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TNF rs1799964 as a Predictive Factor of Acute Toxicities in Chinese Rectal Cancer Patients Treated With Chemoradiotherapy

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Abstract: Acute toxicity is the main dose-limiting factor in the chemoradiotherapy of rectal cancer patients and depends on several pro-inflammatory factors, including interleukin-1 (IL-1), IL-6, and tumor necrosis factor-alpha (TNF-α). It is unknown whether genetic factors, such as single-nucleotide polymorphisms (SNPs) in the *IL-1*, *IL-6*, and *TNF* genes, are also associated with acute toxicity in the process.

We genotyped 5 potentially functional SNPs in these 3 genes (*TNF* rs1799964, *TNF* rs1800629, *IL-6* rs1800796, and *IL-1* rs1143623, *IL-1* rs1143627) and estimated their associations with severe acute radiation injury (grade ≥2) in 356 rectal cancer patients.

We found a predictive role of the *TNF* rs1799964 T variant allele in the development of acute injury (for CT vs CC: adjusted odds ratio [OR] = 4.718, 95% confidence interval [CI] = 1.152–19.328, $P = 0.031$; for TT vs CC: adjusted OR = 4.443, 95% CI = 1.123–17.581, $P = 0.034$). In the dominant model, for CT/TT vs CC, the adjusted OR = 4.132, 95% CI = 1.069–15.966, and $P = 0.04$.

Our results suggested that genetic variants in the *TNF* gene may influence acute injury in rectal cancer patients treated with chemoradiotherapy and may be a predictor for personalized treatment. Additional larger and independent studies are needed to confirm our findings.

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Abbreviations: 5-FU = 5-fluorouracil, AEs = adverse events, CHB = Chinese Han Beijing, CIs = confidence intervals, IL-1 = interleukin-1, IMRT = intensity modulated radiation therapy, LD = linkage disequilibrium, MAF = minor allele frequency, ORs = odds ratios, SNPs = single-nucleotide polymorphisms, TNF-α = tumor necrosis factor-alpha, TRG = tumor response grade.

INTRODUCTION

Rectal cancer is one of the most common malignancies and often presents with a poor prognosis.[1] Radiotherapy with or without concurrent chemotherapy is a major modality in the treatment of rectal cancer.[2,3] Radiotherapy reduces local recurrence and possibly improves overall survival but with increased radiation-related morbidity,[4–7] because of the damage to the surrounding normal tissues that primarily manifests as radiation intestinal injury, including acute toxicities and chronic fibrosis. Mucositis, vomiting, diarrhea, pain, tenesmus, bleeding, and hematologic dysfunction are the most common acute adverse effects.[8–10] Many studies have focused on late radiation-induced injury.[11,12] However, severe acute toxicities also impair the quality of life in rectal cancer patients, in addition to chronic complications. We were particularly interested in early normal tissue injury and attempted to explore additional molecular markers that predict acute chemoradiation-induced injury in rectal cancer patients.

Acute reactions to radiotherapy either are related to inflammation or occur through target cell depletion. Genes that affect early processes in the DNA repair or inflammation pathways may lead to a wide range of acute reactions after radiotherapy.[13–15] The associations between DNA repair and radiation injury have been extensively investigated,[16,17] though with inconsistent results, and recent studies have increasingly focused on the relationship between inflammation-related factors and radiation-induced injury.[18] Ionizing radiation can activate the pro-inflammatory signal, which is then amplified by the recruitment and transmigration of monocytes and activation of resident mast cells and result in the production of pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor-alpha (TNF-α).[19,20]

However, only a subset of patients develops severe radiation injuries, and little information is available to identify such individuals. A predictive tool to identify radiosensitive patients based on host factors such as genetic variants may be beneficial to personalized cancer treatment. Those genetic variants in key inflammatory-related genes may modulate the balance of inflammation and result in a change in radiation-induced normal tissue injury. Previous studies have demonstrated that variations in the circulating levels of IL-1, IL-6, and TNF-α were associated with the risk of radiation-induced pneumonitis and toxicities in breast cancer and head and neck cancer.[21–24] Hence, we hypothesized that inter-individual variability in inflammatory cytokines may modulate the phenotype of radiosensitivity in rectal cancer patients.

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This study was designed to determine whether the genotypes of the inflammatory-related genes *IL-1*, *IL-6*, and *TNF* were predictive of acute adverse events in patients with rectal cancer treated with pelvic radiotherapy.

MATERIALS AND METHODS

Study Subjects

The patients recruited in this study had received pelvic irradiation between January 2012 and October 2013 at Fudan University Shanghai Cancer Center (Shanghai, China). Totally, there were 398 eligible patients during the timespan. However, there were 42 patients whose blood samples were not collected. Thus, this study included 356 rectal cancer patients. The eligible patients were histopathologically confirmed with rectal adenocarcinoma, and other histological types and all metastases to the rectum were excluded. Blood samples of all patients were collected and processed by the Institutional Tissue Bank at Shanghai Cancer Center. Written informed consent was obtained from each patient. This study was approved by the Institutional Review Board of Fudan University Shanghai Cancer Center.

Treatment and Toxicity Evaluation

All patients received pelvic radiation with 6-MV (million volt) X-rays from linear accelerators (Elekta, Stockholm, Sweden; Varian, Palo Alto, CA). The intensity-modulated radiation therapy (IMRT) technique was used in all patients, and the IMRT plans were generated using the inverse planning module. A total dose ranging from 45 to 55 Gy was given with 1.8 or 2 Gy per fraction, 5 days a week. More than half of the patients had undergone pre-operation radiotherapy (Table 1). Over 90% of the patients also received concurrent chemotherapy, which was 5-fluorouracil (5-FU)/capecitabine or combined with oxaliplatin or irinotecan. Monotherapeutic capecitabine and 5-FU were administered at a dose of 825 mg/m^2 twice a day by p.o. (per os) and 1000 mg/m^2 5 days per week during the course of irradiation, respectively. In the Capecitabine + Oxaliplatin group, patients were administered oxaliplatin at a dose of 50 mg/m^2 by 2-hour intravenous infusion once a week, and capecitabine was administered p.o. at 625 mg/m^2 bid for 5 days per week. In the Capecitabine + Irinotecan group, irinotecan was administered at 50 to 70 mg/m^2 by intravenous infusion once a week. All of the patients, who received surgery in our center, were treated by total mesorectal excision (TME) procedure.

Acute adverse events, including gastrointestinal (vomiting, diarrhea and incontinence), dermatitis, and hematologic toxicity were evaluated weekly. The Common Terminology Criteria (CTC) toxicity criteria version 3.0 scale[25] was used to score acute adverse events (AEs) as follows: grade 0 means no AE, grade 1 indicates mild AE without the need for therapeutic intervention, grade 2 to 4 suggests AE with the need for therapeutic intervention, and grade 5 represents the most severe toxicity of adverse event-related death. The patients were grouped into 2 subgroups according to the CTC v3.0 score system: grade $\geq$2 patients were categorized as the case group (obvious toxicity), and grade 0 to 1 patients were categorized as the control group (mild toxicity). Grade 2 was set as a cutoff value because developing grade 2 toxicity was considered to impair quality of the patient's life.[26,27] The total toxicity grade indicated the highest toxicity grade among the hematologic toxicity, vomiting, diarrhea, incontinence (anal reaction), and dermatitis.

TABLE 1. Frequency Distribution of Clinical Characteristics by Total Toxicity*

Variables	Grade $\geq$2 No (%)	Grades 0–1 No. (%)	$P^{\dagger}$
All subjects	264(100.0)	92(100.0)	
Age (years)			
Range	18–83	33–87	
Mean‡	54.3 ± 11.8	56.5 ± 11.6	0.132
$\leq$55	132 (50.0)	41 (44.6)	0.369
>55	132 (50.0)	51 (55.4)	
Sex			0.126
Males	154 (58.3)	62 (67.4)	
Females	110 (41.7)	30 (32.6)	
Smoking status			0.210
Yes	55 (20.8)	25 (27.2)	
No	209 (79.2)	67 (72.8)	
Drinking status			0.742
Yes	31 (11.7)	12 (13.0)	
No	233 (88.3)	80 (87.0)	
Radiation pattern			0.718
Pre-operative	153 (58.0)	56 (60.9)	
Postoperative	89 (33.7)	27 (29.3)	
Recurrent	22 (8.3)	9 (9.8)	
Total radiation dose			0.273
$\leq$50 Gy	158 (59.8)	61 (66.3)	
>50 Gy	106 (40.2)	31 (33.7)	
Concurrent chemo§			0.184
No	16 (6.1)	11 (12.0)	
Fluorouracil based	167 (63.3)	56 (60.9)	
Irinotecan combined	30 (11.4)	6 (6.5)	
Single drug	51 (19.3)	19 (20.7)	
Tumor stage‖			0.237
I	4 (1.7)	1 (1.2)	
II	31 (13.0)	17 (20.7)	
III	172 (72.3)	58 (70.7)	
IV	31 (13.0)	6 (7.3)	

* Total toxicity: indicated the highest toxicity grade among the hematologic toxicity, vomiting, diarrhea, feces incontinence (anal reaction), and dermatitis.
† Two-sided χ^2 test for distribution between cases and controls.
‡ Data were mean ± SD.
§ ''No'' means radiation alone without concurrent chemotherapy; Fluorouracil-based regimen including capecitabine or 5-FU combined with Oxaliplatin; Irinotecan combined regimen means Irinotecan add capecitabine; Single drug including 5-FU, capecitabine.
‖ According to TNM staging of AJCC 2010 version.

SNP Selection and Genotyping

The single-nucleotide polymorphisms (SNPs) were screened and selected from the NCBI dbSNP (http://www.ncbi.nlm. nih.gov/snp) and HapMap databases (http://www.hapmap.org) for potentially functional SNPs in the *IL-1*, *IL-6*, and *TNF* genes on the basis of 3 criteria: at the 2 ends of these genes, such as near the 5'-end, 3'-end, 5'untranslated regions (UTRs), or 3'UTR, which may be the regulatory regions of the genes; minor allele frequency (MAF) $\geq$5% in Chinese Han Beijing (CHB) descendants; and potentially affect functions as predicted by SNPinfo (http://snpinfo. niehs.nih.gov/), such as transcription factor binding site (TFBS) and microRNA

(miRNA) binding site activity. In addition, linkage disequilibrium (LD) analysis was performed to optimize SNP selection using the Haploview software (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview). As a result, 5 SNPs (rs1799964 and rs1800629 in *TNF*, rs1800796 in *IL-6*, rs114362, and rs1143627 in *IL-1B*) met these criteria and were selected for genotyping. SNPs, *TNF*rs1799964C/T and rs1800629A/G, *IL-6* rs1800796C/G, and *IL-1* rs1143623C/G and rs1143627G/A locate near the 5'-end of the genes that are predicted to affect TFBS activity.

Blood samples of all patients were collected before treatment and processed by the Institutional Tissue Bank at Shanghai Cancer Center. Genomic DNA was extracted from white blood cells. DNA purity and concentration were determined using a UV spectrophotometer (BioTek, Winooski, VT). The Taqman universal PCR master mix and SNP-genotyping probes were purchased from Applied Biosystems by Life Technologies (Applied Biosystems, Foster City, CA), and the genotyping was performed using the Taqman real-time PCR method on a 7900HT sequence detector system of ABI. A strict quality control procedure was used in each 384-well plate, in which 4 duplicated positive controls (duplicated 4 wells) and 4 negative controls (ddH_2O instead of DNA), and the concordant rate was 100%.

TNF Gene Expression by Genotypes in the HapMap Data

The biological plausibility of our findings was further investigated by assessing the correlation between genotypes and the mRNA expression levels of the same gene in 270 lymphoblastoid cell lines. In the present study, the *TNF* rs1799964 SNP was obviously associated with chemoradiotherapy-induced total toxicity; thus, the mRNA expression levels were further evaluated for their associations with the genotype data from the HapMap website (http://www.hapmap.org), and the mRNA expression information was retrieved using the SNPexp online tool (http://app3.titan.uio.no/biotools/help.pjp?app=snpexp)[28] for the genotype–phenotype association analysis. The genotyping data in the HapMap phase II release 23 data set were used, which was consisted of 3.96 million SNP genotypes of 270 individuals from 4 populations (Utah residents with ancestry from northern and western Europe: 90 cases; Han Chinese in Beijing: 45 cases; Japanese in Tokyo: 45 cases; Yoruba in Ibadan, Nigeria: 90 cases).[29] The mRNA expression data were from the same 270 individuals.[30]

TNF mRNA Expression in Tumor Tissues of Rectal Cancer Patients

Further, 212 primary tumor tissues by biopsy from rectal cancer patients before treatment were available at our institutional bank. These patients had received pre-operative rectal irradiation at Fudan University Shanghai Cancer Center between September 2007 and January 2014. The patients underwent conventional pelvic radiation with concurrent 5-fluorouracil-based chemotherapy and were re-evaluated 4 to 6 weeks after irradiation using magnetic resonance imaging (MRI). Consequently, 156 patients received primary cancer resection at Fudan University Shanghai Cancer Center. We evaluated the pathologic tumor response using the AJCC TRG (tumor response grade) score system, in which grade 0 indicates a complete response without viable cancer cells, and grade 3 indicates a poor response with a minimal evidence of a tumor response. According to the TRG score, 31 of the 156 patients reached a pathologically complete response (CR; TRG = 0), and 34 achieved a poor response (TRG = 3). We extracted the total RNA of biopsy tumor tissue from patients with TRG 0 and TRG 3, and compared the different TNF mRNA expression levels between these 2 tumor response groups. We used TRI Reagent (Sigma Co., St. Louis, MO) to extract RNA, and synthesized cDNA using the PrimeScript RT Master Mix system (TAKARA, Osaka, Japan) according to the manufacturer's instructions. Quantitative SYBR Green PCR was carried out using a Light Cycle 480 Real-Time PCR System (Roche applied Science, Mannheim, Germany), with β-actin as an internal reference gene. The primers used were as follows: 5'-TTGTTACAGGAAGTCCCTTGCC-3' (forward) and 5'–ATGCTATCACCTCCCCTGTGTG–3' (reverse) for β–actin and 5'–GAAGATAG GGTGTCTGGCACA–3' (forward) and 5'–TTAGCCCTGAGGTGTCTGGT–3' (reverse) for TNF. The mRNA expression level of TNF was calculated using $2^{-\Delta\Delta}$CT (ΔCT = CTTNF $-CT_{\beta}$-actin,CT: threshold cycle).

Statistical Methods

Patients were categorized according to their genotyping results. Chi-squared (χ^2) test was used to compare clinical characteristics and frequencies of genotypes between the 2 groups. Univariate and multivariate logistic regression analyses were used to calculate the odds ratios (ORs) and 95% confidence intervals (95% CIs), in order to assess the associations between severe radiation injury and genotypes. Multivariate adjustments were made for age, sex, drinking and smoking status, radiation dose, and concurrent chemotherapy. Student *t*-test was used to compare the mRNA expression levels between the groups. The trend of the transcript expression levels in genotypes was evaluated by general linear regression model. Statistical significance was established at $P < 0.05$, and all tests were 2-sided and were performed using SPSS 20.0 software.

RESULTS

Clinical Characteristics

The baseline clinical characteristics of the patients are summarized in Table 1. The analysis included 356 rectal cancer cases with a median age of 55 years (range: 18–87 years). More than 50% of the patients received radiation before surgery, approximately 30% of the patients received postsurgery radiotherapy, and the others received radiotherapy because of local recurrence after surgery but without previous pelvic radiation. Over 90% of the patients had received concurrent chemotherapy, which primarily comprised 2-drug regimens and included fluorouracil or capecitabine. The fluorouracil-based regimens contained capecitabine + oxaliplatin or fluorouracil + oxaliplatin, and the irinotecan-containing regimens comprised irinotecan + capecitabine. The radiation dose was also comparable between the case and control groups. There were also no significant differences ($P > 0.05$) in the distributions of age, sex, smoking, drinking, the pattern of radiation, and chemotherapy between the groups based on the total toxicity.

Association Between Genotypes and Radiation-Induced Acute Toxicity

The associations of SNPs in *IL-1*, *IL-6*, and *TNF* genes with the occurrence of normal tissue injuries were examined. The crude ORs (95% CI), *P* values, and the corrected data for age, sex, radiation dose, radiation pattern, smoking, drinking, and the regimens of concurrent chemotherapy were obtained.

The genotyping of several SNPs was not successfully examined in some samples, so the total cases used to analyze the association between the genotypes and toxicities, as shown in the subsequent tables, did not reach 356. The allele and genotype frequencies of the 5 SNPs are summarized in Table 2. For *TNF* rs1799964, a positively and statistically significant association was found between the presence of 1 or both variant alleles and the risk of obvious (grade ≥2) total toxicity (CT vs CC: adjusted OR = 4.718, 95% CI = 1.152–19.328, $P = 0.031$; TT vs CC: adjusted OR = 4.433, 95% CI = 1.123–17.581, $P = 0.034$). Assuming a dominant model (the combination of heterozygous and variant homozygous genotypes vs the wild-type genotype),

TABLE 2. Logistic Regression Analysis of Association Between the Genotypes and Total Toxicity in Rectal Cancer Patients

Variant	Genotypes	Grade ≥2 No. (%) (N = 264)	Grades 0–1 No. (%) (N = 92)	P^*	Crude OR (95% CI)	P	Adjusted OR (95% CI)	$P^\dagger$
TNF rs1799964				0.114				
	CC	4(1.5)	5(5.6)		1.000		1.000	
	CT	87(33.5)	28(31.1)		3.884(0.975–15.470)	0.054	4.718(1.152–19.328)	0.031
	TT	169(65.0)	57(63.3)		3.706(0.962–14.276)	0.057	4.443(1.123–17.581)	0.034
Dominant model	CT/TT	256(98.5)	85(94.4)	0.052‡	3.765(0.988–14.341)	0.052	4.132(1.069–15.966)	0.040
	CC/CT	91(35.0)	33(36.7)		1.000		1.000	
Recessive model	TT	169(65.0)	57(63.3)	0.776§	1.075 (0.653–1.770)	0.776	1.076 (0.648–1.787)	0.766
Allele	C	95(18.3)	38(21.1)	0.402‖	1.000		1.000	
	T	425(81.7)	142(78.9)		1.197(0.785–1.825)	0.403	1.207(0.787–1.852)	0.388
TNF rs1800629				0.446				
	AA	2(0.8)	1(1.1)		1.000		1.000	
	AG	37(14.2)	18(19.6)		1.028(0.087–12.098)	0.983	0.923(0.076–11.150)	0.950
	GG	222(85.1)	73(79.3)		1.521(0.136–17.014)	0.734	1.454(0.126–16.750)	0.764
Dominant model	AG/GG	259(99.2)	91(98.9)	1.00‡	1.423(0.128–15.881)	0.774	1.327(0.115–15.245)	0.820
	AA/AG	39(14.9)	19(20.7)		1.000		1.000	
Recessive model	GG	222(85.1)	73(79.3)	0.204§	1.482(0.806–2.723)	0.206	1.568(0.839–2.931)	0.159
Allele	A	41(7.9)	20(10.9)	0.211‖	1.000		1.000	
	G	481(92.1)	164(89.1)		1.431(0.815–2.513)	0.213	1.485(0.837–2.633)	0.177
IL-6 rs1800796				0.319				
	CC	147(56.3)	44(48.4)		1.000		1.000	
	CG	93(35.6)	36(39.6)		0.773(0.464–1.289)	0.324	0.721(0.428–1.217)	0.221
	GG	21(8.0)	11(12.1)		0.571(0.256–1.276)	0.172	0.571(0.253–1.287)	0.176
Dominant model	CG/GG	114(43.7)	47(51.6)	0.189‡	0.726(0.450–1.171)	0.190	0.686(0.421–1.118)	0.130
	CC/CG	240(92.0)	80(87.9)		1.000		1.000	
Recessive model	GG	21(8.0)	11(12.1)	0.248§	0.636(0.294–1.377)	0.251	0.653(0.299–1.426)	0.285
Allele	C	387(74.1)	124(68.1)	0.118‖	1.000		1.000	
	G	135(25.9)	58(31.9)		0.746(0.516–1.078)	0.118	0.722(0.497–1.049)	0.087
IL-1 rs1143623				0.764				
	CC	68(25.8)	21(22.8)		1.000		1.000	
	CG	133(50.4)	46(50.0)		0.893(0.493–1.616)	0.708	0.938(0.511–1.721)	0.836
	GG	63(23.9)	25(27.2)		0.778(0.397–1.527)	0.466	0.804(0.400–1.616)	0.541
Dominant model	CG/GG	196(74.2)	71(77.2)	0.576‡	0.853(0.487–1.492)	0.576	0.892(0.503–1.584)	0.697
	CC/CG	201(76.1)	67(72.8)		1.000		1.000	
Recessive model	GG	63(23.9)	25(27.2)	0.526§	0.840(0.490–1.441)	0.526	0.840(0.479–1.473)	0.543
Allele	C	269(50.9)	88(47.8)	0.466‖	1.000		1.000	
	G	259(49.1)	96(52.2)		0.883(0.631–1.235)	0.466	0.905(0.642–1.276)	0.570
IL-1 rs1143627				0.616				
	GG	61(23.2)	17(18.5)		1.000		1.000	
	GA	133(50.6)	48(52.2)		0.772(0.411–1.451)	0.422	0.826(0.434–1.572)	0.561
	AA	69(26.2)	27(29.3)		0.712(0.354–1.431)	0.340	0.771(0.378–1.569)	0.473
Dominant model	GA/AA	202(76.8)	75(81.5)	0.347‡	0.751(0.412–1.367)	0.348	0.806(0.438–1.485)	0.489
	GG/GA	194(73.8)	65(70.7)		1.000		1.000	
Recessive model	AA	69(26.2)	27(29.3)	0.563§	0.856(0.506–1.449)	0.563	0.883(0.515–1.512)	0.650
Allele	G	255(48.5)	82(44.6)	0.360‖	1.000		1.000	
	A	271(51.5)	102(55.4)		0.854(0.610–1.197)	0.360	0.896(0.636–1.263)	0.531

The results were in bold, if the 95% CI on one side of 1 or $P < 0.05$. CI = confidence interval, OR = odds ration.

* Chi-square test for genotype distributions between cases and controls.

† Adjusted for age, sex, smoking and drinking status, total radiation dose, the pattern of radiation, and the regimen of concurrent chemotherapy in logistic regression models.

‡ For dominant genetic models (the combination of heterozygous and variant homozygous genotypes vs wild-type genotype).

§ For recessive genetic models (the variant homozygous genotypes vs variant hetrozygous combined with wild-type genotype).

‖ At allelic level.

the genotype distribution between grade ≥2 and grade 0 to 1 was significantly different for the SNP rs1799964 in *TNF* (CT/TT vs CC: adjusted OR = 4.132, 95% CI = 1.069–15.966, $P = 0.040$).

mRNA Expression of *TNF* by Genotype in Lymphoblastoid Cell Lines

The structural characteristics of the *TNF* gene are shown in Figure 1A, which indicated 4 exons in this gene and rs1799964 labeled at the 5'-end. The correlation analysis of *TNF* rs1799964 genotypes and mRNA expression in 270 HapMap lymphoblastoid cell lines provided additional data to support our findings. As summarized in Table 3, the *TNF* mRNA expression levels did not show a statistically significant difference among the 3 genotypes of rs1799964 in all populations. In the Chinese population, however, 16 cell lines with the CT genotype and 25 cell lines with the TT genotype seemed to have higher TNF mRNA expression levels than the cell lines with the CC genotype, although neither reached statistical significance ($P = 0.082$ and 0.054, respectively). In a dominant model, 41 cell lines with the CT or TT genotypes also had statistically nonsignificant higher levels (6.31 ± 0.05, $P = 0.064$) than the AA genotype (6.21 ± 0.01). However, the trend of the mRNA expression levels of 45 cell lines according to the T allele from unrelated CHB indicated statistical significance ($P = 0.002$).

TNF mRNA Expression in Patients with a Different Response to Pre-Operative Radiotherapy

Radiation-induced normal tissue injury has been suggested to be associated with radiosensitivity in tumor tissue. To analyze whether the naive TNF expression in tumor tissues would indicate the treatment response of radiotherapy, we tested the *TNF* mRNA expression levels in tumor tissues biopsied from patients with pre-treatment primary tumors. The response of tumors to radiotherapy was graded by pathologists in postsurgery tumor tissues according to the extent of alive tumor cells. The *TNF* mRNA expression in 31 patients who achieved a complete response was lower than that in 34 patients who had a poor response to radiotherapy ($P = 0.041$, Fig. 1B).

TABLE 3. *TNF* mRNA Expression by the Genotype of rs1799964 Using Data from the Hapmap

		mRNA expression		
Genotype	**No**	**Mean ± SD**	P^*	$P^{\dagger}_{trend}$
All population	270	6.29 ± 0.07		
rs1799964‡				
CC	4	6.26 ± 0.09		
CT	80	6.30 ± 0.07	0.575	
TT	166	6.29 ± 0.08	0.595	0.192
CT + TT	246	6.29 ± 0.07	0.607	
CC + CT	84	6.30 ± 0.07	0.706	
CHB		6.31 ± 0.05		
rs1799964				
CC	2	6.21 ± 0.01		
CT	16	6.33 ± 0.05	0.082	
TT	25	6.30 ± 0.05	0.054	0.002
CT + TT	41	6.31 ± 0.05	0.064	
CC + CT	18	6.32 ± 0.06	0.214	

TNF rs1799964 genotyping data and mRNA expression levels for TNF by genotypes were obtained from the HapMap phase II release 28 data from EBV-transformed lymphoblastoid cell lines from 270 individuals, including 45 Han Chinese in Beijing (CHB).

* Two-sided Student *t* test.

† *P* values for the trend test of TNF mRNA expression among 3 genotypes for SNP rs1799964 from a general linear model.

‡ There were missing data because genotyping data for 20 individuals were not available.

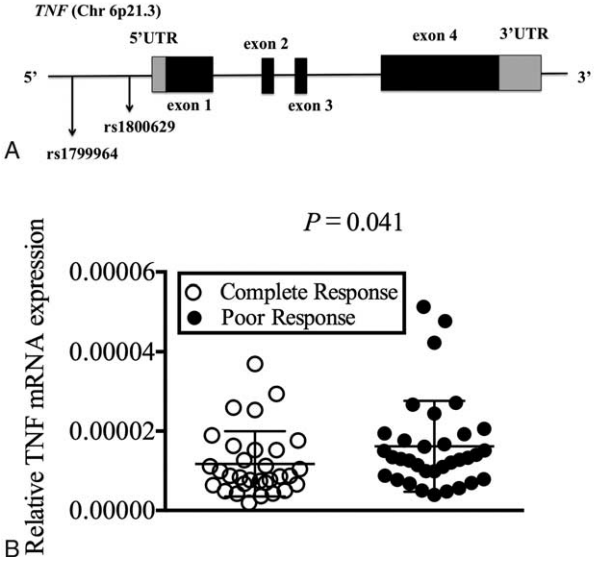

FIGURE 1. Structural characteristics of the *TNF* gene and *TNF* mRNA expression in patients with different responses to pre-operative irradiation. (A) The *TNF* gene consists of 4 exons with 2 SNPs at the 5'-end as labeled; (B) *TNF* mRNA expression levels in primary tumor tissues in rectal cancer patients. All of the patients received pre-operative radiotherapy. In addition, the response to radiotherapy was evaluated using alive residual tumor cells according to AJCC (American Joint Committee on Center) criteria with a 4-point scale. Thirty-one patients achieved a complete response, and 34 showed a poor response.

DISCUSSION

It has long been recognized that cancer patients exhibit a heterogeneous response to chemoradiotherapy and that these responses manifest as variations in normal tissue toxicity. Genes that affect early processes in DNA repair or inflammation pathways are expected to be responsible for early chemoradiation-induced reactions. As inflammation-related factors, IL-1, IL-6, and TNF-α are known to be extensively involved in acute inflammation reactions.[31] In this study, we investigated 5 SNPs in the *IL-1*, *IL-6*, and *TNF* genes for their associations with radiation-induced acute normal tissue injury in rectal cancer patients treated with pelvic radiotherapy. We considered grade ≥2 toxicity as the toxicity group and grade 0 to 1 as the control group because developing grade ≥2 toxicity will influence the patients' quality of life. We found for the first time that variant genotypes of rs1799964 of *TNF* were associated with acute injury in rectal cancer patients with pelvic radiation—that is, patients with the variant genotypes were more likely to develop grade ≥2 toxicity, which may influence the patients' quality of life.

The incidence of severe radiation-induced injury (grade $\geq$2) in our study was 74.2%. Some publications have suggested that combined chemotherapy will influence the toxicity of radiation.[32,33] In our study, for patients who received radiotherapy alone, 6.1% developed grade $\geq$2 toxicity, whereas more patients developed grade $\geq$2 toxicity (12%) in the group with a single or combined chemotherapy; however, the results did not reach statistical significance ($P = 0.184$). Because TNF-α, IL-1, and IL-6 participate in the radiation response, associations between the variants of these genes and risk of developing radiation injury are biologically plausible. Although little is known concerning the relationship between *TNF*, *IL-1*, and *IL-6* variants and the risk of chemoradiation injury in rectal cancer, several studies examining the association between *TNF*, *IL-1*, and *IL-6* variants and radiosensitivity have been published, and their results can be used for comparison.

Our findings demonstrated that patients with the variant genotypes (CT or TT) of *TNF* rs1799964 had a higher probability of developing severe total toxicity than those with the wild-type (CC) genotype. TNF-α is a pro-inflammatory cytokine and is produced by activated macrophages. It is well known that TNF-α has both beneficial and harmful effects, playing a role as a ''double-edged sword.''[34] The pro-inflammatory cytokine TNF-α may be responsible for initiating inflammation in response to tissue injury. The importance of TNF-α in inflammation has made this cytokine one of the most widely studied molecules in relation to its genetic variants and inflammation. The molecular mechanisms involved in the process of radiation-induced inflammation are somewhat similar to infectious or wound inflammation; as a result, TNF-α is considered very important in radiation-induced injury.[28,35,36] In some reports, elevated levels of TNF-α were documented in the acute-phase response after the radiation of head and neck tumors,[37,38] which suggests that TNF-α might be useful for monitoring this acute response in patients receiving radiotherapy. It was found that the variants in *TNF* were associated with radiation-induced esophagitis and pneumonitis in nonsmall cell lung cancer patients who received thoracic radiotherapy.[39] It was also reported that SNPs near *TNF* were associated with radiation toxicity in breast cancer.[23] These studies suggest that *TNF* variants may play a role in both the efficacy and side effects of radiotherapy. In addition to radiotherapy, TNF-α was associated with severe chemotherapy-induced gastrointestinal toxicity.[40] More than 90% of the patients in our study received concurrent chemotherapy. Thus, TNF-α may be an effective indicator of toxicity in patients who receive chemoradiotherapy.

We could not detect any correlation between SNPs in *IL-1* or *IL-6* and radiation-induced injury in our study, although it was reported that early variations of circulating IL-6 levels were significantly associated with the risk of radiation inflammation.[21,24,41] In another study, *IL-6* rs1800795 was shown to be associated with an increased risk of esophagitis in nonsmall cell lung cancer patients with thoracic radiation,[39] and 2 SNPs in *IL-1* were associated with a higher risk of pneumonitis in Caucasian patients.[39] The cause may be that SNPs in *IL-1* and *IL-6* are organ and histology-specific regarding radiation-induced injury; alternatively, our study may not have had sufficient study power to detect the weaker difference in Chinese patients.

Whether SNPs in *IL-1*, *IL-6*, and *TNF* genes influence the expression level of mRNA and protein in serum and tissue is controversial. A meta-analysis did not find a relationship between the variant and IL-6 serum levels.[42] We analyzed the *TNF* mRNA expression by different genotypes of SNP rs179964 using data from the HapMap database, including 270 individuals from different populations. Although there was no difference in the mRNA expression levels in all 270 individuals, *TNF* mRNA expression tended to be higher in subjects with the CT and TT genotypes than in those with the CC genotype in 45 Chinese populations. Combined with our result that patients with the CT and TT genotypes have an increased risk of developing acute injury, the underlying mechanism may be that the CT and TT genotypes of SNP rs1799964 may positively modulate the mRNA expression level of *TNF* and consequently increase the acute injury after radiotherapy. The results of the present study are consistent with our current opinion that the acute inflammatory factor TNF-α may increase acute radiation-induced injury.

Several studies have investigated the toxicity as radiosensitivity,[43–46] considering that severe toxicity indicated increased sensitivity to radiation, partly because it is difficult to evaluate the sensitivity to radiation in postsurgery radiotherapy without the gross tumor volume. We examined the *TNF* mRNA expression levels in tumor tissues before radiotherapy and analyzed the correlation between *TNF* mRNA expression and the tumor response to radiotherapy. The *TNF* mRNA expression level was found to be lower in the complete response group than in the poor response group. In addition, the result seemed to be in contrast to previous results. However, it should be clarified whether tumor tissues and normal tissues show a consistent response to radiotherapy. Furthermore, the present result should be confirmed in further studies with a larger sample size and different ethnicities.

Over half of the patients in our study received pre-operative radiotherapy, which is considered the standard treatment for T3 to T4 rectal cancers. Whether radiotherapy combined with chemotherapy improves survival further is controversial. The results of clinical trials FFCD9203 and EORTC22921 showed that preoperative chemoradiotherapy had no impact on overall survival but significantly improved local control,[47,48] with moderate increased acute toxicity. Adding oxaliplatin to fluorouracil-based preoperative chemoradiotherapy did not affect the primary tumor response but significantly increased the toxicity in the STAR-01 and ACCORD12/0405 trials.[49,50] However, in our center and another cancer center in China, the combination with oxaliplatin increased the pathological response in local advanced rectal cancer patients,[51,52] although some patients showed obvious toxicity. If *TNF* rs1799964 is validated as an indicator for predicting acute toxicity in Chinese populations, it may determine which patients will benefit from additional oxaliplatin therapy without severe toxicity and which group of patients is not suited for 2-drug concurrent chemoradiotherapy. Furthermore, the treatment intensity for elderly patients is controversial. Although it was reported that elderly patients with rectal cancer could be safely treated with radiotherapy or chemoradiotherapy, toxicities must be carefully monitored.[53,54] *TNF* rs1799964 may be an additional predictor for acute toxicities that can allow treatments to be carefully considered in elderly patients.

A limitation of our study is that only a few potentially functional SNPs were analyzed. Therefore, we might omit SNPs such as intronic SNPs that may also be functional and thus associated with the risk of acute injury after chemoradiotherapy by modifying the mRNA and protein expression. In addition, our results should be validated in larger samples and at multicenter institutes. Finally, the ethnicities included in the study should also be considered. The SNPs reported in Caucasian or

Japanese populations may not always be consistent with those in Chinese populations.

In summary, we found that SNP rs1799964 in *TNF* was associated with the risk of total acute chemoradiotherapy-induced injury in Chinese rectal cancer patients. Alternatively, a patient possessing the variant CT or TT genotype of SNP rs1799964 may be an ideal candidate for radiotherapy because this genetic variant may render this patient's cancer radiosensitive. It is possible that such an individual could be treated using a lower radiotherapy dose or lower intensity of concurrent chemotherapy that is less likely to cause injury. For patients, who do not possess such genetic variants, higher radiation doses or combined 2-drug chemotherapy may improve their curable chance without injury to the surrounding normal tissues. In this regard, once validated, genotyping of *TNF* variants among rectal cancer patients may help identify individuals who are at a high or low risk of radiation injury, which in turn allows for the design of personalized radiotherapy with maximal doses to the tumor and minimal toxicity to normal tissues. Therefore, our findings may support the value of these SNPs in assessing the risk of development radiation-induced normal tissue injury. However, our findings require further validation in subsequent studies.

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