ORIGINAL PAPER

RJME
Romanian Journal of Morphology & Embryology
http://www.rjme.ro/

Determination of autophagy gene ATG16L1 polymorphism in human colorectal cancer

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Abstract

Autophagy has emerged not only as an essential repair mechanism to degrade damaged organelles and proteins but also as a major player in protection of tumor cells from multiple stresses. It was shown that autophagy gene polymorphisms are correlated with development of chronic inflammatory lesions, which represent a risk factor for colorectal tumors. In this study, we aimed to determine if ATG16L1 +898A>G (Thr300Ala) polymorphism is associated with an increased risk of developing colorectal cancer (CRC) and to establish correlations between ATG16L1 genotypes and the major clinical and morphological parameters. We observed that subjects carrying GG genotype were at a higher risk for CRC (OR 1.99, 95% CI: 1.02–3.91, p=0.039) when compared with the more frequent AA genotype, furthermore this was even more consistent in male subjects (OR 2.72, 95% CI: 1.11–6.63, p=0.019) but not in female subjects (OR 1.29, 95% CI: 0.43–3.86, p=0.652). In addition, we noticed a correlation between ATG16L1 GG genotype and tumor stage in moderately and poorly differentiated CRC cases. GG genotype carrying patients were at a higher risk for CRC (OR 5.19, 95% CI: 1.50–17.87, p=0.002) when compared with the more frequent AA genotype. Such correlation suggests a possible role of autophagy gene polymorphisms in the development of human colorectal cancer.

Keywords: ATG16L1, gene polymorphism, colorectal cancer, autophagy.

Introduction

It has been accepted that inflammatory bowel disease (IBD) is associated with an increased risk of developing advanced colorectal neoplasia, including colorectal high-grade dysplasia and colorectal cancer (CRC) [1]. The risk of developing CRC is increased in both main types of IBD and it has been associated with prolonged duration of disease, extent of disease, associated primary sclerosing cholangitis and active inflammation [2]. Some recent studies suggest that the risk of CRC in IBD is present from the early phases of the disease [1].

Autophagy is a catabolic intracellular process in which cytoplasmic components are sequestered within vesicles and delivered to the lysosomes [3]. It is the pathway used by cells during nutrient starvation to break down non-vital components and use them as nutrients. Autophagy is involved in infectious processes by helping get rid the cell of foreign antigens by breakdown of the pathogen [3]. This pathway can also be implemented as a repair mechanism to degrade damaged organelles and proteins. Autolysosomes, formed by the fusion of lysosomes and autophagic vacuoles, are also involved in processing intracellular bacteria. A mutation within an autophagy gene can cause a change in normal flora and many gastrointestinal (GI) problems, representing a possible cause of Crohn's disease (CD) [4]. If the cells cannot regain nutrients or fight off foreign antigens within the GI tract, they will undergo a high rate of apoptosis leading to extensive tissue damage from superficial lesions to ulcers within the intestines, creating dead infected patches of tissue [3]. These GI damages represent a key

factor in pathogenesis of IBDs like CD. When they appear at a young age (less than 30 years of age), the affected individuals have a high risk of developing colorectal cancer (CRC) [5].

Alteration of the autophagy mechanisms lead to diseases such as neurodegenerative diseases or cancer through cellular destructions and genetic instability [6]. Among the autophagy genes frequently reported as mutated in gastric and colorectal human cancers are: BECN1, UVRAG, SH3GLB1 (Bif-1), Atg2B, Atg5, Atg9B, Atg12, and RAB7A [7–10].

For cancer cells, autophagy is a critical survival pathway under conditions of cell stress or nutrient limitation [11, 12]. Autophagy is activated in colorectal cancers, in both *in vitro* and *in vivo* studies, and may contribute to the cancer cells survival [13]. Loss of the autophagy protein ATG16L1 (autophagy-related 16-like 1) enhances the endotoxin-induced cytokine production, acting as a modulator factor of inflammation [14], with an effect on different cancer cell types survival [15].

To test whether the ATG16L1 +898A>G polymorphism contributes to human colorectal cancer onset and/or evolution, we have compared the genotype frequencies detected in two Romanian groups: patients diagnosed with CRC and healthy control subjects. This polymorphism was selected based on functional data depicting its potential role in autophagy and carcinogenesis, mainly related to the gastrointestinal tract [4, 5, 16–18].

Materials and Methods

Patients and samples

A total number of 466 Romanian subjects (109 patients diagnosed with sporadic CRC and 357 healthy controls) were included in this study, following a standard diagnostic procedure at the Emergency County Hospital of Craiova, Romania. Matched controls patients of the same ethnic and geographical background were recruited, based on a negative history of tumor or inflammatory bowel diseases. Blood samples were obtained from both groups. Demographic data, age, gender, clinical information (family history of CRC and patient history of chronic diseases) were also collected and recorded both their medical history records and questionnaires for each patient. The Ethics Committee of University of Medicine and Pharmacy of Craiova, Romania approved this study and all the enrolled subjects have signed the informed consent.

The rectal cancer subgroup included the patients with tumors localized on rectum and rectosigmoid junction. Tumors from the cecum to the splenic flexure were defined as proximal cancers, whereas the ones on splenic flexure, descending colon and sigmoid colon were classified as distal colon cancers.

DNA extraction and genotyping

Genomic DNA was extracted from the peripheral blood leukocytes using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), following the manufacturer's protocol. Polymorphism genotyping was performed with a predesigned TaqMan assay from Applied Biosystems (Foster City, CA, USA): C_9095577_20 (ATG16L1, +898A>G, rs2241880).

Genotyping was carried out in a 5-μL reaction volume using the 5' nuclease assay by minor groove binder (MGB) probes fluorescently labeled with FAM or VIC and using the protocol recommended by the supplier (Applied Biosystems, Foster City, CA, USA).

Real Time PCR cycling conditions (RotorGene 6200 HRM – Qiagen) for the denatured reactions were 95^0C for 10 minutes, followed by 50 cycles of 92^0C for 15 seconds and 60^0C for one minute annealing temperature.

Interpretation of samples was done using Rotor-Gene 6000 Series Software 1.7 with the option for Allelic Discrimination.

Statistical analysis

Associations between genotypes and CRC were calculated as odds ratios (OR) with 95% confidence intervals (CIs) using unconditional logistic regression analysis. In addition to the overall association analysis, the analysis was performed stratified by tumor localization, stage and histology to further explore the association between autophagy genotypes and the risk of CRC in each stratum. In all cases, homozygous genotype for the most common allele in Caucasians was used as reference. A *p*-value less than 0.05 was considered statistically significant.

Results

Patients' characteristics

All 466 samples harvested from colorectal cancer patients during surgery and healthy controls were genotyped. Genotyping was performed in 109 CRC patients and 357 controls. The characteristics of the patients enrolled in this study are shown in Table 1.

Table 1 – ***Patients' characteristics***

	Colorectal cancer	Control
n	109	357
Males/Females	66/43	267/90
Age [years], mean±SD	67±8.81	60.69±7.94
Location:		
▪ Proximal	25	
▪ Distal	50	
▪ Rectum	34	
Tumor stage (Dukes' stage):		
▪ A + B	56	
▪ C + D	53	
▪ Unknown/Not defined	0	
Histological grade:		
▪ G1 – well-differentiated	26	
▪ G2 – moderate differentiated	63	
▪ G3 – poorly differentiated	20	

There were no differences in median age and gender between cases and controls. CRC cases were characterized by tumor site (proximal, distal and rectum), Dukes' stage (A, B, C, D), and histological grade (G1 – well-differentiated, G2 – moderate differentiated, G3 – poorly differentiated). In 75 cases, the location was within colon

and in 34 cases within rectum. The tumor was limited to mucosa (A) in three cases, in 53 cases, the tumor had invaded the muscularis propria (B), in 35 cases, the tumor had invaded lymph nodes (C), and in 18 cases, tumor metastases were found (D). Based on histological grading, 26 CRC cases were well-differentiated, 63 moderately differentiated, and 20 poorly differentiated.

Genotype frequency and the risk of CRC in Romanian population

Due to the fluorescent signal given by the TaqMan probe, the alleles could be identified as being the allele G the one given by the signal FAM and allele A the one from signal VIC, in this way forming the signals for the three genotypes: homozygote – GG, heterozygote – AG, and homozygote AA. Genotype frequency is shown in Figure 1 and Table 2. We did not find any statistical significant difference between ATG16L1 +898A>G genotype frequencies by gender in our healthy control subgroups (Table 2).

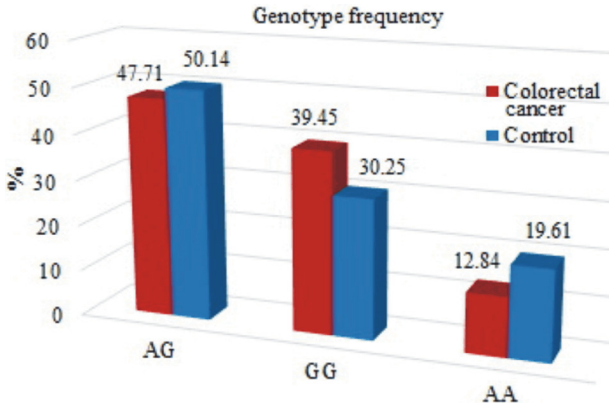

Figure 1 – *Genotype frequency for ATG16L1 +898A>G (Thr300Ala) polymorphism.*

Table 2 – *ATG16L1 +898A>G (Thr300Ala) genotype frequencies in healthy male and female patients*

Genotype	Males (*n*=267)	Females (*n*=90)	OR (95% CI)	*P*-value
AA	52 (19.48%)	18 (20%)	Reference	–
AG	133 (49.81%)	46 (51.11%)	1.00 (0.57–1.88)	0.998
GG	82 (30.71%)	26 (28.89%)	1.09 (0.55–2.19)	0.805

A significant association was observed for patients carrying GG genotype that were at a higher risk for CRC (OR 1.99, 95% CI: 1.02–3.91, p=0.039) when compared with the more frequent AA genotype. Furthermore, this was consistent in male subjects carrying GG genotype that showed also a higher risk for CRC when compared with the more frequent AA genotype (OR 2.72, 95% CI: 1.11–6.63, p=0.019). There were found no correlation between CRC cases and controls for female patients carrying GG genotype (OR 1.29, 95% CI: 0.43–3.86, p=0.652) or AG genotype (OR 1.29, 95% CI: 0.47–3.52, p=0.621), results are shown in Table 3.

Association of ATG16L1 +898A>G (Thr300Ala) polymorphism with Dukes' stage and histological subtype was examined separately (A, B, C, D). In a stratified analysis, the only association between CRC and autophagy polymorphism was found for carriers of GG allele and was restricted to moderate and poorly differentiated cases (OR 5.19, 95% CI: 1.50–17.87, p=0.002) (Table 4). No significant differences were observed between tumor stage (A + B) (Table 4), localization (Table 5) or histological grading and controls in stratified analysis (Table 6).

Table 3 – *Comparative analysis between genotype frequencies and the risk of CRC for ATG16L1 +898A>G (Thr300Ala) polymorphism in male and female patients*

Polymorphism	Colorectal cancer	Control	OR (95% CI)	*P*-value
ATG16L1:	(*n*=109)	(*n*=357)		
AA	14 (12.84%)	70 (19.61%)	Reference	–
AG	52 (47.71%)	179 (50.14%)	1.45 (0.76–2.79)	0.251
GG	43 (39.45%)	108 (30.25%)	**1.99 (1.02–3.91)**	**0.039**
ATG16L1 in male patients:	(*n*=66)	(*n*=267)		
AA	7 (6.42%)	52 (19.48%)	Reference	–
AG	29 (26.61%)	133 (49.81%)	1.62 (0.66–3.93)	0.269
GG	30 (27.52%)	82 (30.71 %)	**2.72 (1.11–6.63)**	**0.019**
ATG16L1 in female patients:	(*n*=43)	(*n*=90)		
AA	7 (6.42%)	18 (20%)	Reference	–
AG	23 (21.1%)	46 (51.11%)	1.29 (0.47–3.52)	0.621
GG	13 (11.93%)	26 (28.89%)	1.29 (0.43–3.86)	0.652

Table 4 – *Comparative analysis between genotype frequencies and the risk of CRC in tumor stage subgroups (Dukes' stages)*

Tumor stage (Dukes)	Colorectal cancer	Control	OR (95% CI)	*P*-value
A + B:	(*n*=56)	(*n*=357)		
AA	11 (10.09%)	70 (19.61%)	Reference	–
AG	26 (23.85%)	179 (50.14%)	0.92 (0.43–1.97)	0.839
GG	19 (17.43%)	108 (30.25%)	1.12 (0.50–2.49)	0.782
C + D:	(*n*=53)	(*n*=357)		
AA	3 (2.75%)	70 (19.61%)	Reference	–
AG	26 (23.85%)	179 (50.14%)	3.39 (0.99–11.56)	0.025
GG	24 (22.02%)	108 (30.25%)	**5.19 (1.50–17.87)**	**0.002**

Table 5 – *Comparative analysis between genotype frequencies and the risk of CRC in the tumors site subgroups*

Tumor site (localization)	Colorectal cancer	Control	OR (95% CI)	*P*-value
Proximal:	(*n*=25)	(*n*=357)		
AA	2 (1.83%)	70 (19.61%)	Reference	–
AG	13 (11.93%)	179 (50.14%)	2.54 (0.56–11.55)	0.182
GG	10 (9.17%)	108 (30.25%)	3.24 (0.69–15.23)	0.097

Tumor site (localization)	Colorectal cancer	Control	OR (95% CI)	*P*-value
Distal:	(*n*=50)	(*n*=357)		
AA	8 (7.34%)	70 (19.61%)	Reference	–
AG	25 (22.94%)	179 (50.14%)	1.22 (0.53–2.84)	0.637
GG	17 (15.60%)	108 (30.25%)	1.38 (0.56–3.36)	0.476
Rectal:	(*n*=34)	(*n*=357)		
AA	4 (3.67%)	70 (19.61%)	Reference	–
AG	14 (12.84%)	179 (50.14%)	1.37 (0.44–4.30)	0.582
GG	16 (14.68%)	108 (30.25%)	2.59 (0.83–8.08)	0.077

Table 6 – ***Comparative analysis between genotype frequencies and the risk of CRC in the histological grade subgroups***

Histological grade	Colorectal cancer	Control	OR (95% CI)	*P*-value
G1*:*	(*n*=26)	(*n*=357)		
AA	4 (3.67%)	70 (19.61%)	Reference	–
AG	11 (10.09%)	179 (50.14%)	1.08 (0.33–3.49)	0.903
GG	11 (10.09%)	108 (30.25%)	1.78 (0.55–5.82)	0.322
G2*:*	(*n*=63)	(*n*=357)		
AA	9 (8.26%)	70 (19.61%)	Reference	–
AG	29 (26.61%)	179 (50.14%)	1.26 (0.57–2.80)	0.564
GG	25 (6.42%)	108 (30.25%)	0.50 (0.18–1.42)	0.191
G3*:*	(*n*=20)	(*n*=357)		
AA	1 (0.92%)	70 (19.61%)	Reference	–
AG	12 (11.01%)	179 (50.14%)	4.69 (0.60–36.77)	0.070
GG	7 (6.42%)	108 (30.25%)	4.54 (0.55–37.68)	0.099

Discussion

Autophagy is a complex pathway, modulated by several molecular mechanisms that still remain elusive. There have been approximately 30 autophagy-related (Atg) genes identified, with two proteins having ubiquitin-like characteristics, Atg12 and Atg8 [19]. These proteins covalently modify their target protein with molecules such as ubiquitin-like proteins to tag them for degradation. Both proteins also contain a conserved ubiquitin-fold region [20]. Autophagosomes use two conjugation systems, the Atg12 and LC3-II systems [21]. These systems were first discovered during yeast genetic studies revealing a set of 17 ATG genes involved in the autophagy pathway [22]. In the Atg12 conjugation system, an Atg12-Atg5-Atg16L complex forms, and dissociates from the membrane just before or after completion of the autophagosome [19, 23, 24].

The ATG16L1 protein is expressed in the colon, small intestine, intestinal epithelial cells, leukocytes, and spleen [21]. The official name of the ATG16L1 gene is "autophagy-related 16-like 1 (*S. cerevisiae*)." Location: 2q37.1; sequence: chr. 2, NC_000002.11 (234160217..234204320) [25, 26]. This the 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 [27].

An USA study by Baldassano *et al.* reported in a query of their dataset for an association of the T300A non-synonymous variant of the ATG16L1 gene with susceptibility to pediatric Crohn's disease, a healthy control group of 281 out of which 27.8% were GG genotype, 23.8% were AA genotype and the rest of 48.4% were identified as being AG genotype [28]. A Dutch study from 2012, by Huijbers *et al.*, about the effect of the ATG16L1 Thr300Ala polymorphism on susceptibility and outcome of patients with epithelial cell-derived thyroid carcinoma shows, in the healthy control group of 1964 subjects, that the frequency of the GG genotype (Ala/Ala) is 28% and the AA genotype (Thr/Thr) is of 19%, the rest of the subjects 52% were AG genotype [18]. Also, another study 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 [29]. Büning *et al.* study in three European IBD cohorts searching for an confirmation of ATG16L1 898A>G (Thr300Ala) variant as being a susceptibility factor for Crohn's disease, used a healthy control cohort of 707 subjects out of which 25.88% (183) were GG genotype, 24.04% (170) were AA genotype and half of the cohort was AG genotype (50.07% – 354) [30]. This study was realized with a cohort from Germany of 285 healthy control subjects from which were 23.85% (68) GG genotype, 25.96% (74) AA genotype and 50.17% (143) AG genotype. Another sub-study within this research project had a Hungarian healthy control cohort (207 subjects) with: GG genotype – 23.67% (49), AA genotype – 23.67% (49) and AG genotype – 52.65% (109). The third cohort from this study was from The Netherlands, with a total number of 215 healthy control individuals that was formed of 30.69% (66) GG genotype subjects, 21.86% (47) AA genotype subjects and 47.44% (192) AG genotype subjects [30]. In an Italian study, the evaluation of the ATG16L1 T300A polymorphism (rs2241880) by Latiano *et al.*, in 2008, made available a cohort of 749 healthy control individuals with the following results: 29% (214) for GG, 21% (159) for AA and 50% (376) for AG [31].

When comparing ATG16L1 898A>G genotype frequencies in healthy control cohorts from different studies, including our healthy control group, it can be noticed that half of them are made off heterozygous AG genotype and the rest consist of a quarter with GG genotype with a slightly higher tendency than the other quarter formed by AA genotype.

In 2010, a study has shown that an ATG16L mutation: ATG16L1 – Thr300Ala (rs2241880), located on chromosome 2, is associated with the onset of ileal CD, and is therefore a key molecule in elucidating the genetic aspects of this disease [32]. Many studies have

been performed since, each resulting in the same conclusion that ATG16L is associated with chronic inflammation status of gastrointestinal tract, which increases the risk for colorectal cancer [16, 17]. During a genome-wide survey of 19 779 non-synonymous single nucleotide polymorphisms (SNPs), Thr300Ala within the N terminus of ATG16L it was found to be highly associated with CD by using a haplotype and regression analysis. This study used a total of 735 CD patients, 368 controls and 72 SNPs [33].

A second report from the North American CD genome-wide study also shows an association with ATG16L using a case-control analysis in 988 CD patients and 1007 controls [25]. Another group of German and British collaborators demonstrated that rs2241880, another non-synonymous variant of the ATG16L gene on chromosome 2q37.1, is implicated in the autophagy pathway [21]. In a study performed on an Italian cohort, the same polymorphism, rs2241880, was observed in 667 CD. Both the frequency of the G allele and number of carriers of the G allele were increased in CD patients when compared to the controls [34]. A study from Oxford compared 645 CD patients with 1190 controls and showed an association of ATG16L1 with CD [35].

It has been noticed that autophagy can also be involved in tumorigenesis inducing senescence and cell death but also tumor preservation and drug resistance can be modulated by autophagy [36–39]. Taking into account that patients with IBD, especially CD have a six-fold higher risk of developing colorectal cancer, several studies targeting to reveal if there is a link between autophagy genes, and susceptibility to this type of cancer, were conducted [40]. Thus, a study conducted by Kang *et al.*, in 2009, indicates that frameshift mutations in ATG genes with mononucleotide repeats appear in gastric and colorectal carcinomas suggesting that these mutations may contribute to cancer development by deregulating the autophagy process [8]. Also, Yoshioka *et al.* showed that microtubule-associated protein 1 light chain 3 (LC3) expression is linked with gastrointestinal cancer development especially in early-phase carcinogenesis [41].

In our hospital-based case-control study was assessed whether ATG16L1 898A>G polymorphism influence sporadic CRC development and progression in Romanian population.

There are recent scientific discussions regarding the roles of autophagy bridging both the innate and adaptive immune systems, and autophagic dysfunction associated with inflammation and cancer [42]. It is necessary to study also if there is a link between ATG16L1 gene, CD course and colorectal cancer predisposition. It is clear that variants of this gene have been proven to be directly associated with CD because autophagy plays a critical role in disease pathogenesis. Further research needs to focus on understanding how ATG16L1 variants contribute to disease susceptibility in CRC patients, and their possible therapeutic implications, and, allows, the link between the ATG16L1 variants in IBD and colorectal cancer.

Conclusions

In this study, it was found that ATG16L1 +898A>G (Thr300Ala) polymorphism influences the risk of CRC in Romanian population mainly for the male carriers of GG genotype that are at a higher risk for CRC. While this study indicates a strong association in the moderately and poorly differentiated CRC cases, the small subgroups size oust drawing conclusions. Further research is required in different ethnic populations to improve the level of knowledge of autophagy in colon cancer tumorigenesis.

Acknowledgments

Elena-Raluca Nicoli acknowledges the support of the research grant POSDRU/107/1.5/S/82705 project, supported by Sectorial Operational Programme Human Resources Development, financed by the European Social Fund and the Romanian Government. First author also acknowledges that this work was supported by a grant of the Romanian National Authority for Scientific Research, CNCS–UEFISCDI, project ID PNII-CT-ERC-2012-1.

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Received: November 6, 2013 *Accepted: February 3, 2014*