Original Article
Association between polymorphism of CD20 gene and chronic lymphocytic leukemia in Chinese population

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Abstract: Rituximab was widely used in clinical practice. Some chronic lymphocytic leukemia (CLL) patients were primary or secondary resistance to rituximab, but the mechanism has not been yet clear. CD20 gene coding region was amplified by PCR in 92 cases of newly diagnosed CLL patients and 200 healthy donors. The expression of CD20 was conducted in peripheral blood specimens of CLL patients. Proportions of CD20 expression and fluorescence intensity were detected by flow cytometry. Exon-3 c.246C>T (rs17155019) and Exon-4 c.632C>T (rs2070770) were present in 4.35% (4/92) and 9.78% (9/92) of newly diagnosed CLL patients. The mutations were not found in remaining exons. The frequency of C/C genotype and C allele of rs2070770 were significantly higher than the normal control population (90.22% vs 81.00%, P=0.04; 95.11% vs 90%, P=0.04). There was no significant relationship between genotypes with CLL development (P>0.05), however, C allele of rs2070770 may be associated with CLL (P=0.04, OR=0.46, 95% CI=0.22-0.98). The expression CD20 mRNA, proportion and intensity of CD20 were no significant different between genotypes of two polymorphic loci (P>0.05). Low expression of CD20 for CLL was not associated with mutation of CD20 gene coding region. Other mechanisms, such as promoter methylation, may result in low expression of CD20.

Keywords: Chronic lymphocytic leukemia, CD20, rituximab, single nucleotide polymorphism, mutation

Introduction

Heterogeneity of CD20 expression was existed in chronic lymphocytic leukemia (CLL); the prognostic significance of CD20 expression in Chinese patients with CLL had been reported. High level of CD20 expression in CLL appeared to be associated with a good prognosis, but not an independent prognostic factor [1].

Rituximab was a chimeric mouse-human monoclonal antibody consisting of a human kappa constant region, a human IgG1 Fc portion, and a murine variable region which recognizes human-CD20 [2]. As with most immunoglobulin G1 (IgG1) therapeutic antibodies, rituximab can mediate complement-dependent cell cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and direct apoptosis with a cross-linking antibody [3]. Rituximab has been emerged as a vital component of the therapy for CLL. However, we found that some CLL patients were primary or secondary resistance to rituximab in clinical practice. The exact mechanisms of rituximab resistance remain poorly understood. Potential mechanisms of tumor resistance have been described in each of the three major pathways of proposed rituximab action (CDC, ADCC, and apoptosis induction).

The CD20 antigen was a membrane-bound protein that contains four trans-membrane domains, functions as a calcium channel, and it has been shown to play an important role in B cell activation and differentiation. The CD20 molecule is expressed across most of the committed stages of normal B-cell development, from pre-B cell to mature, activated, and memory B cells, as well as by virtually all B-cell malignancies. The level of CD20 expression in CLL was lower than that in normal B cells and the other B-cell leukemia, which was firstly reported by Ginaldi et al. [4]. Two amino acid sequenc-

Table 1. The sequences of PCR primers of CD20 gene

Primer	Sequence	Product length (bp)
CD20-Exon-3 F	5'-CAAGGTGTCCTCTACAAAGATAAAG-3'	604 bp
CD20-Exon-3 R	5'-CCACTGTGTTAGACATAAAGAAGAC-3'	
CD20-Exon-4 F	5'-AAAAGACAAAATTCTTGGCACCTCC-3'	348 bp
CD20-Exon-4 R	5'-GAAATAATCTGGCATATCCCTGTGG-3'	
CD20-Exon-5 F	5'-ACTGATAAAAATGGGTGGATGGTTG-3'	346 bp
CD20-Exon-5 R	5'-AATAACATTGTGGAGGGTCTTTGCT-3'	
CD20-Exon-6 F	5'-AGAGGCTAAAAACAACTGAGAGAAC-3'	512 bp
CD20-Exon-6 R	5'-TTCCCAAAGCCACACAGACAGTAAC-3'	
CD20-Exon-7 F	5'-AGAGTTAGGGTTATAAAGATGCTGT-3'	370 bp
CD20-Exon-7 R	5'-CAACTCATCAGATTACATTCTCCAT-3'	
CD20-Exon-8 F	5'-GCAATGTTCTTTTCCCAATACCACG-3'	696 bp
CD20-Exon-8 R	5'-AAAGAAGAAGCGTGACAACACAAGC-3'	

es, ANPS and YCYSI at positions 170 to 173 and 182 to 185, were determined to be the epitope of rituximab. The rituximab epitope were coded by Exon-5 of the CD20 gene. Terui et al. [5] have shown that C-terminal deletion mutations were related to the decline of CD20 expression and poor patient outcome. C-terminal deletion mutations were found in one case of diffuse large B-cell lymphoma (DLBCL) that without treatment (1/19), one case of recurrence of DLBCL (1/3), and one case (1/1) in patients with recurrent mantle cell lymphoma (MCL). The replacement of an amino acid (F125L) which altered the third transmembrane domain was reported in one CLL patient.

Whether CD20 coding region mutations were related with low CD20 expression or not is still unknown. Mutations in CD20 could potentially lead to Rituximab resistance. To our knowledge, there have been no reports of CD20 mutational status in Chinese CLL patients so far.

Material and methods

Patients

Between January 2003 and December 2011, 92 consecutive newly-diagnosed CLL patients were enrolled in the present study. All patients provided informed consent and the study was approved by the ethics committee of the Third Affiliated Hospital of Soochow University and the First Affiliated Hospital of Nanjing Medical University. The study also carried out according to the Declaration of Helsinki. Fresh peripheral blood (PB) samples of all the patients were collected at diagnosis. The percent of CD19+ cells in PB were higher than 80%. The diagnosis of CLL was based on clinical characteristics, peripheral blood morphologies, immunophenotype, and B-lymphocytes ≥ 5.0×10^9/L [6]. Clinical stage was determined using the Binet staging system according to the IWCLL criteria. The control group consisted of 200 healthy blood donors, 110 (55%) males and 90 (45%) females, covering comparable age range to CLL patients. The population controls were unrelated ethnic Han Chinese and residents in Jiangsu Province, People's Republic of China.

Analysis of CD20 mutations

Genomic DNA was isolated from mononuclear cells stored at -80°C using the TIANamp genomic DNA kit (Tiangen Biotech, Beijing, China) according to the recommended procedure. Primers to amplify Exons-3, 4, 5, 6, 7 and 8 of the human CD20 gene (GenBank accession NG_023388.1) were designed using the Primer 5 program (http://www.premierbiosoft. com). **Table 1** shows the sequence of the primers. Samples were amplified using the following PCR reaction conditions: Exon-3, 6 and 7: 94°C for 4 min followed by 35 cycles at 94°C for 30 s, 68°C for 45 s, and 72°C for 60 s; Exon-4 and 5: 94°C for 4 min followed by 35 cycles at 94°C for 30 s, 61.2°C for 45 s, and 72°C for 60 s; Exon-8: 94°C for 4 min followed by 35 cycles at 94°C for 30 s, 64.6°C for 45 s, and 72°C for 60 s; final extension time of 10 min at 72°C. PCR products of 92 samples were purified by standard methods (Invitrogen, Shanghai, China) and directly sequenced with primer CD20 Exon-3-F, Exon-4-F, Exon-5-F, Exon-6-F, Exon-7-F and Exon-8-F using the ABI3730XL 96-capillary DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

qRT-PCR analysis for CD20 mRNAs expression

Using the Trizol reagent, total RNA was extracted from peripheral blood mononuclear cells. qRT-PCR analysis was carried out as previously described [7]. Expression of CD20 was analyzed using 1 mg of purified total RNA, 5×M-MLV buffer, M-MLV, DTT, specific primer sets

Table 2. Clinical and biological characteristics of 92 CLL patients

Characteristic	Value (%)
Sex (n=92)	
Male	57 (61.96)
Female	35 (38.04)
Age (n=92)	
≥60	50 (54.35)
<60	42 (45.65)
Binet stage (n=92)	
A	37 (40.22)
B	22 (23.91)
C	33 (35.87)
CD38 (n=88)	
≥30%	22 (25.00)
<30%	66 (75.00)
ZAP70 (n=87)	
≥20%	23 (26.44)
<20%	64 (73.56)
IGHV (n=78)	
Mutated (>2% deviation from a germline)	51 (65.38)
Unmutated (≤2% deviation from a germline)	27 (34.62)
p53 mutation status (n=86)	
Presence	17 (19.77)
Absence	69 (80.23)
Cytogenetics (n=92)	
del (17p13) or del (11q22.3)	26 (28.26)
del (13q14) as the sole abnormality	6 (6.52)
others	60 (65.22)

sciences, San Jose, CA) flow cytometer with simultaneous assessment of CD20, CD38 and ZAP-70 as previously described [1]. Data analysis was carried out with CellQuest software, cell subpopulations of interest were delineated using CD45/side scatter dot plots, and after subgating CD19-positive tumor cell, percent of CD20 expression positivity and mean fluorescence intensity (MFI) were calculated. At least 10,000 events were measured from each sample. Cutoff points of 30% and 20% were used to define positivity for CD38 and ZAP70, respectively.

Detection of molecular cytogenetic aberrations by fluorescence in situ hybridization (FISH)

FISH analysis was performed on the sample for conventional cytogenetic studies from 92 CLL patients. In order to detect prognostic relevant abnormalities of chromosomal regions, the following fluorescent labeled probes were used in interphase cytogenetic analyses: LSI MYB (6q23), LSI ATM (11q22.3), LSD 13S319 (13q14), LSI IGHC/IGHV (14q32), LSI p53 (17p13) and CEP12 (centromere12) (all probes purchased from Vysis, Downers Grove, IL, USA). FISH was performed as previously described [8].

and SYBR Green I (all were purchased from Invitrogen, Shanghai, China). qRT-PCR was performed on an Stratagene Mx3000P Instrument using the following primers: CD20 forward, 5'-ATGACAACACCCAGAAATTC-3'; CD20 reverse, 5'-TTAAGGAGAGCTGT-CATTTTCT-3'. β-actin forward, 5'-AGCGAGCATCCCCCAAAGTT-3'; β-actin reverse, 5'-GGGCACGAAGGCTCATCATT-3'. Each amplification reaction was performed in a final volume of 20 μl containing 1 μl of the cDNA, 1 μl of 0.5 μmol/l of each primer, 1×SYBRGreen PCR Master mix 10 μl and 7 μl of deionized water. The reaction was first incubated at 95°C for 5 min, followed by 35 cycles of 95°C for 5 s, 58°C for 30 s and 72°C for 30 s. All reactions were run in triplicate. Sequences of amplified products were verified by DNA sequencing.

Analysis CD20, CD38 and ZAP-70 expression by flow cytometry

Three-color immunophenotypic analysis was performed using a FACSCanto II (BD Bio-

Analysis of p53 mutation and IGHV mutation status

Genomic DNA was isolated from peripheral-blood mononuclear cell preparations stored at -80°C. Primers to amplify Exons-2/3, 4, 5/6, 7, 8/9, 10 and 11 of the human p53 gene and adjacent intronic sequences (GenBank accession NG_017013.1) were designed using the Primer 5 program. PCR products were purified using standard methods (Invitrogen, Shanghai, China) and directly sequenced with primer p53 ex2-3-F, ex4-R, ex5-6-F, ex-7-F, ex8-9-F, ex-10-F, ex-11-F using the ABI3730XL 96-capillary DNA Analyzer (Applied Biosystems, Foster City, CA, USA). IGHV sequencing was performed as previously described [9]. A germline homology of 98% was used as the cutoff between IGHV mutated and unmutated cases.

Statistical analysis

All statistical analyses were performed with SPSS version 17.0 (SPSS, Chicago, IL, USA).
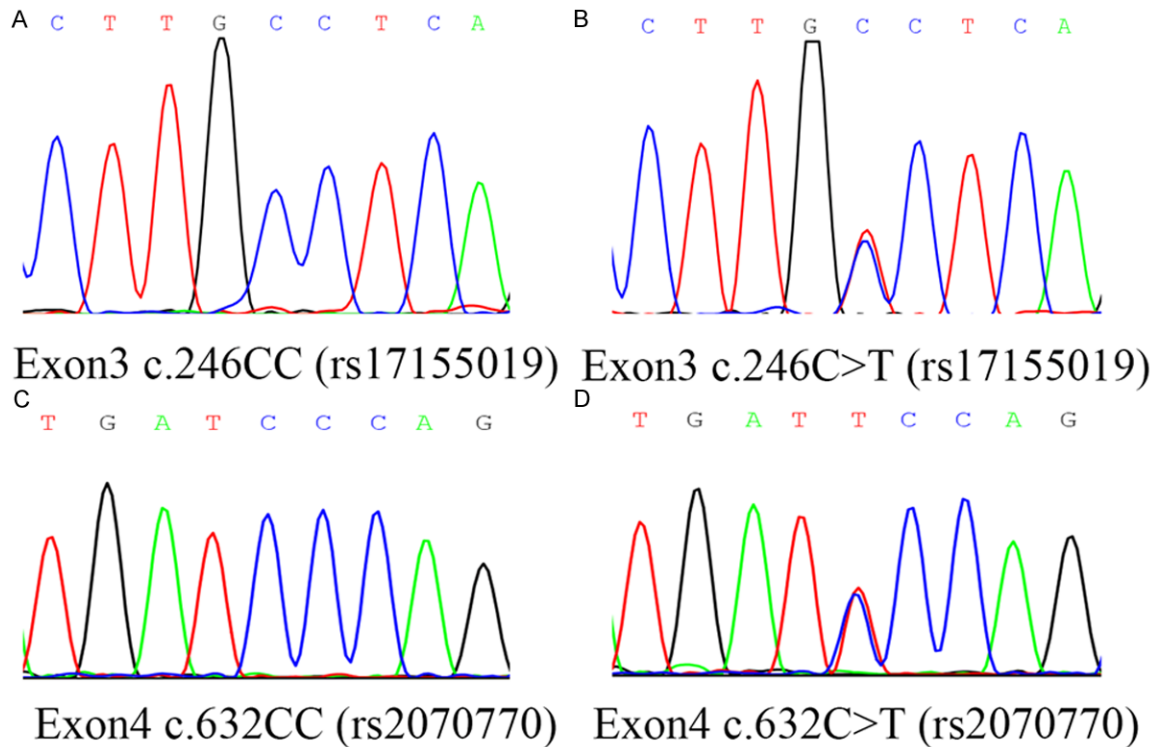
Figure 1. Sequence heterogeneity of the CD20 gene. Wild type or heterozygosity of Exon-3 for C246T (rs17155019) (A, B) and Exon-4 for C632T (rs2070770) (C, D).

The relative amount of CD20 mRNA was calculated by the equation $2^{-\Delta Ct}$. CD20 mRNA expression levels were compared using Mann-Whitney U-test. The distributions of clinical parameters in the cohorts with C/C and C/T genotype were compared using the χ^2 test. The association between the CD20 SNP and the risk for CLL was estimated by odds ratios (OR) with the 95% confidence intervals (CI). A $P<0.05$ was considered statistically significant, and all tests were two-tailed.

Results

Patient's characteristics

Patient's characteristics are summarized in **Table 2**. A total of 92 Chinese CLL patients were recruited into this study. Fifty-seven patients were male and 35 were female (male: female ratio, 1.63:1.00), and the median age was 60.5 years (range, 34-86 years). According to the Binet clinical staging system, 37 patients (40.22%) were classified as Binet stage A, 22 (23.91%) as Binet stage B, and 33 (35.87%) as Binet stage C. In a total of 92 CLL cases, the median expression percent of CD20 was 88.05% (range, 29.13%-100%), and MFI of

CD20 on CLL cells was 1083.39 (range, 87.93-4682.64).

CD20 genomic sequencing

The sequence showed a heterozygous C246T mutation in Exon-3, this site was located in the 5'UTR of CD20. We also found heterozygous C632T mutation in Exon-4. The synonymous mutation was not introducing amino acid substitution in the expressed gene product. After the NCBI SNP database (http://www.ncbi.nlm. nih. gov/projects/SNP) retrieval, two mutation site were confirmed for polymorphism loci. Four patients' specimens (4.35%) showed a heterozygous allele, c.246C>T (rs17155019) in Exon-3; Nine patients' specimen (9.78%) showed a heterozygous allele, c.632C>T (rs2070770) in Exon-4 (**Figure 1**).

CD20 SNP246 and SNP632 polymorphism in patients and controls

The frequencies of CD20 SNP246 and SNP632 genotypes for the healthy controls (n=200) and CLL patients (n=92) were consistent with the Hardy-Weinberg equilibrium distribution ($P=$ 0.48 and $P=1.00$ vs $P=0.77$ and $P=0.49$). The

Table 3. Comparison of allele frequency and distribution of CD20 gene SNPs in CLL patients with healthy controls and Logistic regression analysis of CD20 gene SNPs on CLL risk

CD20 variants	CLL, n=92	Controls, n=200	χ² test	Logistic regression*		
			P	OR	95% CI	P
rs17155019						
Genotype, n (%)			0.38			
246CC	88 (95.65%)	186 (93.00%)		1.00	-	
246CT	4 (4.35%)	14 (7.00%)		0.60	0.19-1.89	0.38
Allele, n (%)			0.39			
246C	180 (97.83%)	386 (96.50%)		1	-	
246T	4 (2.17%)	14 (3.50%)		0.61	0.20-1.89	0.39
HWE: p	0.77	0.48		-	-	-
rs2070770						
Genotype, n (%)			0.04			
632CC	83 (90.22%)	162 (81.00%)		1.00	-	
632CT	9 (9.78%)	36 (18.00%)		0.49	0.22-1.06	0.07
632TT	0 (0.00%)	2 (1.00%)		0.39	0.02-8.20	0.31
Allele, n (%)			0.04			
632C	175 (95.11%)	360 (90.00%)		1.00	-	
632T	9 (4.89%)	40 (10.00%)		0.46	0.22-0.98	0.04
HWE: p	0.49	1.00		-	-	-

*P values were adjusted for age, gender by binary logistic regression; OR: Odds ratio.

genotype distributions of CD20 polymorphism in the cases and the controls are shown in **Table 3**. CD20 C246T genotype frequencies were 95.65% (CC), 4.35% (CT), and 0.00% (TT) among the cases, and 93.00% (CC), 7.00% (CT), and 0.00% (TT) in the control subjects (P=0.38); CD20 C632T genotype frequencies were 90.22% (CC), 9.78% (CT), and 0.00% (TT) among the cases, and 81.00% (CC), 18.00% (CT), and 1.00% (TT) in the control subjects (P=0.04). The frequencies of variant alleles were as follows: for CD20 SNP246T, 2.17% in patients and 3.50% in controls (P=0.39); for 632T allele, 4.89% in patients with CLL and 10% in controls (P=0.04); Logistic regression analysis did not show a significant impact of variant genotypes on the likelihood of CLL development. However, CD20 SNP632T allele was associated with a significantly reduced risk of CLL (OR=0.46; 95% CI 0.22-0.98; P=0.04) (**Table 3**).

Clinical and biological characteristics among CD20 SNP genotypes in all patients

In the entire cohort, no correlation was shown between the CD20 SNP genotypes and age at diagnosis, gender, Binet stage, *IGHV* mutation-

al status, CD38 expression or ZAP-70 expression, *p53* mutation and cytogenetic abnormalities (P>0.05) (**Table 4**). The median mRNA expression levels of CD20 were 0.0824 (0.0064-1.3950). We did not found correlations of CD20 SNP genotypes with CD20 mRNA expression levels in all patients (P=0.73 and P=0.65). There were no differences in the percentage of CD20-positive cells in the CD20 SNP genotypes groups (P= 0.99 and P=0.97), however, there were also no differences in the MFI of CD20-positive cells in the CD20 SNP genotypes groups (P=0.53 and P=0.99) (**Table 5**).

Discussion

CD20 expression is quite heterogeneous in different lymphoma types, as well as among cells of an individual tumor sample. This can be visualized when examining the distribution in a flow cytometric histogram of a suspension of lymphoma cells stained with anti-CD20 [10]. Typically, CLL and small lymphocytic lymphoma have dim CD20 staining, and a corresponding lower rituximab response rate than follicular lymphoma [11]. Recent several reports have suggested that CD20-negative could develop in B cell lymphomas [12, 13]. In our center, we also found some CLL patients who experienced disease relapsed after rituximab treatment, but some CLL patients were CD20-negative. As the CD20 molecule was the physical target of rituximab, we hypothesized that mutations in the CD20 gene might be the reason for the treatment failures of rituximab in CLL.

It has been reported that the blocking capacity of CD20 extracellular proteins by rituximab was strongly reduced by mutation of the CD20ANPS or YCYSI protein sequences [14]. Terui et al. [15] studied fresh CD19-positive cells which were isolated from the lymph nodes or bone marrow of 68 patients, and evaluated CD20

Table 4. Clinical and biological characteristics among CD20 SNP genotypes in CLL patients

Characteristic	rs17155019		P	rs2070770		P
	C/C	C/T		C/C	C/T	
Sex (n=92)			1.00			0.47
Male	54 (94.74%)	3 (5.26%)		50 (87.72%)	7 (12.28%)	
Female	34 (97.14%)	1 (2.86%)		33 (94.29%)	2 (5.71%)	
Age (n=92)			0.62			1.00
≥60	41 (97.62%)	1 (2.38%)		38 (90.48%)	4 (9.52%)	
<60	47 (94.00%)	3 (6.00%)		45 (90.00%)	5 (10.00%)	
Binet stage (n=92)			0.89			0.63
A	35 (94.59%)	2 (5.41%)		33 (89.19%)	4 (0.81%)	
B	21 (95.45%)	1 (4.55%)		21 (95.45%)	1 (4.55%)	
C	32 (96.97%)	1 (3.03%)		29 (87.88%)	4 (12.12%)	
CD38 (n=88)			1.00			0.44
≥30%	21 (95.45%)	1 (4.55%)		21 (95.45%)	1 (4.55%)	
<30%	63 (95.45%)	3 (4.55%)		58 (87.88%)	8 (12.12%)	
ZAP70 (n=87)			1.00			0.44
≥20%	22 (95.65%)	1 (4.35%)		22 (95.65%)	1 (4.35%)	
<20%	61 (95.31%)	3 (4.69%)		56 (87.50%)	8 (12.50%)	
IGHV (n=78)			1.00			1.00
Mutated (>2% deviation from a germline)	49 (96.08%)	2 (3.92%)		46 (90.20%)	5 (9.80%)	
Unmutated (≤2% deviation from a germline)	26 (96.30%)	1 (3.70%)		25 (92.59%)	2 (7.41%)	
p53 mutation status (n=86)			0.17			0.20
Presence	15 (88.24%)	2 (11.76%)		17 (100.00 %)	0 (0.00%)	
Absence	67 (97.10%)	2 (2.90%)		60 (86.96%)	9 (13.04%)	
Cytogenetics (n=92)			0.53			0.10
Del (17p13) or del (11q22.3)	24 (92.31%)	2 (7.69%)		22 (84.62%)	4 (15.38%)	
Del (13q14) as the sole abnormality	6 (100.00%)	0 (0.00%)		6 (100.00%)	0 (0.00%)	
others	58 (96.67%)	2 (3.33%)		60 (96.77%)	2 (3.23%)	

expression by flow cytometry. Point mutations in three CD20 domians (extracellular/cytoplasmic domains, the third transmembrane domain and the C-terminal cytoplasmic domain) have been found in 13 out of the 68 non-Hodgkin's lymphoma (NHL) patients treated with rituximab. Furthermore, patients with point mutation in the C-terminal cytoplasmic domain were shown with weak CD20 expression while point mutation in the transmembrane domain with increased CD20 expression.

In our study, we found two mutations of the CD20 gene in open reading frame sequences of the genes for 92 CLL patients. However, two mutation sites were confirmed for polymorphism loci. 4.35% showed c.246C>T (rs171-55019) mutation in Exon-3; This site was located in the 5'UTR of CD20. 9.78% showed c.632C>T (rs2070770) mutation in Exon-4, this synonymous mutation would not introduce

amino acid substitution in the expressed gene product. The frequency of C/C genotype of rs17155019 and rs2070770 were higher than the normal control population, 95.65% and 93%; 90.22% and 81.00%. CD20 SNP632T allele was associated with a significantly reduced risk of CLL. No statistically significant differences in the CD20 mRNA, proportion and intensity of CD20 expression were found in the different genotypes of two polymorphic loci.

Some literatures indicated that CD20 was regulated by epigenetic such as 5-azacytidine [16], trichostatin A [12], and histone deacetylase (HDAC) inhibitors [17]. Moreover, Czuczman et al. [18] suggested that proteasome inhibition partially reversed rituximab resistance. However, further study revealed that bortezomib (a proteasome inhibitor) could lead to a significant decrease the surface CD20 levels [19]. Our preliminary research found that demethylation

Table 5. Comparison of the CD20 (%), CD20MFI and CD20 mRNA between CD20 SNP genotypes in CLL patients

	rs17155019		P	rs2070770		P
	C/C	C/T		C/C	C/T	
CD20 (%)			0.99			0.97
M (P5-P95)	88.05 (28.63-100)	90.95 (63-100)		89.10 (28-100)	86.90 (63-100)	
CD20MFI			0.53			0.99
M (P5-P95)	1054.87 (80.72-4812.25)	1231.76 (731.64-3596.40)		1111.90 (74.43-4254.49)	751.56 (217.05-9028.22)	
CD20 mRNA			0.73			0.65
M (P5-P95)	0.0824 (0.0149-0.8299)	0.1596 (0.0346-0.2902)		0.0774 (0.0143-0.3887)	0.2901 (0.0412-1.3947)	

drugs could increase the expression of CD20 in CLL cells. There was partially methylation in six CpG of CD20 promoter regions in CLL cells by bisulfite genomic sequencing. Epigenetic mechanisms might be associated with low CD20 expression in CLL (data not published). In conclusion, the results of our study indicated that CD20 SNP632C allele may be associated with CLL and the low CD20 expression of CLL were not related to CD20 coding region mutations. However, due to the limitation of the relatively small number of tested cohorts include in the present study, our results need to be confirmed in further independent prospective studies involving larger patient cohorts.

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Disclosure of conflict of interest

None.

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References

[1] Fang C, Zhuang Y, Wang L, Fan L, Wu YJ, Zhang R, Zou ZJ, Zhang LN, Yang S, Xu W and Li JY. High levels of CD20 expression predict good prognosis in chronic lymphocytic leukemia. Cancer Sci 2013; 104: 996-1001.

[2] Reff ME, Carner K, Chambers KS, Chinn PC, Leonard JE, Raab R, Newman RA, Hanna N and Anderson DR. Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. Blood 1994; 83: 435-445.

[3] Jaglowski SM, Alinari L, Lapalombella R, Muthusamy N and Byrd JC. The clinical application of monoclonal antibodies in chronic lymphocytic leukemia. Blood 2010; 116: 3705-3714.

[4] Ginaldi L, De Martinis M, Matutes E, Farahat N, Morilla R and Catovsky D. Levels of expression of CD19 and CD20 in chronic B cell leukaemias. J Clin Pathol 1998; 51: 364-369.

[5] Terui Y, Mishima Y, Sugimura N, Kojima K, Sakurai T, Mishima Y, Kuniyoshi R, Taniyama A, Yokoyama M, Sakajiri S, Takeuchi K, Watanabe C, Takahashi S, Ito Y and Hatake K. Identification of CD20 C-terminal deletion mutations associated with loss of CD20 expression in non-Hodgkin's lymphoma. Clin Cancer Res 2009; 15: 2523-2530.

[6] Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Dohner H, Hillmen P, Keating MJ, Montserrat E, Rai KR, Kipps TJ. International Workshop on Chronic Lymphocytic Leukemia. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. Blood 2008; 111: 5446-5456.

[7] Dong HJ, Fang C, Fan L, Zhu DX, Wang DM, Zhu HY, Zhuang Y, Miao KR, Liu P, Xu W and Li JY. MDM2 promoter SNP309 is associated with an increased susceptibility to chronic lymphocytic leukemia and correlates with MDM2 mRNA expression in Chinese patients with CLL. Int J Cancer 2012; 130: 2054-2061.

[8] Xu W, Li JY, Pan JL, Qiu HR, Shen YF, Li L, Wu YF and Xue YQ. Interphase fluorescence in situ hybridization detection of cytogenetic abnormalities in B-cell chronic lymphocytic leukemia. Int J Hematol 2007; 85: 430-436.

[9] Chen L, Zhang Y, Zheng W, Wu Y, Qiao C, Fan L, Xu W and Li J. Distinctive IgVH gene segments usage and mutation status in Chinese patients with chronic lymphocytic leukemia. Leuk Res 2008; 32: 1491-1498.

[10] Sar A, Perizzolo M, Stewart D, Mansoor A, Difrancesco LM and Demetrick DJ. Mutation or polymorphism of the CD20 gene is not associated with the response to R-CHOP in diffuse large B cell lymphoma patients. Leuk Res 2009; 33: 792-797.

[11] Smith MR. Rituximab (monoclonal anti-CD20 antibody): mechanisms of action and resistance. Oncogene 2003; 22: 7359-7368.

[12] Tomita A, Hiraga J, Kiyoi H, Ninomiya M, Sugimoto T, Ito M, Kinoshita T and Naoe T. Epigenetic regulation of CD20 protein expression in a novel B-cell lymphoma cell line, RRBL1, established from a patient treated repeatedly with rituximab-containing chemotherapy. Int J Hematol 2007; 86: 49-57.

[13] Alduaij W and Illidge TM. The future of anti-CD20 monoclonal antibodies: are we making progress? Blood 2011; 117: 2993-3001.

[14] Binder M, Otto F, Mertelsmann R, Veelken H and Trepel M. The epitope recognized by rituximab. Blood 2006; 108: 1975-1978.

[15] Terui Y, Mishima Y, Yokoyama M, Hatake K, Sugimura N, Kojima K, Sakurai T and Takeuchi K. Point mutation of C-terminal region of CD20 molecule predicts rituximab-induced complement-dependent cytotoxicity and clinical response to rituximab in non-Hodgkin's lymphoma. J Clin Oncol (Meeting Abstracts) 2006; 24: 7563.

[16] Hiraga J, Tomita A, Sugimoto T, Shimada K, Ito M, Nakamura S, Kiyoi H, Kinoshita T and Naoe T. Down-regulation of CD20 expression in B-cell lymphoma cells after treatment with rituximab-containing combination chemotherapies: its prevalence and clinical significance. Blood 2009; 113: 4885-4893.

[17] Shimizu R, Kikuchi J, Wada T, Ozawa K, Kano Y and Furukawa Y. HDAC inhibitors augment cytotoxic activity of rituximab by upregulating CD20 expression on lymphoma cells. Leukemia 2010; 24: 1760-1768.

[18] Czuczman MS, Olejniczak S, Gowda A, Kotowski A, Binder A, Kaur H, Knight J, Starostik P, Deans J and Hernandez-Ilizaliturri FJ. Acquirement of rituximab resistance in lymphoma cell lines is associated with both global CD20 gene and protein down-regulation regulated at the pretranscriptional and posttranscriptional levels. Clin Cancer Res 2008; 14: 1561-1570.

[19] Bil J, Winiarska M, Nowis D, Bojarczuk K, Dabrowska-Iwanicka A, Basak GW, Sulek K, Jakobisiak M and Golab J. Bortezomib modulates surface CD20 in B-cell malignancies and affects rituximab-mediated complement-dependent cytotoxicity. Blood 2010; 115: 3745-3755.