ioana Streata[1] · Ionica Dan Vilcea[2] · Ion Rogoveanu[1] · Mihai Ioana[1]

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Abstract Gastric cancer is a major leading cause of cancer-related death in both sexes in Europe. The role of autophagy process in carcinogenesis remains unclear and there is increasing evidence that Helicobacter pylori is a key player in modulating autophagy in gastric carcinogenesis. The aim of this study was to assess the potential association of ATG16L1 T300A polymorphism with susceptibility of gastric cancer, and further to analyze the expression profile of ATG16L1 gene in paired tumoral and peritumoral gastric tissue. A total of 108 patients diagnosed with gastric cancer and 242 healthy controls were enrolled. ATG16L1 T300A polymorphism was detected using TaqMan genotyping assay containing primers and specific probes for A and G allele, respectively. ATG16L1 mRNA level was evaluated in 34 paired tumoral and peritumoral tissues using qRT-PCR. We found a significant association for both carriers of AG (OR 0.52, 95 % CI: 0.30–0.91, $p = 0.02$) and GG genotype (OR 0.53, 95 % CI: 0.28–0.98, $p = 0.043$), these were at a lower risk for gastric cancer when compared with the wild-type AA genotype. The strongest association was observed in a dominant model, the carriers of G allele were protected against gastric cancer (OR 0.52, 95 % CI: 0.13–0.88, $p = 0.013$). In a stratified analyse, the association was limited to non-cardia type and intestinal type. ATG16L1 gene expression was detected in both tumor and peritumoral tissues, with the mRNA-ATG16L1 levels significantly higher in tumor sample. Our results suggest that ATG16L1 T300A polymorphism may be associated with gastric carcinogenesis.

Keywords Gastric cancer · Autophagy · ATG16L1 gene · Polymorphism · Gene expression

Introduction

Gastric cancer is a major health problem worldwide despite advances in diagnosis and treatment, the prognosis being often poor, mainly because a large proportion of gastric cancer patients are detected at a late stage. Although decreases in stomach cancer incidence were seen in many European countries [1], gastric cancer still is one of the most commonly diagnosed cancers in Europe, occupying the sixth position. Furthermore, it is the fourth leading cause of cancer-related death in both sexes after lung, colorectal and breast cancers, accounting for 6.1 % of total deaths from cancer, with an estimated 107.000 deaths in 2012 [2]. The genetic and environment factors are major players in the multifactorial etiology of gastric cancer. Helicobacter pylori (H. pylori) represents the strongest known risk factor for development of gastric cancer, being classified as a class I carcinogen [3, 4]. Infection with H. pylori can induce innate and adaptive immune responses in the host, with the autophagy process as modulator for both of them [5, 6].

Autophagy is an evolutionarily cytoplasmic process that initially uses a double membrane, known as an autophagosome, to engulf cellular components (e.g. long-lived proteins, damaged organelles), as well as pathogens [7]. Subsequently, an

Florin Burada and Marius Eugen Ciurea contributed equally to this article.

✉ Florin Burada
buradaflorin@gmail.com

[1] Research Center of Gastroenterology and Hepatology, University of Medicine and Pharmacy of Craiova, 1 May street no 66, Craiova, Romania

[2] Department of Surgery, University of Medicine and Pharmacy from Craiova, Petru Rares street no 2-4, 200349 Craiova, Romania

autolysosome is generated by the autophagosome fusion with the lysosome, the autophagic being then degraded through the activity of acid hydrolases [8]. Autophagy process is controlled by a complex genetic network involving multiple autophagy-related genes (ATGs). ATG16L1 is a key regulatory autophagy gene, involved in the elongation and closure steps of autophagosomes as a member of ATG5–ATG12–ATG16L1 complex [9]. The ATG16L1 presence is required for the initiation of the autophagosomes formation via the conjugation of microtubule-associated protein 1-light chain (LC3) to the lipid phosphatidylethanolamine, bringing LC3 to the site of lipidation [10]. As a consequence, the absence of ATG16L1 severely affects autophagosome formation, degradation of long-lived proteins and the clearance of bacteria within both immune and epithelial cells [11]. ATG16L1 gene was mapped to chromosome 2q37.1 [12], and the single-nucleotide polymorphism (SNP) in the ATG16L1 c.898A > G (rs2241880) gene results to a change in polarity of amino acids by substitution of threonine to alanine (T300A/Thr300Ala). This above mentioned SNP has been shown to affect the autophagy process [13], and the G allele has been identified as a risk allele in Crohn's disease, a common form of chronic inflammatory bowel disease [14–16].

The role of autophagy process in carcinogenesis is unclear with a polymorphic tissue-type dependent pattern. The autophagy process seems to have two opposite functions: prooncogenic as well as tumor-suppressant. Autophagy promotes achievement of nutrients critical for tumor growth, inhibits cellular death and increases drug resistance. On the contrary, autophagy limits inflammation, tissue damage, genetic instability, and discards damaged organelles and pathogen [7, 17].

There is increasing evidence that H. pylori plays a critical role in modulating autophagy in gastric carcinogenesis [6, 18]. Initially, infection with H. pylori promotes autophagy as a self defense mechanism against pathogen in gastric epithelial cells, but long-time exposure to vacuolating cytotoxin A (VacA) impairs autophagy [19]. The presence of ATG16L1 T300A polymorphism is reported to increase the risk of H.pylori infection in Caucasian individuals from Scotland and Germany [20].

We aimed to assess the association of ATG16L1 T300A (c.898A > G, rs2241880) polymorphism with susceptibility of gastric cancer, and further to analyze the expression profile of ATG16L1 gene in paired tumoral and peritumoral gastric samples obtained by upper endoscopy.

Material and Methods

Subjects

In this hospital-based case-control study, we included a total of 350 Romanian subjects (108 patients with gastric adenocarcinoma and 242 healthy controls). The upper endoscopy was used to diagnose gastric cancer and all cases were histologically confirmed by biopsy specimens, at the Emergency Clinical County Hospital of Craiova, Romania. All included patients were H. pylori positive by histological examination or rapid urease test. Age and gender matched controls with no positive history of a tumor, autoimmune, inflammatory or infectious chronic disease were recruited among unrelated volunteers admitted to the same hospital. Blood samples were obtained from both groups and demographic characteristics, age, sex, and family history of cancer or other diseases were recorded for each participant. The Ethics Committee of University of Medicine and Pharmacy of Craiova, Romania approved this study and a written informed consent was provided by the included participants.

ATG16L1 T300A (rs2241880) Genotyping

The human genomic DNA was isolated from the peripheral blood leukocytes using Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA), according to manufacturer's protocol. The variants of ATG16L1 T300A were detected using predesigned TaqMan genotyping assay (C___9095577_20; Applied Biosystems, Foster City, CA, USA), containing primers and specific probes for A allele and G allele, respectively. Real Time PCR cycling conditions (7300; Applied Biosystems, Foster City, CA, USA) were 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min annealing temperature, and ATG16L1 rs2241880 genotyping was carried out in a 8 μl reaction volume. All samples were randomly distributed and blindly genotyped for quality control. All samples that did not yield a reliable result in the first round were resubmitted for an additional round of genotyping. We included in each run a negative control sample and three positive controls (homozygous for the wild-type allele, and heterozygous and homozygous for the mutant allele).

Table 1 Subject characteristic

	Gastric adenocarcinoma	Control
n	108	242
Male/Female	68/40	152/90
Age (years), mean ± SD	64,22 ± 5,65	60,69 ± 4,94
H.Pylori +	108	
Location		
cardia	29	
noncardia	79	
Histologic type		
intestinal	63	
diffuse	44	
mixed	1	

Table 2 Risk of gastric cancer by genotype

Polymorphism ATG16L1 T300A	Gastric cancer (n = 108)	Control (n = 242)	OR (95%CI)	p value
AA	34 (31.48 %)	47 (19.42 %)	Ref	-
AG	46 (42.59 %)	122 (50.41 %)	0,52 (0.30–0.91)	0.02
GG	28 (25.93 %)	73 (30.17 %)	0.53 (0.28–0.98)	0.043
G carriers	74 (68.52 %)	195 (80.58)	0.52 (0.13–0.88)	0.013

ATG16L1 mRNA Evaluation

ATG16L1 mRNA level was evaluated in tumoral and peritumoral tissue biopsy specimens obtained during endoscopy from 34 gastric adenocarcinoma patients. The samples were collected in RNA later solution (Ambion, Austin, USA) and stored at −80 °C, until RNA isolation. On histopathological examination none of the peritumoral tissues microscopically showed invasion with malignant cells. SV Total RNA Isolation System (Promega, Madison, USA) was used for the isolation and purification of total RNA from tumor and peritumor mucosa samples; the RNA concentration and purity were measured spectrophotometrically using a Biophotometer (Eppendorf, Hamburg, Germany) and the quality of RNA was assessed using the Agilent 2010 Bioanalyzer (Agilent Technologies, Santa Clara, USA). The reverse-transcription was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA). ATG16L1 mRNA was evaluated using quantitative reverse transcription polymerase chain reaction (qRT-PCR) with specific TaqMan qene expression assay (Hs01003142_m1, Applied Biosystems, Foster City, USA). The reactions were performed on a ViiA™ 7 Real Time PCR System (Life Technologies, Carlsbad, USA) using GAPDH (Hs99999905_m, Applied Biosystems, Foster City, USA) as the selected house-keeping gene.

Statistical Data Analysis

Hardy-Weinberg equilibrium was tested by comparing the observed genotype frequencies with those expected using chi–squared test. Associations between genotypes and gastric cancer were calculated as odds ratios (OR) with 95 % confidence intervals (CIs) by logistic regression. The homozygous genotype for the wild type allele in Caucasians has been used as the reference category (codominant and dominant models). A p value <0.05 was considered as significant. Expression of ATG16L1 was normalized to GAPDH mRNA and variations in paired samples were expressed as fold changes, being considered significant when the mRNA levels varied more than 1.8 fold (>1.8 greater expression in tumor; <0.55 greater expression in peritumor tissue; 0.55–1.8 = irrelevant difference between tumorand peritumor tissue). $2^{-\Delta\Delta Ct}$ method was used for calculating fold changes between paired samples. The SPSS statistical software package version 17 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5 software (GraphPad, San Diego, USA) were used for statistical analyses.

Results

The characteristics of participants are shown in Table 1, and there were no significant differences in the distribution of age and gender between gastric cancer and control groups. Based on the origin site of cancer, the gastric cancer group was classified into two subgroups: cardia (29) and non-cardia (79). According to the Lauren classification, 63 (58 %) were intestinal-type, 44 (41 %) were diffuse-type and 1 (1 %) was mixed type.

The genotype frequencies of ATG16L1 T300A SNP in controls were distributed in accordance with Hardy–Weinberg equilibrium. In a codominat model, we observed a significant association for both carriers of AG (OR 0.52, 95 % CI: 0.30–0.91, p = 0.02) and GG genotype (OR 0.53, 95 % CI: 0.28–0.98, p = 0.043), these were at a lower risk for gastric cancer when compared with the wild type AA genotype (Table 2). Furthermore, the strongest association was found

Table 3 Risk of cardia and non-cardia adenocarcinoma by genotype

ATG16L1 T300A	Cardia n(%)	OR (95%CI); p	Non-cardia n (%)	OR (95%CI); p
AA	9 (31.03 %)	Ref	25 (31.65 %)	Ref
AG	13 (44.83 %)	0.56 (0.22–1.39); 0.2	33 (41.77 %)	0.51 (0.27–0.94); 0.03
GG	7 (24.14 %)	0.50 (0.18–1.44); 0.19	21 (26.58 %)	0.54 (0.27–1.07); 0.07
G carriers	20 (68.97 %)	0.54 (0.23–1.25); 0.14	54 (68.35 %)	0.52 (0.29–0.92); 0.023

in a dominant model, the carriers of G allele were protected against gastric cancer (OR 0.52, 95 % CI: 0.13–0.88, $p = 0.013$). Association of ATG16L1 T300A SNP with tumor site and histologic subtype was examined separately. In a stratified analyse, the association between gastric cancer and polymorphism was limited to non-cardia type (OR 0.52, 95 % CI: 0.29–0.92, $p = 0.023$) (Table 3) and intestinal type (OR 0.52, 95 % CI: 0.28–0.96, $p = 0.035$) (Table 4) in a dominant model.

The expression of total ATG16L1 was assessed in 34 paired samples. qRT-PCR analysis revealed that ATG16L1 was detected in both tumor and peritumoral tissues, with the mRNA-ATG16L1 levels significantly higher in tumor sample ($p = 0.0001$) (Fig. 1). ATG16L1 expression was greater in tumor tissues when compared with the non-invaded peritumor paired samples in 12 cases (35.29 %). For the remaining 22 cases (64.71 %) the difference between paired samples was biological insignificant. No association between ATG16L1 dysregulation and tumor site, histologic type or genotype was depicted.

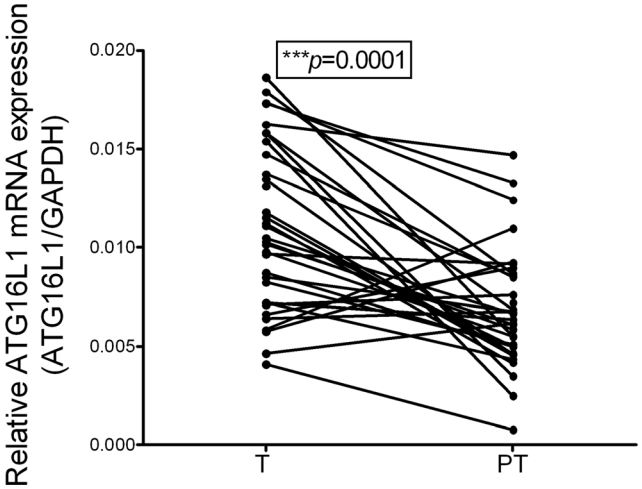

Fig. 1 Comparative expression of ATG16L1 mRNA in paired tumoral and peritumoral mucosa. Data are presented as relative mRNA expression of target gene to GAPDH. Wilcoxon matched-pairs signed rank test

Discussion

To our knowledge, this is the first report showing that ATG16L1 T300A polymorphism might influence susceptibility to gastric cancer, the carriers of the G allele seem to be more protected against gastric cancer development, mainly for intestinal and non-cardia types. We found that ATG16L1 gene expression was significantly upregulated in gastric tumor samples compared to paired normal samples, but not significant differences between ATG16L1 expression induced by allelic variants in both tumor and peritumoral samples was found. ATG16L1 is a key gene, not only for autophagosome formation and induction of autophagy, but also in modulation of inflammation [21], a well known risk factor for gastric adenocarcinoma. Published findings on the ATG16L1 T300A SNP produced inconsistent results. ATG16L1 deficient macrophages showed an abnormal inflammatory reaction with an increased levels of the IL-1β and IL-18 cytokines following lipopolysaccharide stimulation [21]. ATG16L1 T300A stimulated production of IL-1β in mononuclear cells of subjects carrying homozygous GG genotype compared with AA genotype [22]. Moreover, a higher level of the pro-inflammatory cytokine IL-1β and a decreased antibacterial autophagy was found in mice bearing ATG16L1 T300A variant [23]. A higher susceptibility to H. pylori infection was observed for ATG16L1 T300A carriers, and peripheral blood monocytes from individuals with the ATG16L1 risk variant showed impaired autophagic responses to VacA exposure [20].

Furthermore, it has been suggested that both alleles seem to have an equal contribution of mediating bulk autophagy levels. The presence of risk G allele did not significantly affect canonical autophagy against Salmonella, with no impaired basal autophagy in human intestinal cells, suggesting that a non-autophagic function of T300A may underlie its association with Crohn's disease, as well as other human diseases [24].

Similar to our findings, the presence of ATG16L1 G allele has been associated with a protective effect against epithelial thyroid carcinoma. Both heterozygous AG and homozygous GG patients have been associated with a better prognosis than homozygous AA patients [22]. In a recent study, ATG16L1 T300A was associated with increased overall survival and reduced metastasis in colorectal cancer patients, showing an improved survival for GG genotype carriers [25]. In the same study, ATG16L1 T300A variant does not affect canonical autophagy, but stimulates type I interferon production, suggesting that disruption of cytokine balance can modify the disease outcome.

Table 4 Risk of intestinal and diffuse adenocarcinoma by genotype

ATG16L1 T300A	Intestinal n(%)	OR (95%CI); p	Diffuse n(%)	OR (95%CI); p
AA	20 (31.75 %)	Ref	13 (29.55 %)	Ref
AG	28 (44.44 %)	0.54 (0.28–1.05); 0.07	18 (40.90 %)	0.53 (0.24–1.17); 0.11
GG	15 (23.81 %)	0.48 (0.22–1.04); 0.058	13 (29.55 %)	0.64 (0.27–1.51); 0.31
G carriers	43 (68.25 %)	0.52 (0.28–0.96); 0.035	31 (70.45 %)	0.58 (0.28–1.18); 0.13

We propose several hypotheses to explain our results. On one hand, the ATG16L1 gene-related epistasis exists due to the fact that autophagy is a complex mechanism involving various genes that regulate different processes (e.g., inflammation, immunity). On the other hand, ATG6L1 T300A influences IL-1β production and it may affect cancer susceptibility through modulation of the cytokine response in a context-dependent manner. Autophagy tumor promoting roles could be disrupted by ATG16L1 genetic changes with a protective effect in a specific tissue-types. Other possible explanation might be that the autophagy process is further modulated by the interactions between various SNPs within haplotypes. The haplotype context influenced by ethnicity background and past selective pressures unique to different populations may explain the controversial published results on populations of different geographic origins. To attenuate the small size of subgroups we used both codominat and dominant models.

In conclusion, we found that ATG16L1 T300A SNP can influence susceptibility to gastric cancer, the presence of G allele is associated with a protective effect against gastric cancer development. ATG16L1 gene tends to be upregulated in tumor compared to normal gastric tissue. Further investigations are required to confirm our results, and furthermore to unravel the underlying autophagy mechanisms in gastric carcinogenesis.

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Author Contributions F.B., I.R. and M.I. conceived the study; F.B, R. N. and I.S. performed the experiments; E.M.C., I.D.V., I.R. and I.M. provided resource and materials; F.B, E.M.C., I.S., R.N., and I.D.V analyzed the data; F.B., E.M.C. and I.M. wrote the manuscript; I.R. and I.M. supervised the project.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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