Single nucleotide polymorphisms of CD20 gene and their relationship with clinical efficacy of R-CHOP in patients with diffuse large B cell lymphoma

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Abstract
Background: R-CHOP has significantly improved survival rates of patients with diffuse large B cell lymphoma (DLBCL) by ~20% as compared to CHOP. CD20 antigen, highly expressed on more than 80% of B-cell lymphomas, is the target for rituximab. The goal of our study was to examine polymorphism in the CD20 gene in Chinese DLBCL population and whether CD20 gene polymorphism is associated with clinical response to R-CHOP.

Method: CD20 gene polymorphism was detected in the entire coding regions including 6 exons by polymerase chain reaction (PCR)-sequencing assay in 164 patients with DLBCL. Among them, 129 patients treated with R-CHOP as frontline therapy (R ≥ 4 cycles) were assessable for the efficacy.

Results: Polymorphisms at three single nucleotides (SNP) were identified in the entire coding regions of the CD20 gene in the 164 patients. One of them, CD20 Exon2[216] was found to be highly correlated with response to R-CHOP. Patients with homozygous C genotype showed a trend toward higher overall response rate than others with CT plus TT genotype (90.6% vs. 79.5%; P =0.166). A trend toward higher complete remission (CR) rate was observed in patients with homozygous C genotype (67.4%) compared with CT plus TT genotype (47.1%) (P = 0.091).

Conclusion: These results suggest that there are 3 SNPs in CDS of the CD20 gene in Chinese DLBCL population. The CC genotype at Exon2[216] appears to be associated with favourable response to R-CHOP.

Introduction
Genetic polymorphisms are variants in individual genomes and remain constant throughout a person’s lifetime. Many genetic polymorphisms contribute to variability in drug pharmacokinetic and pharmacodynamic processes [1]. The relationship between therapeutic efficacy and gene polymorphism has been extensively studied for a few monoclonal antibodies [2,3].

Rituximab is a chimeric monoclonal antibody targeting the CD20 antigen on normal and neoplastic B cells [4-7]. R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone) has significantly improved survival rates of patients with diffuse large B cell lymphoma (DLBCL) [8-11]. CD19 and other monoclonal antibodies are also being explored for novel lymphoma therapies [12].

CD20 was first identified as a B-cell specific marker in 1980 [13], which was highly expressed on more than 80% of B-cell lymphomas but not on stem cells, pro-B cells, normal plasma cells, or other normal tissues [14]. The CD20 gene, namely MS4A1 gene, is located on chromosome 11q12-q13.1 with 6 exons in its coding sequence (CDS) [15,16]. To date, 509 single-nucleotide polymorphisms (SNPs) have been reported for the CD20 gene. Among them, 57 SNPs are located in CDS, but there were no genotype and allele frequency data for most SNPs. In particular, there has been no report on the relationship between CD20 gene SNPs and their impacts on the response to R-CHOP in DLBCL patients. Several clinical studies offered scanty information about
the CD20 gene polymorphism when they researched the CD20 mutations in tumour tissues. Johnson et al. described that the CD20 gene mutations in the rituximab epitope are rare and no SNPs were detected in exon 5 of the CD20 gene [17]. Another study found no mutation in the CDS of the CD20 gene in tumours from 23 patients, with only one case showing a SNP [16].

Therefore, this study examined polymorphism of the CD20 gene in Chinese DLBCL population and the relationship between the polymorphism and clinical efficacy in patients with DLBCL treated with R-CHOP.

Methods
Study population
The clinical research protocol had been approved by our Institutional Review Board (IRB). This study had been approved by the Research and Ethical Committee of Peking University School of Oncology. A written informed consent had been obtained from each patient participated in this study.

This study included 164 patients with CD20+ DLBCL confirmed by our Department of Pathology according to the World Health Organization classification. All patients received R-CHOP (120 patients) or R-CHOP-like (44 patients) chemotherapy regimen between June 2007 and December 2010 at Beijing Cancer Hospital, Peking University School of Oncology. For elderly patients or patients with other complications, the dosage of CHOP was changed, which is not standard dosage of CHOP, so called R-CHOP-like. R-CHOP chemotherapy was administered as follows: one course of chemotherapy consisted of an intravenous infusion of Cyclophosphamide 750 mg/m², adriamycin 50 mg/m², vincristine 2 mg, and an oral administration of 100 mg prednisone on days 1 to 5, which was repeated every 3 weeks. Rituximab 375 mg/m² was infused over 4 to 6 hours on day 1 before CHOP or CHOP-like chemotherapy was started. Among the 164 patients, 129 received frontline R-CHOP and were evaluable for clinical efficacy. Of the 129 patients, 31 patients received involved-field radiation. The response to R-CHOP therapy was evaluated after completion of 2 to 3 courses of therapy and 1 to 2 months after completion of all therapy plans, then every 3 months for the first year and every 6 months thereafter until progression.

Results
Patient characteristics
The general features of the patients in this study are summarized in Table 1, including 81 female and 83 male. The median age at diagnosis was 53 years (range, 15–90 years). Eighty nine (54%) patients were in stages 3 or 4 and 50 (30%) patients had intermediate-to-high or high International Prognostic Index (IPI) scores. Bone
marrow was involved by lymphoma in 6 patients (4%) at diagnosis. R-CHOP followed by involved-field radiation was given to 31 (19%) patients. R-CHOP as a front-line regimen was administrated to 129 patients whose clinical efficacy was evaluable for this study. A median of 6 rituximab doses were given (range, 4–14), and a median of 6 cycles of chemotherapy was given (range, 2–8 cycles).

**CD20 gene polymorphism**

Three SNPs were identified in the entire coding regions (6 exons) of the CD20 gene in this DLBCL patient population (Table 2). All three SNPs were located in exon1 and exon2. Exon 3–6 showed no SNPs. The c.111G > C in exon 1, the c.208C > T and c.216C > T (CD20 Exon2 [216]) in exon2 have been previously reported separately as rs200805059, rs79703274 and rs2070770 (www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=931). Genotype and allele frequencies of the CD20 gene polymorphism in 164 patients with DLBCL were analyzed (Table 3). The frequency of the CD20 Exon2 [216] C allele among the 164 patients was 0.869, whereas the frequency of the CD20 Exon2 [216] T allele was 0.131. Seventy-five percent (123 of 164) of patients were homozygous for CD20 Exon2 [216] C allele, 23.8% (39 of 164) were heterozygous (C/T), and 1.2% (2 of 164) were homozygous for CD20 Exon2 [216] T allele (Table 3). The genotype distribution of DLBCL population enrolled in this study was in Hardy-Weinberg equilibrium with regard to the CD20 Exon2 [216] polymorphism examined ($P = 0.57$).

**Patient characteristics according to Exon2 [216] allele status**

There was no significant difference in patients’ disease features between the CD20 Exon2 [216] CC and CT plus TT polymorphism groups (Table 1).

**Clinical responses and Exon2 [216] Polymorphism**

Although not statistically significant, the patients with homozygous C genotype showed a trend toward higher overall response rate than those with CT plus TT genotype (90.6% vs. 79.5%; $P = 0.166$, Table 4). The trend seems to be largely due to a better complete response rate (CR). Higher CR rate was observed in patients with homozygous C genotype (67.4%) compared to those with CT plus TT genotype (47.1%) ($P = 0.091$). When the rate of CR was compared with that of non-CR (PR + SD +

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**Table 1 Patient’s characteristics and their correlations with CD20 Exon2 [216] genotype**

| Clinical parameters | No. | Genotype | $P^*$ | Clinical parameters | No. | Genotype | $P^*$ |
|---------------------|-----|----------|-------|---------------------|-----|----------|-------|
| Gender              |     | CC       | CTplusTT | Bulky mass          |     | CC       | CTplusTT |       |
| Male                | 83  | 63       | 20     | 0.787               | 18  | 11       | 7      | 0.249   |
| Female              | 81  | 60       | 21     |                     | 146 | 112      | 34     |         |
| Age                 |     |          |        |                     |     |          |        |         |
| ≤60                 | 102 | 76       | 26     | 0.852               | Yes | 25       | 19     | 6       | 0.900   |
| >60                 | 62  | 47       | 15     |                     | No  | 139      | 104    | 35      |         |
| B symptoms          |     |          |        |                     |     |          |        |         |
| Positive            | 62  | 46       | 16     | 0.852               | ≤1  | 122      | 90     | 32      | 0.535   |
| Negative            | 102 | 77       | 25     |                     | >1  | 42       | 33     | 9       |         |
| LDH                 |     |          |        |                     |     |          |        |         |
| Positive            | 77  | 55       | 22     | 0.320               | 93  | 68       | 25     | 0.524   |
| Negative            | 87  | 68       | 19     |                     | 71  | 55       | 16     |         |         |
| β2-MG               |     |          |        |                     |     |          |        |         |
| Positive            | 49  | 35       | 14     | 0.684               | 0-2 | 114      | 84     | 30      | 0.557   |
| Negative            | 106 | 79       | 27     | 3-5                 | 50  | 39       | 11     |         |         |
| Stage               |     |          |        |                     |     |          |        |         |
| I-II                | 75  | 55       | 20     | 0.651               | GCB| 28       | 21     | 7       | 0.868   |
| III-IV              | 89  | 68       | 21     |                     | Non-GCB| 113    | 83     | 30      |         |

Abbreviations: LDH lactate dehydrogenase level, MG macroglobulin, IPI international prognostic index, GCB germinal centre B cell, *χ² test.

**Table 2 CD20 polymorphisms in 164 Chinese people with DLBCL**

| Nucleotide change* | Effect on protein | location | Ref    |
|--------------------|-------------------|----------|--------|
| c.111G > C         | p.L37L            | Exon1    | rs200805059 |
| c.208C > T         | p.L70L            | Exon2    | rs79703274  |
| c.216C > T         | p.I72I            | Exon2    | rs2070770  |

Abbreviations: G Guanine, C Cytosine, T Thymine, L Leu, leucine, I: Ile isoleucine. * Position in CD20 cDNA is according to Reference Sequence: NM_021950.3. The mutation nomenclature follows the instructions provided by the HGVS (http://www.hgvs.org). The DNA polymorphisms numbering is based on cDNA sequence with +1 corresponding to the A of the ATG translation initiation codon.
Table 3 Genotype and allele frequencies of CD20 polymorphisms in 164 Chinese patients with DLBCL

| SNP         | Genotype frequencies | Allele frequencies |
|-------------|----------------------|--------------------|
|             | Genotype freq | count | Genotype freq | count | Genotype freq | count | Genotype freq | count |
| c.111G>C    | GG       | 0.988  | 162       | GC     | 0.012  | 2       | CC     | 0       | 0       | 164 |
|             | G        | 0.994  | 163       | C       | 0.006  | 1       | TT     | 0       | 0       | 164 |
| c.216C>T    | CC       | 0.750  | 123       | CT     | 0.238  | 39      | TT     | 0.012  | 2       | 164 |
|             | C        | 0.869  | 285       | T       | 0.131  | 43      |        |        |        |    |

Table 4 Clinical response to R-CHOP therapy according to CD20 Exon2[216] Polymorphism

| Response | CD20 exon2[216] genotype n (%) | P Value* |
|----------|--------------------------------|----------|
|          | No.(N = 129)                   | CC No.(%)| CT + TT No.(%)  | 95       | 34 |
| CR       | 64(67.4)                      | 16(47.1) | 0.091 |
| PR       | 22(23.2)                      | 11(32.4) |
| SD/PD    | 9(9.4)                        | 7(20.5)  |
| ORR(CR + PR) | 8(69.0)  | 27(77.9) | 0.166* |
| CR       | 64(67.4)                      | 16(47.1) | 0.033* |
| Non-CR   | 31(32.6)                      | 18(52.9) |

Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; ORR, overall response; non-CR, PR + SD + PD.

*#1 P = 0.166 when comparing ORR with SD/PR; #2 P = 0.036 when comparing CR with non-CR (PR + SD + PD); *P-test.
Figure 1 Progression-free survival (PFS) in DLBCL subjects with CD20 Exon2(216) polymorphism. Kaplan-Meier curve of PFS was plotted by CD20 Exon2(216) CC and CT plus TT genotype.

Figure 2 Disease specific survival (DSS) in DLBCL subjects with CD20 Exon2(216) polymorphism. Kaplan-Meier curve of DSS was plotted by CD20 Exon2(216) CC and CT plus TT genotype.
deletion mutations of CD20 gene changed the expression level of CD20 antigen, which may be related to the resistance after rituximab therapy [20]. Therefore, the CD20 Exon2[216] may potentially alter the level of CD20 antigen expression, thus affecting the clinical response to R-CHOP. We have used immunohistochemical method (IHC) to detect correlation of CD20 expression intensity with CD20 Exon2 [216]; no significant difference was found (Data was not shown). The CD20 Exon2[216] C for T substitution is a synonymous SNP of the third base of the codon for Ile72. Because synonymous SNPs encode a change in the DNA sequence without altering the resultant protein sequence, such “silent” changes were long assumed to be inconsequential. However, there is clear and accumulating evidence from recent work that synonymous SNPs can alter the expression, conformation, or function of a protein by a variety of mechanisms, including altering the efficiency of gene translation, affecting the stability of mRNAs and regulating the splicing process of mRNAs [21]. For example, a synonymous SNP, the C3435T polymorphism in the Multidrug Resistance 1 (MDR1) gene affects MDR1 gene product p-glycoprotein (P-gp) activity through altering its conformation [18,22]. Synonymous SNPs within the DRD2 transcript can reduce the stability of the mRNA and thus the expression of the dopamine receptor [23].

Synonymous SNPs in CHRNA4 alter the receptor response to Ach [24]. To date, silent SNPs have been reported in association with more than 40 diseases that have genetic bases [25]. Meanwhile, according to biased codon usage, synonymous SNP substituting a rare codon for a common codon encoding the same amino acid may directly impact the translation kinetics of a protein, resulting in its function alteration [22,26]. In the case of CD20 Exon2[216], the ATT > ATC transversion represents a change from a rare (ATT, 13.0 per thousand) codon encoding isoleucine to a more frequently used codon (ATC, 29.9 per thousand; frequencies obtained from the Codon Usage Databasehttp://www.kazusa.or.jp/codon/). Alternatively, CD20 Exon2[216] may be commonly inherited as part of a haplotype and exist in linkage disequilibrium with other disease-associated molecular markers [20], such as SNPs from a key gene involved in ADCC or cell-dependent cellular cytotoxicity (CDCC).

While we explored a correlation between clinical outcome and CD20 Exon2 [216], we found there are no statistically significant correlation between CD20 Exon2[216] genotypes and PFS and DSS (Figures 1 and 2). In addition, the CD20 Exon2[216] was not an independent predictor for prognosis. The possible explanation is that as a target, CD20 antigen is an important factor for initial response, but not for duration of R-CHOP response in DLBCL patients. The latter has different mechanisms and may be more dependent on immunity status of DLBCL patients. Meanwhile, patients with DLBCL, GCB lymphoma have better survival than non-GCB lymphoma. In our study, the difference of the overall survival between homozygous C patients, and T carriers did not reach statistical significance. This difference was neither statistically significant between GCB and non-GCB lymphoma groups in subgroup analysis (data not shown). Maybe it is a prognostic factor independent of molecular subtypes (GCB or non-GCB). Or it is possible that this may be due to the small number of patients that preclude reliable analysis.

Conclusion

In conclusion, this study points to a possible association between polymorphism in the CD20 gene and the response to R-CHOP in DLBCL patients. Ongoing studies will explore the impact of this SNP in a larger population of DLBCL and verify it with laboratory experiment.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
ZJ and SYQ designed the study and reviewed the final manuscript. DHR and JX performed and evaluated the experiments. DN helped to perform the experiments. FZY helped to collect the samples. JX collected and analyzed data. JX, SYQ and DHR wrote the manuscript. DHR and JX contributed equally to this work. All authors read and approved the final manuscript.

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