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Original Article

ATG16L1 rs2241880 polymorphism predicts unfavorable clinical outcomes for colorectal cancer patients in the Chinese population

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Abstract: Numerous studies have shown that the single-nucleotide polymorphism (SNP) rs2241880 (Thr300Ala) of autophagy related 16-like 1 (*ATG16L1)* is strongly associated with development of Crohn's disease, which represents a risk factor for colorectal cancer (CRC). To date, the role of *ATG16L1* rs2241880 polymorphism in CRC remains unclear. In this study, we aim to determine if the rs2241880 SNP is correlated with the risk of developing CRC and to investigate the prognostic value of this SNP in patients with CRC in the Chinese population. No significant association was found between the *ATG16L1* genotypes and clinicopathological parameters. We observed that *ATG16L1* rs2241880 was not associated with the risk of CRC. However, compared to those with AA and AG genotypes, CRC patients carrying the GG genotype at *ATG16L1* showed a marginal trend to be diagnosed with CRC at younger age (recessive model) (P=0.067). Besides, overall median follow-up time was significantly decreased in patients with the GG genotype (recessive model) (P=0.027). Kaplan-Meier survival analysis exhibited that patients carrying the GG genotype of rs2241880 had worse overall survival than those with the AA and AG genotype (P<0.001). In multivariate analyses, rs2241880 GG genotype was revealed as an independent prognostic marker for overall survival (HR=4.70, P<0.001). These results suggest that GG genotype of rs2241880 may predict unfavorable outcome of patients with colorectal cancer. Further investigations are required to explore the role and mechanism of the polymorphism rs2241880 at *ATG16L1* in colorectal cancer.

Keywords: *ATG16L1*, SNP, rs2241880, colorectal cancer, survival

Introduction

Autophagy, a process involved in the elimination and recycling cytoplasmic materials through lysosomal degradation, is essential for survival, differentiation, development and homeostasis. Impairment of autophagy is associated with numerous diseases including infections, aging, neurodegenerative and myodegenerative diseases, cardiomyopathies and cancers [1]. ATG16L1 (autophagy-related protein 16-like 1 (S. cerevisiae)) is an important autophagy related protein which interacts with ATG12-ATG5 to mediate the formation of vesicular autophagosomes [2]. Of interest, a plenty of genome-wide association studies (GWASs) and candidate-gene based association studies performed have consistently identified single-nucleotide polymorphisms (SNPs) in *ATG16L1* associated with Crohn's disease in different populations [3-7]. The rs2241880 is one of the most common and widely studied SNPs in *ATG16L1* and encodes a missense variant leading to a threonine-to-alanine substitution at amino acid position 300 (Thr300Ala) in exon 9 [3].

The Thr300Ala variant enhances ATG16L1 degradation by caspase 3, resulting in decreased autophagy [8]. Carriers of the G allele of rs2241880 have been reported to be associated with several disease such as palmoplantar pustulosis [9] and Paget disease of bone [10], which may be related to a deregulation of

Table 1. Demographic Characteristics of the Study Population

	Colorectal cancer	Control
n	964	891
Males/Females	530/434	464/427
Age (year), mean ± SD	62.38±12.15	55.16±11.97

SD, stand deviation.

autophagy. ATG16L1 is also required for bacterial clearance and the generation of antigen-specific T-cell responses [11]. The Thr300Ala variant leads to decreased antibacterial defense and elevated production of pro-inflammatory cytokines (such as TNF-α, IL-6 and IL-1β), which could drive the chronic inflammation observed in Crohn's disease [12-14]. And many *in vitro* studies show that patients with *ATG16L1* rs2241880 GG genotype have a higher risk of Crohn's disease [15]. It has been well recognized that the patients with Crohn's disease, a major type of inflammatory bowel disease (IBD), are associated with susceptibility to colorectal cancer (CRC) [16, 17].

Colorectal cancer is the third most common cancer and the fourth leading cause of cancer death in the world [18]. Over the past few decades, the incidence of CRC has rapidly increased in previously low-risk countries, including China [19, 20]. The 5-year survival rate of patients with CRC at a localized stage is 90%, while the survival rate of primary CRC involving adjacent organs or lymph nodes drops to 70% [21]. Once CRC patients present distant metastasis (such as liver), the 5-year survival rate is <10% [22]. Given this, finding novel prognostic biomarkers of CRC are needed. It has been reported that GG genotype of rs2241880 was significantly related to a higher risk of developing CRC among Romanian population, in accordance with its accepted role in Crohn's disease [23]. Nevertheless, the function of autophagy in cancer is very intricate and still somewhat controversial. It seems to be tumor suppressive during cancer initiation, but contributes to tumor cell survival during cancer progression [24]. As an important autophagy related protein, the role of ATG16L1 in metastasis and survival of cancer patients has also not been fully elucidated. A very recent study has showed that GG genotype of rs2241880 is associated with improved overall survival and reduced metastasis in CRC patients from Caucasian and African-American populations [25]. And given the ethnic differences in disease susceptibility genes, replication of these findings in populations with a different genetic background is essential to confirm the disease-related genes [26].

To date, few studies have been done on the role of this SNP in CRC patients from the Chinese population. Thus, this study aims to examine the association between *ATG16L1* rs2241880 SNP and the development of CRC, and to confirm the role of this SNP as a predictor of the outcomes for CRC patients in Chinese population. To test this, we compare the genotype frequencies detected in patients with CRC and control subjects and investigate the prognostic value of this SNP in CRC patients.

Materials and methods

Patients

The study population (n=1856) consisted of 965 CRC patients of Chinese origin and 891 healthy, unrelated controls. One of the samples was failed in the genotyping and removed. These subjects were collected at the Taizhou Hospital of Zhejiang Province, the First Affiliated Hospital of Zhejiang University School of Medicine and the Shaoyifu Affiliated Hospital of Zhejiang University School of Medicine between 2006 and 2011. Pathologic diagnoses of the patients were evaluated by well-trained pathologists via biopsy reports. Patients with other malignant diseases in their medical history were excluded. 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 for genotyping were obtained from all the subjects. A compressive demographic data, age, gender, clinical information (family history of CRC and patient history of chronic diseases) were collected. Despite this, only 158 CRC patients had prognostic information. Among these, 56 (35%) were confirmed cancer-specific death and 102 (65%) were still alive. The Ethics Committee of Zhejiang University's School of Medicine approved this study and all the enrolled subjects signed the informed consent.

Table 2. Comparative analysis between genotype frequencies and the risk of CRC for *ATG16L1* rs2241880 polymorphism

Polymorphism	Colorectal cancer (n=964)	Control (n=891)	OR (95% CI)	*P*
ATG16L1 genotype				
AA	384 (39.8%)	377 (42.3%)	1*	-
AG	463 (48.0%)	399 (44.8%)	1.14 (0.93-1.39)	0.216**
GG	117 (12.1%)	115 (12.9%)	0.98 (0.72-1.33)	0.891**

OR, odds ratio; CI, confidence interval; *Reference; **The *P* value was adjusted for age.

DNA extraction and genotyping

Genomic DNA was extracted from the peripheral blood leukocytes using a TACO automatic nucleic acid extraction apparatus (GeneReach Biotechnology Corp., Taiwan, China), following the manufacturer's recommendations. NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA) was used to measure the DNA concentration and purity. Genotyping data was obtained from our previous GWAS data, which was performed using Illumina Human-Omni Express Bead Chip (Illumina Inc., San Diego, CA, USA). Genotyping procedures were carried out according to the protocol recommended by the supplier. Appropriate positive/negative and internal controls were included.

Statistical analysis

All the statistical analyses were performed by using SPSS software (Version.19.0; SPSS Inc., Chicago, IL, USA) and the two-sided *P* value less than 0.05 was considered to indicate statistical significance. Hardy-Weinberg equilibrium was tested by χ^2 tests (*P*=0.235). Associations between genotypes and CRC were calculated as odds ratios (OR) with 95% confidence intervals (CIs) using unconditional logistic regression analysis. Categorical variables were tested using χ^2 tests and Fisher's exact test. Continuous variables were analyzed by one-way ANOVA (age) and Kruskal-wallis H test (follow-up time). The univariate estimates of survival functions were calculated by the Kaplan-Meier method and the Cox regression was used to adjust for potential confounding variables. Prognostic factors were tested by univariate analysis and multivariated models using log-rank test for equality of survival functions. The hazard ratios (HRs) and 95% confidence intervals (CIs) were assessed by Cox proportional hazards model.

Results

Genotype frequencies and the risk of CRC in Chinese population

Genotyping was performed in 1856 samples harvested from CRC patients and controls. One sample was removed due to the failure of genotyping. The demographic characteristics of all the patients and controls enrolled in this study are shown in **Table 1**. There was statistical difference in median age between cases and controls, while no difference in gender and genotype frequencies as shown (see **Table 2**). The genotype frequencies of *ATG16L1* rs2241880 SNP in controls were distributed in accordance with Hardy-Weinberg equilibrium. There was no correlation between CRC cases and controls for the subjects carrying GG genotype (OR 0.98, 95% CI: 0.72-1.33, *P*=0.891) or AG genotype (OR 1.14, 95% CI: 0.93-1.39, *P*=0.216) when compared with AA genotype, which were shown in **Table 2**. The *P* values were adjusted for age. Importantly, these genotypic frequencies were similar to those public databases (dbSNP) described previously in Han Chinese populations with CRC [25].

Association of ATG16L1 rs2241880 with worse overall survival

The clinical characteristics and *ATG16L1* rs2241880 genotypes of the 158 CRC patients who had prognostic information were shown in **Table 3**. No significant association was found between the *ATG16L1* genotype and clinicopathological parameter, including mean age, gender, tumor stage or grade at diagnosis and tumor location (**Table 3**).

Sixty-five (41.1%) patients were homozygous for the Thr300 allele (AA), 77 were heterozygous for AG (48.7%), and 16 (10.1%) were homozygous for the Ala300 allele (GG). Interestingly, compared to those with AA and AG genotypes, patients carrying the GG genotype in *ATG16L1* trended to be diagnosed with CRC at a younger age (63.38 vs 57.31, *P*=0.067, **Table 3**) (recessive model). Overall median follow-up time was significantly decreased in patients with the GG

Table 3. Patient clinicopathologic characteristics

		ATG16L1 genotype					
Characteristic	Total n (%)	AA n (%)	AG n (%)	GG n (%)	*P*	AA or AG n (%)	*P*
Number (%)	158	65 (41.1)	77 (48.7)	16 (10.1)		142 (89.9)	
Age (years)							
Mean ± SD	62.77±12.56	63.28±12.38	63.47±11.94	57.31±15.46	0.186[b]	63.38±12.10	0.067[b]
Gender							
Male	95 (60.1)	36 (55.4)	46 (59.7)	13 (81.3)	0.166[a]	82 (57.7)	0.104[d]
Female	63 (39.9)	29 (44.6)	31 (40.3)	3 (18.8)		60 (42.3)	
Grade							
I-II	130 (82.2)	52 (80.0)	65 (84.4)	13 (81.8)	0.853[a]	117 (84.2)	0.724[a]
III	25 (15.8)	11 (16.1)	11 (14.3)	3 (18.8)		22 (15.8)	
Unknown	3 (1.9)	2 (3.1)	1 (1.3)	0 (0.0)			
AJCC stage							
Stage I	20 (12.7)	9 (13.8)	9 (11.7)	2 (12.5)	0.856[a]	18 (12.7)	0.723[a]
Stage II	48 (30.4)	20 (30.8)	22 (28.6)	6 (37.5)		42 (29.6)	
Stage III	69 (43.7)	30 (46.2)	34 (44.2)	5 (31.3)		64 (45.1)	
Stage IV	21 (13.3)	6 (9.2)	12 (15.6)	3 (18.8)		18 (12.7)	
Tumor location							
Colon	68 (43.0)	26 (40.0)	34 (44.2)	8 (50.0)	0.741[a]	60 (42.3)	0.601[d]
Rectum	90 (57.0)	39 (60.0)	43 (55.8)	8 (50.0)		82 (57.7)	
pT							
T1-T3	88 (55.7)	43 (66.2.)	37 (48.1)	8 (50.0)	0.086[a]	80 (56.3)	0.792[d]
T4	70 (44.3)	22 (33.8)	40 (51.9)	8 (50.0)		62 (43.7)	
pN							
N0	75 (47.5)	30 (46.2)	37 (48.1)	8 (50.0)	0.953[a]	67 (47.2)	1.000[d]
N1	83 (52.5)	35 (53.8)	40 (51.9)	8 (50.0)		75 (52.8)	
pM							
M0	138 (87.3)	60 (92.3)	65 (84.4)	13 (81.3)	0.275[a]	125 (88.0)	0.430[d]
M1	20 (12.7)	5 (7.7)	12 (15.6)	3 (18.8)		17 (12.0)	
Tumor size (cm)							
≤ 5	100 (63.3)	41 (63.1)	47 (61.0)	12 (75.0)	0.573[a]	88 (62.0)	0.415[d]
> 5	58 (36.7)	24 (36.9)	30 (39.0)	4 (25.0)		54 (38.0)	
Follow-up months							
Median (25-75%)	50.92 (36.93-61.57)	52.17 (40.90-60.50)	52.63 (35.40-62.85)	39.38 (16.16-50.63)	0.087[c]	52.40 (38.97-61.77)	0.027[c,*]

Data represented as n (%); [a]Categorical variables analyzed by chi-square test; [b]One-way ANOVA; [c]Kruskal-Wallis H test; [d]Fisher exact test. *P<0.05

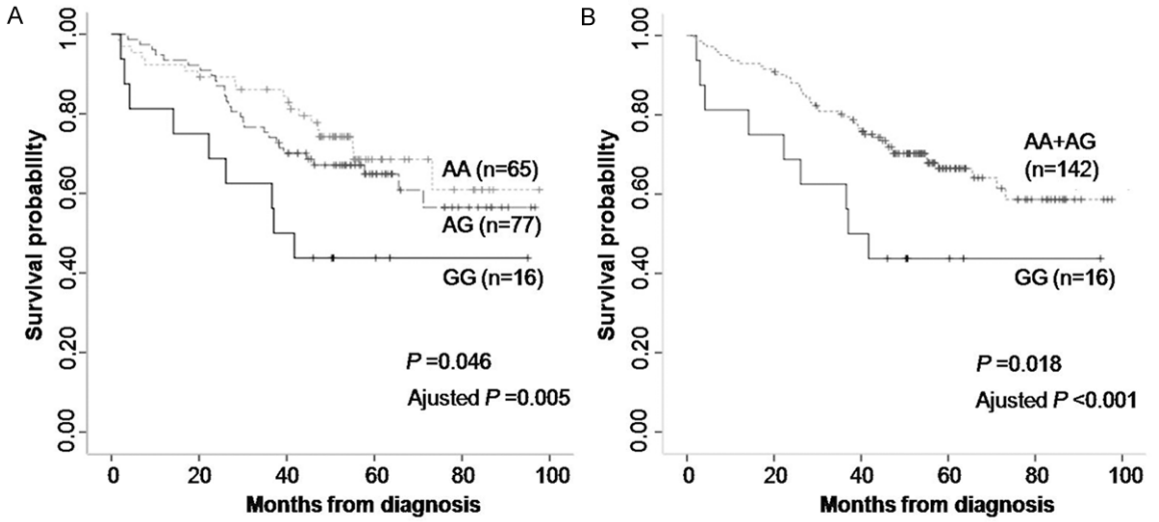

Figure 1. Overall survival of colorectal cancer patients by *ATG16L1* SNP rs2241880. A. The Kaplan-Meier curves were used to estimates of survival probability according to *ATG16L1* genotype. B. Worse overall survival of patients with CRC was observed in GG individuals when using a recessive model. Raw *P*-values were calculated by the log-rank test and then adjusted for age, stage and tumor location by Cox regression.

genotype (recessive model) (*P*=0.027, **Table 3**). Over the period of observation, there were 56 deaths from CRC. The survival was worse for individuals carrying the homozygous GG genotype at rs2241880 (log-rank *P*=0.046, **Figure 1A**), especially when plotted in a recessive model (log-rank *P*=0.018, **Figure 1B**). Moreover, the Cox regression survival analysis showed a higher risk for CRC death in patients with GG genotype while adjusting for age, stage and tumor location (*P*=0.005, **Figure 1A**), especially when using the recessive model (*P*<0.001, **Figure 1B**). These results suggest that estimated survival is worse in CRC patients with GG homozygous in *ATG16L1*.

ATG16L1 rs2241880 genotype as a prognostic factor

A univariate analysis identified a disadvantage of overall survival in patients with CRC carrying the *ATG16L1* GG genotype (HR=2.65, *P*=0.016) in addition to other prognosis factors, including age, stage and tumor size (**Table 4**). A multivariate analysis using Cox proportional hazards model was used for evaluation of the statistically significant variables found in univariate analysis. This method indicated that *ATG16L1* genotype remained to be a significant prognostic factor with respect to overall survival (HR=4.70, *P*<0.001) as well as age, stage and tumor size.

Discussion

In the present study, we investigated if GG carriers at rs2241880 were associated with an increased risk of developing colorectal cancer (CRC) and investigated the impact of this polymorphism on the outcomes of patients with colorectal cancer (CRC) in the Chinese population. We failed to demonstrate any statistical significant associations between genotype frequencies and the risk of CRC for *ATG16L1* rs2241880 polymorphism. However, our observations suggest that the homozygous GG genotype at rs2241880 may predict inferior survival in CRC patients.

Several studies consistently showed that the *ATG16L1* variant rs2241880 is a risk factor in Crohn's disease [27, 28]. However, no association of *ATG16L1* and Crohn's disease has been observed in Asians [29, 30]. The relationship between rs2241880 and cancer susceptibility is less clear. It has shown that the 300Ala (G) allele of *ATG16L1* was associated with decreased risk for epithelial cell-derived thyroid cancer (TC) in a Dutch population [31]. But a previous genome-wide association study demonstrated no effect of this *ATG16L1* polymorphism on susceptibility to TC in patients from Iceland [32]. Also, another study revealed that both carriers of AG and GG genotype were at a lower risk for gastric cancer when compared with the

Table 4. Cox regression analysis of prognostic factors with respect to overall survival

Factor	n	Univariate	*P*	Multivariate	*P*
		HR (95% CI)		HR (95% CI)	
Age	158	1.03 (1.00-1.05)	0.032	1.04 (1.01-1.06)	0.003
Gender					
Male	95	1*		-	-
Female	63	0.94 (0.55-1.61)	0.822	-	-
AJCC stage					
Stage I	20	1*		1*	
Stage II	48	1.69 (0.48-5.99)	0.417	2.34 (0.65-8.43)	0.192
Stage III	69	3.50 (1.06-11.54)	**0.039**	4.77 (1.43-15.91)	**0.011**
Stage IV	21	5.89 (1.68-20.69)	**0.006**	7.54 (2.10-27.1)	**0.002**
Grade					
I-II	130	1*		-	-
III	25	1.64 (0.84-3.18)	0.145	-	-
Location					
Colon	90	1*		-	-
Rectum	68	0.73 (0.42-1.26)	0.258	-	-
Tumor size (cm)					
≤ 5	100	1*		1*	
> 5	58	0.45 (0.24-0.84)	**0.012**	0.45 (0.21-0.77)	**0.006**
ATG16L1 genotype					
AA	65	1*		1*	
AG	77	1.27 (0.71-2.28)	0.416	1.40 (0.77-2.54)	0.277
GG	16	2.65 (1.20-5.86)	**0.016**	4.70 (2.03-10.90)	**< 0.001**

P values in bold, statistically significant; *Reference.

wild-type AA genotype in the Romanian population [33]. It is worth noting that a recent study observed that the subjects carrying GG genotype were at a higher risk for colorectal cancer (CRC) when compared with the more frequent AA genotype [23], which was inconsistent with our findings. A plausible explanation may be represented by the differences between the two populations studied. The degree of population differentiation among these data suggests that they may well reflect local adaptation to environment. And the study sample size was much smaller (n=466) than our study (n=1855). Besides, the genotypic frequencies of rs2241880 in CRC differed significantly in different populations. A similar finding indicated that the GG genotype was less prevalent in Han Chinese compared with European American (16% vs 29%, $P<0.001$) [25]. It remains to be investigated whether such data, which exhibit large allele frequency differences between European American and Asian populations, contribute to inter-ethnic gene expression differences in the colon that translate into differences in CRC risk.

To date, few studies about the role of *ATG16L1* rs2241880 in the outcome of patients with cancers has been conducted. Contrary to our results, the aforementioned study has observed that the GG genotype was associated with reduced metastasis and improved overall survival in CRC patients [25]. Yet, *ATG16L1* GG genotype variant was not an independent factor with respect to overall survival. The tumor characteristics such as racial factor may affect the results. Not only this, the survival data in that study just reached statistical significance only when plotted in recessive model ($P=0.044$). The conflicting findings in patients with CRC may be due in part to the etiological heterogeneity between Asian and Western populations.

Another possible explanation for the discrepancy between the *ATG16L1* rs2241880 genotype and CRC is that the function of Thr300Ala variant is very complex. Several studies have found that autophagy is important for the degradation and elimination of intracellular pathogens and the maintenance of intracellular homeostasis [34]. The Thr300Ala variant dec-

reases antibacterial defense and the generation of antigen-specific $CD4^+$ T-cell responses due to impaired innate immune function [11, 35]. However, a recent finding has uncovered a protective role of *ATG16L1* deficiency against intestinal disease induced by the bacterial model [36]. Furthermore, *ATG16L1* was able to downregulate inflammation responses driven by Nod2, which was involved in the innate immune response to pathogens, in an autophagy-independent manner [37]. More interestingly, the Thr300Ala variant has also been revealed to possess an altered capacity to negatively regulate Nod-dependent pro-inflammatory signaling [37]. Thus, the role of Thr300Ala variant in bacterial clearance and inflammation effects remains elusive. Likewise, the contribution of the variant rs2241880 to the clinical phenotype is not well established and further investigations are warranted.

Statistics show that 40-50% of patients with CRC present with metastasis at the time of diagnosis and suffer poor outcomes [38]. Up to now, how autophagy influences metastasis and survival remains unknown. Autophagy serves both pro- and anti-metastatic functions depending on the contextual demands [39]. Autophagy may inhibit metastasis by promoting anti-tumor inflammatory response or by inhibiting the expansion of dormant tumor cells into macrometastases. Conversely, self-eating may promote metastasis by helping tumor cell adapt to environmental stresses [39]. As the essential protein for canonical autophagy, ATG16L1's role in the prognosis of cancer patients is rarely reported. And the association between *ATG16L1* polymorphisms and prognosis in cancer patients is yet to be further validated. However, we did not find any association between rs2241880 variant and metastasis in our study. This may be due in part to the relatively small sample size in our prognostic analysis, which reduces power to detect significant associations, thereby resulting in a high false negative rate. It is worth noticing that GG homozygous at rs2241880 shows a trend to be associated with a younger age at diagnosis compared with AA/AG genotype in our study (P=0.067). To our best knowledge, rs2241880 was found to be associated with early age-onset for the first time although. More notably, *in vivo* and *in vitro* studies have demonstrated that the Thr300Ala variant was associated with increased pro-inflammatory cytokines production, especially IL-1β [12, 40, 41]. Accumulating evidence has suggested that some cytokines acted as key regulators of the tumor environment and influenced tumor growth, invasion and metastasis [42]. In CRC, these cytokines predicted the overall survival for metastatic CRC patients [43]. Besides, we on-line predicted the SNP rs2241880 may have functions in variants splicing (http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm). ATG16L1 may also have some autophagy-independent functions which wait launching deeper research to seek. Therefore, further investigations and replication of these findings in a larger cohort with more comprehensive survival data is needed.

In conclusion, we provide evidence that the *ATG16L1* rs2241880 polymorphism was not associated with susceptibility to colorectal cancer in the Chinese population. However, GG genotype influenced the clinical outcome of patients with colorectal cancer, suggesting that rs2241880 may be used as a prognosis biomarker in colorectal cancer. Further functional studies are required to elucidate the role of rs2241880 in colorectal cancer.

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Disclosure of conflict of interest

None.

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