CD20 mutations involving the rituximab epitope are rare in diffuse large B-cell lymphomas and are not a significant cause of R-CHOP failure

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ABSTRACT

Rituximab binds an epitope on the CD20 antigen, encompassed in exon 5 of the *MS4A1* gene. We sequenced this region and correlated the presence of mutations with CD20 protein expression and response to R-CHOP in patients with diffuse large B-cell lymphoma: 264 diagnostic biopsies and 15 biopsies taken at the time of relapse were successfully sequenced. CD20 mutations involving the rituximab epitope were detected in only 1/264 (0.4%) and 1/15 (6%) of the biopsies taken at diagnosis and relapse, respectively. No polymorphic sequence variants were detected in this region. Three patients had malignant cells that were CD20 protein-positive at diagnosis but CD20-negative at relapse. Thus, CD20 mutations involving the rituximab epitope are rare in both *de novo* and relapsed diffuse large B-cell lymphoma, and do not represent a significant cause of R-CHOP resistance. CD20 protein-negative relapses occur after R-CHOP therapy but their clinical relevance is unknown.

Key words: CD20 antigen, mutation, DLBCL, R-CHOP.

Citation: Johnson NA, Leach S, Woolcock B, deLeeuw RJ, Bashashati A, Sehn LH, Connors JM, Chhanabhai M, Brooks-Wilson A, and Gascoyne RD. CD20 mutations involving the rituximab epitope are rare in diffuse large B-cell lymphomas and are not a significant cause of R-CHOP failure. Haematologica 2009; 94:423-427. doi: 10.3324/haematol.2008.001024

Introduction

Rituximab is a chimeric monoclonal antibody targeting the CD20 antigen on B lymphocytes.[1] The addition of rituximab to multi-agent chemotherapy has improved survival in patients with diffuse large B-cell lymphoma (DLBCL).[2,3] Because of this, there has been great interest in determining the role of CD20 in the pathogenesis of lymphomas and its function in normal B cells. The CD20 antigen is a membrane-bound protein that contains four trans-membrane domains and a large extra-cellular loop.[4] Two amino acid sequences, ANPS and YCYSI at positions 170 to 173 and 182 to 185, were recently determined to be the critical binding sites for rituximab.[5,6] The cytotoxic effects of rituximab, such as antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and direct induction of apoptosis require that the primary event be the binding of rituximab to CD20.[7] The rituximab epitope and part of the third and fourth trans-membrane domain are all included in exon 5 of the *MS4A1* gene. We sequenced exon 5 of *MS4A1* in primary DLBCL samples taken at diagnosis and relapse to determine the frequency and clinical significance of mutations at that site in R-CHOP treated patients. We correlated the presence of mutations with protein expression using immunohistochemistry (IHC) and flow cytometry (FCM).

Design and Methods

Patient selection

Patients diagnosed with DLBCL according to the World Health Organization (WHO) criteria and who had tissue available at the time of diagnosis and/or relapse between March 1st 2001 and December 1st 2006 were included in this study.[8] Baseline clinical characteristics, treatment regimen and clinical outcome were recorded for all patients. This research was

Funding: NAJ is a research fellow of the Terry Fox Foundation through an award from the National Cancer Institute of Canada (019005) and the Michael Smith Foundation for Health Research (ST-PDF-01793). She was previously supported by a training award from the Canadian Institute of Health Research (# STP-53912) during which part of this work was performed. JMC and RDG receive support through the National Cancer Institute of Canada (NCIC), Terry Fox Foundation Program Project grant # 016003. AB-W is a Senior Scholar of the Michael Smith Foundation for Health Research. This work is in part supported by a research grant from Roche Canada.
Manuscript received August 26, 2008. Revised version arrived on September 22, 2008. Manuscript accepted on October 10, 2008.
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approved by the University of British Columbia and BCCA research ethics board and is in accordance with the Declaration of Helsinki.

Sequencing the MS4A1 *gene*

Two-hundred and seventy-seven patients had initial biopsy tissue and 18 patients had tissue available at the time of relapse for sequencing. Ten patients had paired samples taken both at diagnosis and relapse. DNA was extracted using ALL PREP DNA/RNA mini kit (Qiagen) and PureGene DNA purification kit (Gentra) for frozen tissue and formalin fixed paraffin embedded tissue (FFPET), respectively.

We amplified exon 5 with the following PCR primers: 5'-*TGTAAAACGACGGCCAGT*TTGGAATTCCCTCCCAGATT-3' and 5'-*CAGGAAACAGCTATGAC*GGATCCAGAGTTCATGCTCA-3'. -21M13F and M13R sequencing tag extensions (italics) were incorporated at the 5' ends of the forward and reverse primers respectively to allow sequencing with standardized M13 primers and protocols.[9] The 431 base pair PCR product was purified with AMPure magnetic beads (Agencourt Bioscience Corporation) and bi-directionally sequenced using BigDye® Terminator v3.1 (Applied Biosystems) and an ABI 3730 XL sequencer. The forward and reverse sequence reads were assembled together and analyzed using PolyPhred[10] and displayed using Consed,[11] or analyzed using Mutation Surveyor (SOFTGENETICS, PA, USA). Mutations were considered present if they were found in both forward and reverse reads.

Determining CD20 protein expression

CD20 protein expression by immunohistochemistry (IHC) was determined on all samples using the L26 antibody on FFPET (Dako). CD20 protein expression was determined by FCM on 227 and 5 samples taken at diagnosis and relapse, respectively. Tumor cell suspensions were created by disaggregating cells from fresh tissue and suspending them in phosphate buffer (Dulbecco's PBS, Stem Cell Technologies) at a concentration of 10^7cells/mL. Cells were incubated at 4°C for 30 min with antibodies conjugated to either fluorescein isothiocyanate (FITC), phycoerythrin (PE) or phycoerythrin-Cy5 (PE-Cy5). The following antibody combinations were used: 10 ml of a combination of anti-κ-FITC, anti-lambda-PE and anti-CD19-PE-Cy5 and 10 µL each of anti-CD10-FITC, anti-CD11c-PE and anti-CD20-PE-Cy5 (clone B9E9, B1 epitope). Cells were then treated with 250 µL of Opti-Lyse C and washed once with IsoFlow sheath fluid prior to FCM analysis on a Beckman Coulter Cytomics FC500. 500×10^6 cells from 55 samples of normal peripheral blood lymphocytes (PB) were used as "controls" and were treated using the same method except that a 15 min incubation time was used. All reagents were purchased from Beckman Coulter except for the antibodies against CD19 and light chains which were purchased from Dako. A minimum of 5,000 events were analyzed using Flow Jo software version 8.7.1. The mean fluorescence intensity (MFI) of CD20 in the tumor cells and benign PB lymphocytes were recorded.

The tumor content was defined as the percent cells co-expressing CD19 and the tumor-specific light chain in the total live cell gate, determined using forward scatter and side scatter.

Results and Discussion

Two hundred and sixty-four out of 277 (95%) and 15/18 (83%) DLBCL samples taken at diagnosis and relapse were successfully sequenced. Patients' clinical characteristics are found in Table 1 and were similar to those reported by Sehn *et al.*, suggesting that our results were not biased by a dependence on frozen tissue and are representative of the DLBCL patient population in British Columbia, Canada.[3]

The majority of the patients had nodal disease with a minimum of 80% tumor, sufficient tumor cell content that if mutations were present, they would be detectable by sequencing. Lymphomas progressed or relapsed in 24% of the patients following R-CHOP immunochemotherapy.

One of 264 samples (0.4%) taken at diagnosis showed a 13 base pair (bp) heterozygous deletion at position IVS5(+8) in intron 5. This region is not known to contain regulatory elements or alternate splice sites. Germline DNA was not available to determine if this represented a polymorphism or a somatic mutation. Clinically, this patient achieved a complete response to R-CHOP and remains in remission more than two years after diagnosis.

Table 1. Patients' characteristics.

Clinical characteristics	Initial diagnostic biopsies n=277 (%)	Biopsies at relapse n=18 (%)
Median age, years	64	60
Male sex	171 (62)	12 (67)
PS > 1	110 (40)	6 (33)
LDH > normal	161 (58)	10 (55)
Extranodal sites > 1	100 (36)	7 (39)
Stage III/IV	177 (64)	14 (78)
IPI score at diagnosis		
0	33 (12)	0 (0)
1-2	149 (54)	10 (55)
3-4-5	95 (34)	8 (45)
Pathology of biopsy		
DLBCL	255 (92)	16 (89)
PMBCL	22 (8)	
FL		2 (11)
Site: nodal	219 (79)	10 (55)
extranodal	58 (21)	8 (45)
Flow cytometry performed	227 (82)	5 (28)
> 50% tumor content by FCM	161 (71)	4 (80)
Reduced CD20 protein expression		
MFI < 10 by FCM	13 (5)	1 (20)
CD20-negative by IHC	0	1 (6)
CD20-heterogeneous by IHC		2 (12)
DLBCL relapse or progression after R-CHOP	66 (24)	18 (100)

PS: ECOG performance status; LDH: lactate dehydrogenase; IPI: International Prognostic Index; DLBCL: diffuse large B-cell lymphoma; PMBCL: primary mediastinal B-cell lymphoma; FL: follicular lymphoma; IHC: immunohistochemistry; MFI: mean fluorescence intensity; FCM: flow cytometry.

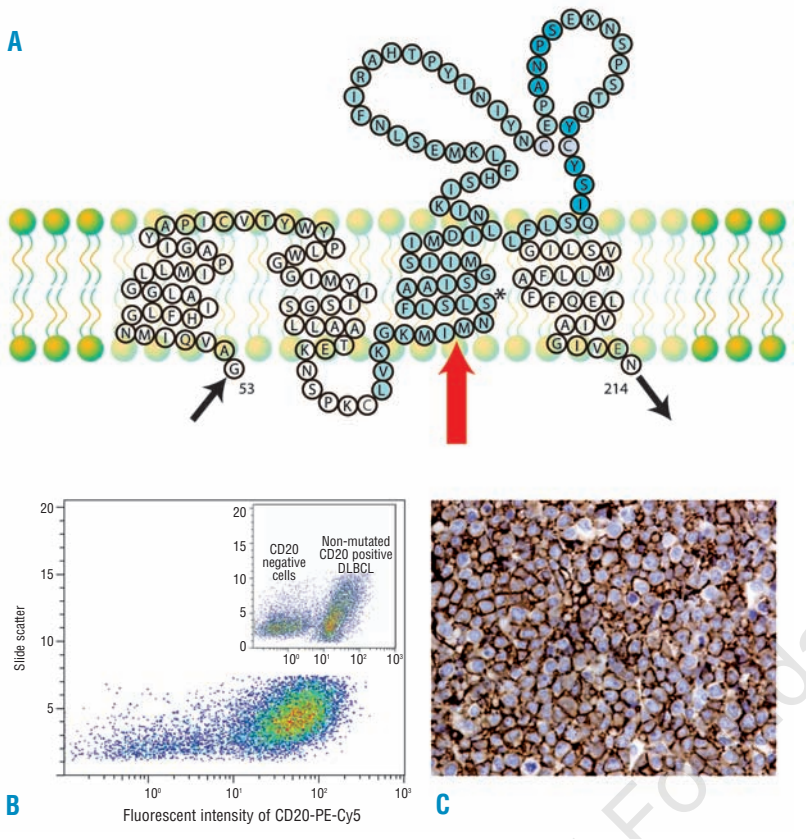

Figure 1. CD20 expression in a diffuse large B-cell lymphoma sample taken at relapse containing a 4 base pair deletion at nucleotide position 353-356. (A) Representation of the amino acid sequence coding for the trans-membrane portion of the CD20 protein (adapted from Binder *et al.*)[5] Light blue amino acids are contained within the sequenced region of exon 5 of the *MS4A1* gene and dark blue amino acids represent the rituximab epitope brought together by a disulfide bond at two cysteine residues. The red arrow represents the location of the 4 base pair deletion leading to premature termination at amino acid 121 (highlighted with a star). (B) Bright CD20 protein expression by flow cytometry in the mutated sample; inset: CD20 expression of a non-mutated diffuse large B-cell lymphoma sample analyzed during the same time frame demonstrating CD20 fluorescent intensity of benign non-B cells (CD20 negative) and tumor cells (CD20 positive) within the sample. (C) Bright CD20 protein expression by immunohistochemistry in the mutated sample (L26 antibody).

Only 1/15 (6%) sample taken one month after completion of 6 cycles of R-CHOP chemotherapy showed a CD20 mutation. This case had a heterozygous 4 bp deletion (TAAT) at nucleotide position 353-356 which predicted for a premature termination at amino acid position 121, well before the critical ANPS binding site (Figure 1). Unfortunately, no pre-treatment biopsy was available to determine if the mutation was present at diagnosis. No single nucleotide polymorphisms (SNPs) were detected in exon 5 of the CD20 gene.

Protein expression of CD20 was assessed in all cases by IHC using the antibody L26 used routinely in most clinical laboratories.[12] This antibody recognizes a cytoplasmic epitope of the CD20 antigen, distinct from the rituximab binding site.[13] All of the initial 277 samples had uniform and bright CD20 protein expression. Three patients had CD20-negative biopsies at the time of relapse where the initial biopsy was CD20-positive. Two of these samples contained malignant cells with different phenotypes; large tumor cells that were clearly CD20-negative while others were positive (Figure 2). The clinical outcome of these patients was poor. Two patients died within six months of relapse and the other is receiving salvage chemotherapy in preparation for an autologous stem cell transplant four months after relapse. Interestingly, the sample taken at relapse showing a CD20 mutation that predicted for a severely truncated protein and loss of the extra-cellular domain, showed strong CD20 protein expression by IHC and FCM (Figure 1B and C). The mean CD20 MFI of this mutated sample was 49.7 compared to the mean CD20 MFI of 55.97 (standard deviation, 105) for 80 DLBCL samples that were analyzed during the same time frame where all instrument settings and analysis protocols remained constant (inset of Figure 1B). The mean CD20 MFI of normal PB lymphocytes during this same time frame was 238 (standard deviation, 105). One patient had a sample taken at relapse that had strong CD20 expression by IHC but weak CD20 expression by FCM (CD20 MFI < 10).

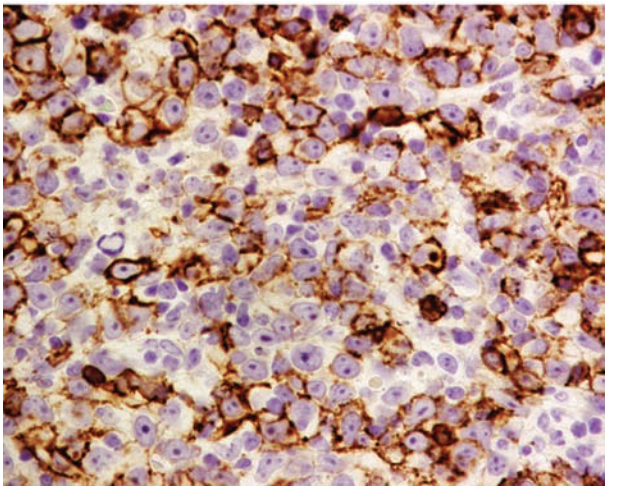

Figure 2. Heterogeneous CD20 protein expression by immunohistochemistry in a diffuse large B cell lymphoma sample taken at relapse following R-CHOP therapy

However, the initial biopsy on this patient had similar CD20 expression by FCM (MFI<10) prior to rituximab exposure.

The recent identification of the rituximab binding site prompted us to determine the frequency of CD20 mutations at that site as a possible cause of primary R-CHOP resistance.[5] We show that the frequency of mutations in the *MS4A1* gene, coding for the extra-cellular domain of the CD20 antigen, is extremely low in DLBCL and is not a significant cause of R-CHOP treatment failure. Indeed, the only mutation found in a biopsy at relapse was heterogeneous and did not result in a change in CD20 protein expression as determined by IHC and FCM, implying that the normal allele may be sufficient to support a normal protein expression level. Furthermore, recent work by Czuczman *et al*. confirms that mutations in the *MS4A1* gene are not the cause of decreased CD20 protein expression in rituximab-resistant cell lines.[14]

Mutations at other sites in *MS4A1* could also potentially lead to R-CHOP resistance but were not addressed in this study. In an unpublished study of CD20 mutations in 50 non-Hodgkin's lymphoma (NHL) samples, which included different lymphoma subtypes and samples taken at relapse, investigators noted a 22% incidence of mutations with cytoplasmic mutations occurring four times more frequently than those in the extra-cellular domain.[15] Mutations at residues 219 to 252 could prevent the formation of lipid rafts in response to binding of rituximab.[16,17] Cross-linking of CD20 monomers onto lipid rafts may be important in enhancing CDC activity and apoptosis.[18] Even if one assumes that these mutations are clinically meaningful, the incidence of *de novo* CD20 mutations is still too low to justify a screening strategy to identify potential rituximab non-responders.

Our finding of reduced CD20 protein expression detected by IHC in three samples taken at relapse indicates that CD20-negative relapses can occur and may be more common than previously described by Davis *et al*.[19] Indeed, since then other case reports and case series have been described but in variably treated patients with different lymphoma subtypes.[20-22] Tissue obtained from patients with primary DLBCL at the time of relapse is rare because most patients undergo fine needle aspiration to confirm relapse or have no biopsy. Thus the true incidence of CD20-negative relapses is unknown. In our cohort, 3 patients who initially had CD20-positive DLBCL had malignant clones in their samples at relapse that were clearly CD20-negative. Unexpectedly, 2 of these patients showed clonal heterogeneity in which only a subset of cells were CD20-negative, a phenotype that to our knowledge has never been described before. Weak CD20 protein expression by FCM but strong CD20 staining by IHC was seen in 13 and one biopsies taken at diagnosis and relapse, respectively.

Recent rituximab exposure can account for apparently CD20-negative B cells by FCM as both rituximab and B1 compete for the same binding site.[23] However, this is not the case in our study. We can not exclude that other genetic alterations in the *MS4A1* gene or post transcriptional regulation of *MS4A1* may have occurred. Aneuploidy and translocations are also common in NHL but unlike chronic lymphocytic leukemia, deletions at chromosome 11q21 in DLBCL are infrequent as assessed by array comparative genomic hybridization.[24] Interestingly, lymphomas progressed or relapsed in 7/13 patients with weak CD20 expression on their primary biopsy by FCM, but these small numbers preclude meaningful conclusions.

Thus, the CD20 antigen may play a role in the pathogenesis of DLBCL, but our data suggest that mutations in the rituximab epitope do not occur with sufficient frequency to account for a meaningful proportion of the observed treatment failures.

Authorship and Disclosures

NAJ designed and performed the research, analyzed the data and wrote the manuscript; SL analyzed the data and performed some of the research (sequencing); BW extracted DNA from the tissues; RJdL extracted DNA from the tissues; AB contributed to manuscript preparation; LS contributed with clinical data collection; JMC contributed with clinical data collection; MC contributed with pathological review of the tissue; AB-W contributed with data analysis; RDG contributed with the design of the research, pathological review of the tissue and writing the manuscript.

The authors reported no potential conflicts of interest.

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