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Association Study of Common Genetic Variants in Pre-microRNAs in Patients with Ulcerative Colitis

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Abstract
Background Common single-nucleotide polymorphisms (SNPs) in microRNAs (miRNA) have been shown to be associated with susceptibility to several human diseases. We evaluated the associations of three SNPs (rs11614913, rs2910164, and rs3746444) in pre-miRNAs (miR-196a2, miR-146a, and miR-499) with the risk of ulcerative colitis (UC) in a Japanese population.
Methods The rs11614913 (T>C), rs2910164 (C>G), and rs3746444 (A>G) SNPs were genotyped in 170 UC and 403 control subjects.
Results The rs3746444 AG genotype was significantly higher among the UC group (odds ratio (OR)=1.51, 95% CI=1.03–2.21, p=0.037). The rs3746444 AG genotype was associated with onset at an older age (OR=1.70, 95% CI=1.04–2.78, p=0.035), left-sided colitis and pancolitis (left-sided colitis, OR=2.10, 95% CI=1.12–3.94, p=0.024; pancolitis, OR=1.81, 95% CI=1.09–3.01, p=0.028, left-sided colitis+pancolitis, OR=1.91, 95% CI=1.26–2.92, p=0.003), higher number of times hospitalized (OR=2.63, 95% CI=1.22–5.69, p=0.017), steroid dependence (OR=2.63, 95% CI=1.27–5.44, p=0.014), and refractory phenotypes (OR=2.76, 95% CI=1.46–5.21, p=0.002) while the rs3746444 AA genotype was inversely associated with the number of times hospitalized (2~, OR=0.36, 95% CI=0.17–0.79, p=0.012), steroid dependence (OR=0.42, 95% CI=0.21–0.88, p=0.021), and refractory phenotypes (OR=0.38, 95% CI=0.20–0.72, p=0.003). The rs1161913 TT genotype also held a significantly higher risk of refractory phenotype (T/T vs. T/C+C/C, OR=2.21, 95% CI=1.17–4.18, p=0.016).
Conclusions Our results provided the first evidence that rs3746444 SNP may influence the susceptibility to UC, and both rs3746444 and rs11614913 SNPs may influence the pathophysiological features of UC.

Keywords Pre-microRNAs · ulcerative colitis · polymorphism · rs11614913 · rs2910164 · rs3746444 · miR-196a2 · miR-146a · miR-499

Abbreviation

UC	Ulcerative colitis
miRNA	MicroRNAs
PCR	Polymerase chain reaction
RFLP	Restricted fragment length polymorphism

Introduction

Ulcerative colitis (UC) affects the colon and rectum and typically involves the innermost lining mucosa, manifesting as continuous areas of inflammation with no segments of normal mucosa [1]. It is characterized by chronic, relapsing colonic inflammation with unknown etiology. Many studies

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have suggested that immune dysregulation and genetic factors play important roles in the pathogenesis of UC.

Ulcerative colitis is a multifactorial, polygenic disease with probable genetic heterogeneity. According to this hypothesis, different genetic backgrounds may explain the various clinical patterns of the disease [2, 3]. A genetic predisposition, through the inheritance of a number of contributory genetic polymorphisms, contributes to the pathogenesis of UC [4–8].

MicroRNAs (miRNA) are 21- to 24-nucleotide-long small non-coding RNA gene products that regulate gene expression by base pairing with target mRNAs at the 3′-untranslated region, leading to mRNA cleavage or translational repression [9–11]. It has been suggested that miRNAs are involved in various biological processes, including cell proliferation, cell death, stress resistance, and fat metabolism [12]. Moreover, a recent report shows that altered expression of miRNAs may be involved in pathogenesis of UC. For example, expressions of miR-192, miR-375, and miR-422b were downregulated in UC, whereas miR-16, miR-21, miR-23a, miR-24, miR-29a, miR-126, miR-195, and Let-7f were reported to be overexpressed in UC [13].

Single-nucleotide polymorphisms (SNPs) or mutations in miRNA sequence may alter miRNA expression and/or maturation. Recently, Hu et al. performed a screening for common SNPs in miRNA sequence and identified four SNPs (rs2910164, rs2292832, rs11614913, and rs3746444) located at the pre-miRNA regions of miR-146a, miR-149, miR-196a2, and miR-499, respectively [14]. They found that among the above four SNPs, the rs11614913 SNP in miR-196a2 was associated with shortened survival time of non-small cell lung cancer through altering the expression of mature miR-196a and binding activity of target mRNA [14]. Consequently, several studies have reported the association between those SNPs and human cancers. Among those SNPs, the rs11614913 in miR-196a2 was associated with lung cancer susceptibility [15]. On the other hand, the rs2910164 within the miR-146a was associated with papillary thyroid carcinoma [16] and hepatocellular carcinoma [17]. Moreover, the rs11614913 in the miR-196a2 and the rs3746444 in the miR-499 were both associated with the risk of having breast cancer [18]. Concerning other human diseases, one recent study has reported that the rs11614913 SNP is associated with congenital heart disease susceptibility [19].

Because functional alteration of miRNAs caused by SNPs may also contribute to the pathogenesis of UC, we evaluated the associations of three selected SNPs (rs11614913, rs2910164, and rs3746444) in pre-miRNAs (miR-196a2, miR-146a, and miR-499), which contributed to human disease susceptibility [14–19] with the risk of UC including its clinical phenotypes in Japanese population.

Materials and Methods

Study Population and Sample DNA Extraction

The studied population comprised 573 subjects, including patients with UC (UC group, n=170), who were enrolled at Fujita Health University Hospital (Aichi, Japan) from January 2005 to September 2009 and unrelated healthy control subjects (HC group, n=403). The diagnosis of UC was based on standard clinical, endoscopic, radiological, and histological criteria [20]. Clinicopathological characteristics such as age of onset and the number of times hospitalized were investigated. UC patients were also classified as having proctitis, left-sided colitis, and pancolitis according to the location and extension of the inflammatory lesions judged by endoscopic findings. Moreover, cases that need continual intravenous or oral steroid therapy were identified as steroid dependent, and those who had one onset over 6 months or two onsets within 1 year were defined as refractory cases. The ethics committee of Fujita Health University School of Medicine approved the protocol, and written informed consent was obtained from all participating subjects.

Genotyping

Using genomic DNA extracted from colonic biopsies or peripheral blood, the rs11614913 (T>C), rs2910164 (C>G), and rs3746444 (A>G) SNPs were determined by PCR-based RFLP assays as described by Hu et al. [14]. All genotypes were determined reading the gel pictures by two independent investigators who were blinded to the names and phenotype of the patients.

Statistical Analysis

Genotype frequencies were calculated by direct counting. The genotype frequencies were compared between two groups by a 2×2 table using the two-sided Fisher's exact test. The strength of association was also assessed by calculating the odds ratio (OR) and 95% confidence intervals (CI). A probability value of less than 0.05 was considered satistically significant.

Results

Study Population, Association Between SNPs in miRNAs with UC Including Its Subtypes

The characteristics of the subjects and prevalence of rs11614913 (T>C), rs2910164 (C>G), and rs3746444 (A>G) SNPs among all UC and HC groups are shown in Table I and Table II, respectively. Overall, prevalence of

Table I Characteristics of subjects

	HC group	UC
Subjects (number)	403	170
Sex (male/female)	195/208	96/74
Mean age±SD (years)	59.8±13.4	40.2±13.9

rs11614913 (T>C) and rs2910164 (C>G) SNPs were not significantly different among those two groups while frequency of rs3746444 AG genotype was significantly higher among the UC group. (27.5% vs. 36.5%, OR=1.51, 95% CI=1.03–2.21, p=0.037). Other genotypes of rs3746444 SNP (AA and GG) did not significantly deviate among the two groups. To further evaluate whether these SNPs might influence the clinicopathologic subtypes of UC, age of onset, extension of the inflammatory lesions, number of times hospitalized, and response to treatment were included in a stratified analysis (Table III). We found that the rs3746444 AG genotype was associated with onset at an older age (≥30 A/G vs. A/A+G/G: OR=1.70, 95% CI=1.04–2.78, p=0.035), left-sided colitis, and pancolitis (left-sided colitis, A/G vs. A/A+G/G: OR=2.10, 95% CI=1.12–3.94, p=0.024, pancolitis, A/G vs. A/A+G/G: OR=1.81, 95% CI=1.09–3.01, p=0.028, left-sided colitis+pancolitis, A/G vs. A/A+G/G: OR=1.91, 95% CI=1.26–2.92, p=0.003), higher number of times hospitalized (2~, A/G vs. A/A+G/G: OR=2.63, 95% CI=1.22–5.69, p=0.017), steroid dependence (A/G vs. A/A+G/G: OR=2.63, 95% CI=1.27–5.44, p=0.014), and refractory phenotypes (A/G vs. A/A+G/G: OR=2.76, 95% CI=1.46–5.21, p=0.002). On the other hand, the rs3746444 AA genotype was inversely associated with the number of times hospitalized (2~, A/A vs. A/G+G/G: OR=0.36, 95% CI=0.17–0.79, p=0.012), steroid dependence (A/A vs. A/G+G/G: OR=0.42, 95% CI=0.21–0.88, p=0.021), and refractory phenotypes (A/A vs. A/G+G/G: OR=0.38, 95% CI=0.20–0.72, p=0.003).

As for, rs1161913 SNP, we found that the rs1161913 TT genotype held a significantly higher risk of refractory phenotype (T/T vs. T/C+C/C: OR=2.21, 95% CI=1.17–4.18, p=0.016).

Discussion

Recent reports show that miRNAs may play a role in regulating inflammation. In macrophages, miR-155 is induced by inflammatory cytokines and toll-like receptor ligands [21]. Similarly in monocytes, miR-146a and miR-146b are induced by inflammatory cytokines and microbial components, including flagellin and lipopolysaccharide [22]. The miR-146a and miR-146b were predicted to bind to the 3′UTR and shown to downregulate the expression of tumor necrosis factor (TNF) receptor-associated factor 6 and interleukin-1receptor-associated kinase 1,2 key molecules involved in toll-like receptor and cytokine signaling. Also, a recent report showing differential expressions of several miRNAs among inflamed colonic mucosa in UC patients indicates that dysfunction of miRNA may play a role in the pathogenesis of UC [13].

Although several associations between the SNPs in miRNAs and human diseases have been investigated, there has been no report concerning the UC susceptibility. Here, we analyzed the influence of three selected SNPs (rs11614913, rs2910164, and rs3746444) in pre-miRNAs (miR-196a2, miR-146a, and miR-499); they contributed to human diseases in the recent association studies [14–19] on individual susceptibility to UC. We found that the AG genotype of rs3746444 in miR-499 was significantly associated with susceptibility to UC. This genotype held about a 1.5-fold increased risk of UC when compared to other genotypes; however, frequencies of other genotypes of rs3746444 (AA and GG) were not different among the UC and controls.

There are three potential speculations for the association that we found. First, it is suggested that the rs3746444 AG heterozygote genotype of miR-499 gene, but not both homozygotes (AA and GG) may alter the function or expression of miR-499 and modify the susceptibility to UC, altering the regulation of target mRNAs related to inflammatory immune responses. Second, speculation for the associations we found is that this polymorphism may be in linkage disequilibrium with other polymorphisms elsewhere in the miR-499 gene, demonstrating biologically relevant

Table II Associations between rs11614913, rs2910164, and rs3746444 SNPs and susceptibility to UC

Variables (n)	rs1161913 genotype (%)			rs2910164 genotype (%)			rs3746444 genotype n(%)		
	T/T	T/C	C/C	C/C	G/C	G/G	A/A	A/G	G/G
HC group (403)	122 (30.3)	206 (51.1)	75 (18.6)	151 (37.4)	178 (44.2)	74 (18.4)	272 (67.5)	111 (27.5)	20 (5.0)
UC (170)	58 (34.1)	82 (48.2)	30 (17.7)	75 (44.1)	67 (39.4)	28 (16.5)	102 (60.0)	62 (36.5)	6 (3.5)

Statistical analysis was performed using the two-sided Fisher's exact test; rs3746444 genotype, A/G vs. A/A+G/G: OR (95% CI) p=1.51 (1.03–2.21) 0.037

n number of samples

Table III Associations between rs11614913, rs2910164, and rs3746444 SNPs and subtypes of UC

Variables (no.)	rs1161913 genotype			rs2910164 genotype			rs3746444 genotype		
	T/T	T/C	C/C	C/C	G/C	G/G	A/A	A/G	G/G
HC group (403)	122	206	75	151	178	74	272	111	20
Age of onset[a]									
<30 (74)	29	32	13	28	34	12	45	26	3
≥30 (84)	26	43	15	36	33	15	50	33	1
Unknown (12)	3	7	2	5	6	1	7	3	2
Extension of the inflammatory lesions[b]									
Proctitis (46)	14	23	9	21	18	7	33	9	4
Left-sided colitis (45)	16	24	5	21	17	7	25	20	0
Pancolitis (76)	28	34	14	32	31	13	43	31	2
Not determined (3)	0	1	2	1	1	1	1	2	0
Number of times hospitalized[c]									
0~1 (134)	44	66	24	60	51	23	83	47	4
2~(28)	10	13	5	11	12	5	12	14	2
Unknown (8)	4	3	1	4	4	0	7	1	0
Response to treatment[d]									
Steroid dependent (32)	12	18	2	15	13	4	15	16	1
Refractory (43)	21	18	4	20	17	6	19	22	2

Statistical analysis was performed using the two-sided Fisher's exact test

[a] ≥30, rs3746444 A/G vs. A/A+G/G; OR (95% CI) p=1.70 (1.04–2.78) 0.035

[b] Left-sided colitis, rs3746444 A/G vs. A/A+G/G: OR (95%CI) p=2.10 (1.12–3.94) 0.024. Pancolitis, rs3746444 A/G vs. A/A+G/G: OR (95%CI) p=1.81 (1.09–3.01) 0.028. Left-sided colitis+pancolitis, rs3746444 A/G vs. A/A+G/G: OR (95% CI) p=1.91 (1.26–2.92) 0.003

[c] 2~, rs3746444 A/G vs. A/A+G/G: OR (95%CI) p=2.63 (1.22–5.69) 0.017, A/A vs. A/G+G/G: OR (95%CI) p=0.36 (0.17–0.79) 0.012

[d] Steroid dependent, rs3746444 A/G vs. A/A+G/G: OR (95% CI) p=2.63 (1.27–5.44) 0.014, A/A vs. A/G+G/G: OR (95%CI) p=0.42 (0.21–0.88) 0.021. Refractory, rs3746444 A/G vs. A/A+G/G: OR (95%CI) p=2.76 (1.46–5.21) 0.002, A/A vs. A/G+G/G: OR (95%CI) p=0.38 (0.20–0.72) 0.003; rs1161913 T/T vs. T/C+C/C: OR (95%CI) p=2.21 (1.17–4.18) 0.016

variability. Finally, there exists the possibility that this polymorphism is in linkage disequilibrium with a genetic variation of another gene located near the miR-499 gene that is related to UC susceptibility.

We have also investigated the association between three miRNA SNPs and subtypes of UC. It was revealed that the rs3746444 AG genotype was associated with onset at older age, left-sided colitis and pancolitis, higher number of times hospitalized, steroid dependence, and refractory phenotypes while the rs3746444 AA genotype was inversely associated with the number of times hospitalized, steroid dependence, and refractory phenotypes. In addition, the rs1161913 TT genotype held a significantly higher risk of refractory phenotype. This observation indicates that different UC subgroups may have different genetic backgrounds. UC is diverse in its clinical course, prognosis, and response to treatment, thus, it was hypothesized that UC is a syndrome in which different pathogenic mechanisms lead to various clinical phenotypes, and it may be necessary to place greater emphasis on the disease heterogeneity. As we know, in extensive colitis phenotypes such as left-sided colitis and pancolitis, patients with higher number of times hospitalized, steroid dependence, and refractory phenotypes, show more severe disease behavior and often need more intensive clinical treatment. In this context, our data suggest that rs3746444 AG and rs1161913 TT genotypes are associated with more severe and rs3746444 AA genotype is associated with rather mild clinical phenotypes of UC, respectively; however, it should be noted that the effect of type II error cannot be excluded in a relatively small sample size in the subgroup analysis. Among the three miRNAs investigated in this study, miR-146a actually has clear evidence that this miRNA would be a negative regulator of inflammatory immune responses [21, 22]. Therefore, it is speculated that SNP in miR-146a is a candidate for UC susceptibility; however, prevalence of rs2910164 genotyes in miR-146a was not significantly different among UC, including its subtypes and HC group. Thus, miR-146a SNP may not be involved in the pathogenesis of UC.

This study is the first to investigate the potential association between miRNA SNPs and UC; replication study will be needed, using larger samples, including ethnically diverse

populations. Moreover, our study might best be viewed as hypothesis-generating rather than hypothesis-testing because the underlying in both vitro and vivo mechanisms of the three selected miRNAs in the pathogenesis of UC are largely unknown, and neither of these genes lie on positions which are not included in UC-susceptible loci [23]. Further characterization of miRNA SNPs, miRNAs, target mRNAs and their compensational or redundancy role in UC are needed to confirm our result.

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