High BCL6 expression predicts better prognosis, independent of BCL6 translocation status, translocation partner, or BCL6 deregulating mutations, in gastric lymphoma

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Abstract

To investigate the role of \textit{BCL6} in the pathogenesis of gastric lymphoma, we analyzed the \textit{BCL6} promoter region for \textit{BCL6} translocations, somatic hypermutations, and deregulating mutations in 43 gastric lymphomas, including 4 MALT (mucosa-associated lymphoid tissue) lymphoma, 33 DLBCL (diffuse large B-cell lymphoma), and 6 DLCLML (composite DLBCL with residual MALT lymphoma). \textit{BCL6} promoter substitutions by immunoglobulin (\textit{Ig}) and non-\textit{Ig} translocation partners, resulting in its deregulation, were frequently involved in DLBCL (36.4\%) and DLCLML (50\%). Two novel \textit{BCL6} translocation partner genes, \textit{28S rRNA} and \textit{DMRT1}, and a new \textit{BCL6} translocation breakpoint in intron 2 were also identified. Deregulating mutations were found only in DLBCL (24.2\%), which correlated significantly with high BCL6 protein expression. Significantly, high BCL6 expression correlated strongly with longer overall survival (OS), independent of mechanism in gastric DLBCL and DLCLML. Gastric DLBCL were further subclassified into GCB (germinal centre B-cell–like) and non-GCB subgroups immunohistochemically. High BCL6 expression was detected in all GCB cases, irrespective of \textit{BCL6} genetic alterations. In the non-GCB subgroup, \textit{BCL6} deregulating mutations correlated significantly with high BCL6 expression level. No significant correlation was found between the BCL6 expression level and OS in the non-GCB subgroup, which had significantly poorer prognosis than the GCB subgroup.
Introduction

Primary gastric lymphoma represents about 80% of all extranodal non-Hodgkin’s lymphoma (NHL) and is the commonest extranodal NHL in Hong Kong Chinese. Based on the World Health Organization (WHO) classification, gastric lymphoma can be further classified into MALT lymphoma (extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue), DLBCL (diffuse large B-cell lymphoma) and DLCLML (composite DLBCL with residual MALT lymphoma; transformed MALT DLBCL). In the West, gastric MALT lymphoma is more prevalent, occurring in up to 75% of cases. However, gastric DLBCL is more frequent in Chinese patients in Hong Kong, accounting for 60% of gastric NHL.

MALT lymphomas show histologic features more in common with those of mucosa-associated lymphoid tissue than those of peripheral lymph nodes. They exemplify the close relationship between chronic inflammation and lymphomagenesis, as a strong association has been found between chronic infection with Helicobacter pylori and gastric MALT lymphoma. Specific karyotypic alterations characterize MALT lymphomas—trisomies 3 and 18, translocations t(11;18)(q21;q21), t(1;14)(p22;q32), t(14;18)(q32;q21), t(3;14)(q27;q32), and t(3;14)(p14.1;q32). In contrast, gastric DLBCL has a similar histology to its nodal counterpart, but a number of observations suggest that the genetic abnormalities of gastric DLBCL are distinct from those of nodal DLBCL.

DLBCL is a heterogeneous entity both clinically and morphologically. Recently, three prognostically important subgroups of DLBCL were identified by cDNA microarray classification (germinal center B-cell–like (GCB), activated B-cell–like (ABC), or type 3), the type 3 group is heterogeneous and behaves in a manner similar to the ABC group. Hans et al subsequently demonstrated that an immunostain panel of CD10, BCL6, and
MUM1 could be used to classify DLBCL into GCB and non-GCB subgroups, with equivalent prognostic significance as gene expression profiling.\textsuperscript{12}

The \textit{BCL6} gene was initially identified through its involvement in the DLBCL translocation associating 3q27 with the immunoglobulin (\textit{Ig}) gene at 14q32.\textsuperscript{13-15} \textit{BCL6} is expressed strongly in B- and T-cells within the GCs, and is required for the development of GCs and Th2-mediated antigen responses.\textsuperscript{16-18} \textit{BCL6} is a transcription repressor.\textsuperscript{19} Downregulation of \textit{BCL6} is necessary for lymphocytes within the GC to differentiate into memory B cells or plasma cells or to undergo selective apoptosis upon antigen stimulation. Among the findings that support this conclusion are the following: 1) \textit{BCL6} protein is rapidly degraded upon antigen receptor signaling through the activation of MAP kinase phosphorylation of \textit{BCL6};\textsuperscript{20} 2) \textit{BCL6} represses \textit{PDCD2}, a gene associated with programmed cell death in thymocytes;\textsuperscript{21} 3) \textit{BCL6} represses a group of genes involving in B-cell terminal differentiation and cell cycle control;\textsuperscript{22} 4) \textit{BCL6} represses p53 by directly binding to its promoter and allows GC B-cells to tolerate the physiological DNA breaks required for \textit{Ig} class-switch recombination and somatic hypermutations (SHM);\textsuperscript{23} 5) \textit{BCL6} interacts with the transcriptional activator Miz-1 upon binding to the promoter of \textit{CDKN1A}, which in turn prevents the p53--independent cell cycle arrest in GC B-cells.\textsuperscript{24}

Structural alterations of \textit{BCL6}, including promiscuous chromosomal translocations and SHM, have been identified in several NHL with various frequencies. \textit{BCL6} translocations are found in approximately 40\% of DLBCL and 5-10\% of follicular lymphoma (FL).\textsuperscript{25-28} \textit{BCL6} translocations typically juxtapose the 5' regulatory region of \textit{BCL6} to the promoter region of a constitutively expressed partner gene, while leaving the coding domain of \textit{BCL6} intact, resulting in constitutive \textit{BCL6} expression. This, in turn,
blocks the normal differentiation of GC B-cells and thus contributes to lymphomagenesis. SHM in the 5’ non-coding region of BCL6 are found at average frequencies of 1.2 x 10^{-3}/bp, in approximately 30% of normal GC B-cells and 70% of DLBCL. The BCL6 SHM region overlaps with the major translocation breakpoints cluster (MTC), within a 2 kb region encompassing the promoter and the first non-coding exon. Several hotspots within the 2 kb region generate shifted bands in a gel mobility shift assay, suggesting that the region includes one or more cis-regulatory elements whose individual mutations may modify the binding of hypothetical BCL6-regulating transcription factors. Although BCL6 SHM are found in normal GC B-cells, a specific subset of these mutations, BSE1A and BSE1B in BCL6 binding motifs in non-coding exon 1, lead to deregulated BCL6 expression and are found only in DLBCL cases (15%). BCL6 mutations accumulate during high-grade transformation of FL, and it is suggested that some SHM (deregulating mutations in this context) may be clonally selected for their effect on the survival of the neoplastic clone.

In this study, we investigated the involvement of BCL6 in the pathogenesis of well characterized primary gastric MALT lymphoma, DLBCL and DLCLML by analyzing the BCL6 promoter region for BCL6 translocations, SHM, and deregulating mutations in BCL6 binding motifs in non-coding exon 1 (BSE1A, BSE1B), and by correlating the results with BCL6 protein expression and prognostic significance. In addition, since BCL6 expression in DLBCL may be more strongly associated with the state of differentiation of the tumour cell (GCB vs. non-GCB) than with BCL6 alterations at the genetic level, cases of gastric DLBCL were further subclassified immunohistochemically into GCB and non-GCB phenotypes according to Hans et al and BCL6 protein expression was correlated with the BCL6 genetic alterations and prognostic significance.
within these two subgroups of DLBCL.

Materials and methods

Gastric lymphoma specimens

Paraffin and frozen tumour blocks were retrieved from recent gastrectomy specimens of 43 cases of primary gastric B-cell NHL from Chinese patients, including 10 MALT (4 low-grade MALT and 6 DLCLML) and 33 DLBCL. The histological diagnosis of gastric lymphoma cases was based on the World Health Organization (WHO) classification scheme.\textsuperscript{1} In 6 DLCLML cases, immunoglobulin light chain restriction\textsuperscript{39} of both the residual MALT and DLBCL components by immunostaining indicated a clonal relationship between them (data not shown). Moreover, using DNA extracted from microdissected MALT and DLBCL components, the clonal link between these two components was further confirmed by sequence analysis of the rearranged IgH gene\textsuperscript{40} in 5 of these 6 DLCLML cases (data not shown). In one DLCLML case, it was not practical to microdissect the residual MALT component from DLBCL lesion. However, immunoglobulin lambda light chain (Ig\(\lambda\)) restriction of both components immunohistochemically was consistent with a clonal relationship (data not shown). The cases were treated with "curative intent" despite the heterogeneity of treatment. Clinical data were available for all of the cases studied; however, no cytogenetic data was available for these cases.

5’ RACE (Rapid amplification of cDNA ends) on mRNA and inverse-PCR on genomic DNA

The analysis of BCL6 transcripts at the 5’end was performed by employing the RACE kit
(Roche Diagnostics GmbH, Mannheim, Germany), using the polymerase chain reaction (PCR) conditions and the BCL6 specific primers as described.\textsuperscript{41} To confirm the presence of chimeric RNA transcripts obtained by 5’ RACE, direct reverse transcription (RT)-PCR was performed on randomly primed cDNA, for each case carrying the translocation.

To amplify BCL6 chromosomal translocations involving intron 1, tumour DNA was digested separately with two restriction enzymes BamHI and XbaI and ligated at low concentration, and inverse-PCR was performed as described\textsuperscript{33} using Expand Long Template System (Roche). To confirm the presence of BCL6 chromosomal translocations detected by inverse-PCR, primers (Supplementary Table 1) were designed across the breakpoint, and direct PCR was performed on genomic DNA for each case carrying the translocation.

**Long-range PCR on genomic DNA**

Long-range PCR was performed to obtain the chromosomal junction sequences and to identify the breakpoints of all BCL6 translocations that were detected by 5’ RACE on mRNA only. The primers were designed based on the sequence of each fusion transcript (Supplementary Table 1) and long-range PCR was performed on genomic DNA for each case carrying the translocation using Expand Long Template System (Roche).

**Mutational analysis of BCL6**

For mutational analysis of the BCL6 promoter region on the DNA level, the genomic fragment spanning non-coding exon 1 and the first intron (encompassing major mutation cluster, MMC) was amplified using primers listed in Supplementary Table 1. To detect somatic mutations in non-coding exon 1 (BSE1A and BSE1B) of the expressed BCL6
mRNA sequence, RT-PCR analysis of BCL6 mRNA spanning exon 1 and part of exon 2 was performed (Supplementary Table 1) on the random primed cDNA of 43 gastric lymphoma cases. Expand High Fidelity PCR System (Roche) was used to minimize the PCR amplification errors.

Cloning and DNA sequencing
The final PCR products of 5’RACE on mRNA, inverse-PCR, and long-range PCR were separated on agarose gels. The dominant bands were excised under ultra-violet light, and DNA was purified from gel slices using QIAEX II Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). For mutational analysis of the BCL6 promoter region, each PCR product was purified using High Pure PCR Product Purification Kit (Roche) to avoid spontaneous mutations generated during the excision of PCR bands from the agarose gel under ultra-violet light. The purified PCR products were then subjected to direct sequencing in both orientations. In addition, PCR products were also subcloned into pGEM®-T Easy vector (Promega, Madison, WI) and sequenced with the forward and reverse pUC/M13 sequencing primers flanking the two sides of the inserts. At least 5 clones were sequenced in both orientations for mutational analysis.

Immunostaining
Forty-two cases of gastric lymphoma were studied for the expression of BCL6 by immunohistochemistry; no adequate paraffin block was available for one DLBCL case. For antigen retrieval before immunostaining, sections were subjected to pressure cooker pre-treatment for 10 minutes in citrate buffer (pH 9.0). The mouse monoclonal anti-BCL6 (Dako Japan Co., Ltd.) was used as the primary antibody. The LSAB system (Dako) was
used for the visualization of the expression of the BCL6 antigen. The level of nuclear BCL6 expression was assessed by a 4-point system based on the proportion of positive cells: <10% (-); 10%-50% (+); 50%-70% (++); >70% (+++). For the subclassification of 32 gastric DLBCL cases into GCB and non-GCB immunophenotypes according to Hans et al,12 the expression pattern of CD10 and MUM1 was also studied immunohistochemically using mouse monoclonal CD10 (Novacastra Laboratories Ltd, Newcastle Upon Tyne, UK) and MUM1 (Abcam, Cambridge, UK) antibodies. A heat-induced antigen retrieval step (95°C for 10 minutes) was performed using citrate buffer (pH 6.0) for CD10 and 1 mM EDTA (pH 9.0) for MUM1 antibodies. The ABComplexes system (Dako) was used for the visualization of the expression of these two antigens and their expression levels were assessed by a 2-point system based on the proportion of positive cells: <30% (-); >30% (+). Immunohistochemical scoring was done independently on separate occasions by two histopathologists (C. C. S. and L. S.), who were blinded to the BCL6 molecular analysis, and was shown to be consistent.

Expression of BCL6 and its translocation partner genes in B-cell lines

Randomly primed cDNA from a series of B-lineage tumour cell lines corresponding to different stages of B-cell development was synthesized from 2 µg of total RNA (PE Applied Biosystems, Foster City, CA). For analysis of the expression of BCL6 and its translocation partner genes (28S rRNA, DMRT1, GAPD, HSP89α, and TFRC), semi-quantitative RT-PCR was performed for each gene on the cDNA. The β-actin mRNA sequence was also amplified as a control to normalize the amount of cDNA used for each sample.
Survival analysis

Statistical analysis was performed using SPSS for Windows Release 13.0. (SPSS Inc, Chicago). Overall survival (OS) of patients was calculated from the date of diagnosis until death or last follow-up. Survival curves were plotted by the Kaplan-Meier method and were compared by the log-rank test.

Results

28S rRNA and DMRT1 are novel translocation partners of BCL6

Two complimentary PCR-based approaches, 5’ RACE on mRNA (Figure 1A (i)) and inverse-PCR on genomic DNA (Figure 1A (ii)), were employed in all cases of gastric lymphoma to detect BCL6 translocations. BCL6 translocations were detected in a total of 15/43 (34.9%) cases of gastric lymphoma, including 12/33 (36.4%) DLBCL and 3/6 (50%) DLCLML. No BCL6 translocations were detected in MALT lymphoma cases. Among these 15 cases with BCL6 translocations, 9/15 (60%) involved Ig: IgH Sγ3 (5 cases), IgH Eμ (3 cases), Igλ J1/C1 (1 case); 5/15 (33.3%) involved non-Ig genes: 28S rRNA, DMRT1, GAPD, HSP89α, and TFRC; and one DLCLML case (#30) was found to harbor two independent BCL6 translocations: IgH Sγ3/BCL6 and Chr.3/BCL6. Of the BCL6 non-Ig fusion partners, GAPD was detected by both 5’ RACE and inverse-PCR while 28S rRNA, HSP89α and TFRC were detected by 5’ RACE and DMRT1 was detected by inverse-PCR only. 28S rRNA and DMRT1 are novel non-Ig fusion partners of BCL6.

To obtain the chromosomal junctions and breakpoints of the Ig/BCL6 or non-Ig/BCL6 translocations detected by 5’ RACE on mRNA only, but not by inverse-PCR on genomic DNA, long-range PCR was performed on genomic DNA (Figure 1A (iii)). Cases #22 and
#29 had been reported previously by our group to have non-Ig/BCL6 chimeric RNA transcripts detected by 5' RACE, and, in the present study, the chromosomal junctions and breakpoints of these two translocations were also characterized using long-range PCR.

The distribution of the BCL6 translocation breakpoints identified in gastric lymphoma is shown in Figure 1B. The majority of these breakpoints (13/15; 86.7%) were located within the MTC region of BCL6, while five were identified in the MMC region (Figure 1B). No translocation breakpoint hyper-cluster region was observed in the MMC region.

**Identifications of a new translocation breakpoint within intron 2 of BCL6**

The nucleotide sequences of the chromosomal junctions of Ig/BCL6 translocations are shown in Figure 1C and the schematic representation of each non-Ig/BCL6 translocation is shown in Figure 1D. In each of the Ig/BCL6 translocations and the non-Ig/BCL6 translocations involving DMRT1, GAPD, HSP89α, and TFRC, the 5' promoter regions of these genes were juxtaposed to the 5' end of the first intron of BCL6, in the MTC region; while in the 28S rRNA/BCL6 translocation, the 5' 1.7 kb of 28S rRNA was juxtaposed to BCL6 intron 2. The involvement of intron 2 in BCL6 translocation has not been described before (Figure 1B).

All of the translocations detected resulted in BCL6 promoter substitution by the corresponding fusion partner genes, thereby placing BCL6 under the regulation of heterologous regulatory sequences. For the GAPD/BCL6 translocation, GAPD coding exon 1 was fused upstream of BCL6 exon 2 in the same orientation (Figure 1D). The chimeric RNA transcript GAPD/BCL6 is expected to code for the first 9 codons of GAPD, followed by a stop codon generated as a consequence of the translocation. This sequence
is in turn followed by the *BCL6* translation initiation codon (ATG) and the complete *BCL6* coding sequence. No *BCL6* protein expression is expected from 28S rRNA/BCL6 fusion RNA transcript as no translation mechanism should act on 28S rRNA.

**Somatic mutations in the *BCL6* MMC region in gastric lymphoma**

All 43 cases of gastric lymphoma were subjected to mutational analysis of the *BCL6* genomic fragment spanning the non-coding exon 1 and the 5’ end of the first intron, encompassing the MMC region. Mutated clones of the *BCL6* MMC region were detected in 24/43 (55.8%) gastric lymphoma: 2/4 (50%) MALT lymphoma, 19/33 (57.6%) DLBCL, and 3/6 (50%) DLCLML. The frequency of mutations ranged from 1.17 to 24.48 x 10^{-3}/bp. The presence of intraclonal heterogeneity in the *BCL6* MMC region, a marker of ongoing somatic mutation in B-cell lymphomas of GC origin, was detected in 6 cases of DLBCL (18.2%). The distribution of somatic mutations detected within the MMC region is shown in Figure 2A. No specific hotspot of somatic mutations within the MMC region was identified. For the 5 *BCL6* translocation breakpoints located within the MMC region, two of them, from IgH/BCL6 and HSP89α/BCL6 translocations, were detected at nucleotide positions where somatic mutations were observed (Figure 2A).

The features of the somatic mutations detected in the *BCL6* MMC region in 24 cases of gastric lymphoma are summarized in Table 1. Of a total of 170 single base-pair substitutions observed, 105 were transitions and 65 were transversions. The overall transition/transversion ratio was 1.63 (expected 0.5). In addition to acquired somatic mutations, two constitutional single-nucleotide polymorphisms (SNPs) within the MMC region at positions 754 (G/C) and 877 (del T) were detected in 4 and 2 cases of gastric lymphoma, respectively. These inherited heterozygous polymorphisms were not counted
as acquired somatic mutations among these cases.

It has been shown that the quadruplet motif RGYW (A/G G C/T A/T) and the inverse repeat WRCY (A/T A/G C C/T) are targets for increased mutational activity in the $BCL6$ MMC region.\textsuperscript{42} To test whether these phenomena also occurred in gastric lymphoma, we analyzed the somatic mutations occurring in these motifs. Together, RGYW/WRCY motifs represented 18.1\% (155/858) of nucleotides within the region studied (Table 1). Mutations of RGYW/WRCY accounted for 25.9\% (44/170) of all nucleotide substitutions, a higher frequency than expected.

**Translocation breakpoints are located within mutational hotspot motifs of $BCL6$ and its partner genes in gastric lymphoma**

In $Ig/BCL6$ translocations, most of the breakpoints on both $Ig$ and $BCL6$ gene sequences were located within or near the mutational hotspot motif RGYW/RGY or its inverse compliment WRCY/RCY (Figure 1C). For the $Ig$ sequence, seven breakpoints were within RGYW motifs, two were within RGY motifs, and one was one bp away from the WRCY motif. For the $BCL6$ sequence, two breakpoints were within RGY motifs, two were within RCY motifs, two were within WRCY motifs and one was flanked by RCY and RGY motifs.

Similar phenomena also occurred in non-$Ig/BCL6$ translocation (Figure 1D). The breakpoint on $DMRT1$ was within a WRCY motif, the $GAPD$ breakpoint was one bp away from an RGYW motif, the $HSP89\alpha$ breakpoint was within an RCY motif, the $TFRC$ breakpoint was within an RGYW motif, and the Chr.3 sequence breakpoint was within an RGYW motif. For the $BCL6$ sequence, the breakpoint on the $GAPD/BCL6$ allele was within an RGY motif, the breakpoint on the $HSP89\alpha/BCL6$ allele was within a WRCY
motif, the breakpoint on the \( TFRC/BCL6 \) allele was within an RGY motif, and the breakpoint on the \( 28S\ rRNA/BCL6 \) allele was flanked by an RGY motif.

**High frequency of \( BCL6 \) somatic mutations is detected in the \( BCL6 \) sequence in \( Ig/BCL6 \) rearranged alleles in gastric lymphoma**

Figures 2B and 2C show \( BCL6 \) somatic mutations detected in \( Ig/BCL6 \) rearranged alleles (9 cases) and non-\( Ig/BCL6 \) rearranged alleles (4 cases), respectively. For each case with \( Ig/BCL6 \) translocation, significantly higher frequencies of \( BCL6 \) mutations were detected in the \( Ig/BCL6 \) rearranged allele when compared with the non-rearranged allele of the same case. However, for each case with non-\( Ig/BCL6 \) translocation, similar frequencies of \( BCL6 \) mutations were detected in both the non-\( Ig/BCL6 \) rearranged and the non-rearranged alleles. A much higher frequency of \( BCL6 \) somatic mutations were detected in the \( Ig/BCL6 \) rearranged alleles than in the non-\( Ig/BCL6 \) rearranged alleles, or the non-rearranged alleles.

**\( BCL6 \) deregulating mutations are identified in gastric lymphoma**

\( BCL6 \) deregulating mutations in the two \( BCL6 \) binding motifs (BSE1A and BSE1B) were analyzed in 43 cases of gastric lymphoma. Mutations at these sites prevented \( BCL6 \) from binding its own promoter, thus disrupting its negative autoregulation. The deregulating mutations were detected in 8/33 (24.2%) cases of DLBCL, with one case (#25) showing these mutations in both alleles (Figure 2D). One DLBCL case (#40) showed both deregulating mutation and \( BCL6 \) translocation. No \( BCL6 \) deregulating mutations were detected in MALT lymphoma and DLCLML cases.
Gastric lymphoma cases with BCL6 deregulating mutations have high BCL6 protein expression

The protein expression of BCL6 in 42 cases of gastric lymphoma was detected by immunohistochemistry using anti-BCL6 mAb. The results of immunostaining for all gastric lymphoma cases with non-Ig/BCL6 translocations, two examples of cases with Ig/BCL6 translocations, and one example of cases with BSE1 mutation in the BCL6 binding motif are shown in Figure 3A. A low percentage of lymphoma cells showed nuclear BCL6 expression in the case with 28S rRNA/BCL6 translocation. Heterogeneous levels of nuclear BCL6 expression was observed in the cases with non-Ig/BCL6 and Ig/BCL6 translocations, while all the cases with BCL6 deregulating mutations expressed a high level of nuclear BCL6 expression. Low BCL6 protein expression was observed in the 4 gastric MALT lymphoma cases.

The BCL6 protein expression levels were next correlated between the gastric DLBCL and DLCLML cases (1) with and without BCL6 translocations, (2) with Ig/BCL6 and non-Ig/BCL6 translocations, and (3) with and without BCL6 deregulating mutations. A two-tailed $\chi^2$-test was used for statistical comparison. The BCL6 expression levels of the cases detected with deregulating mutations were significantly higher than the cases without deregulating mutations ($p<0.05$), while no significant differences of BCL6 protein expression were found between the cases with other chromosomal alterations (Figure 3B).

Gastric lymphoma cases with high BCL6 protein expression have better prognosis

The correlation of BCL6 chromosomal alterations with OS was next assessed in 43 gastric lymphoma patients. The OS of BCL6-high DLBCL and DLCLML cases were
significantly better than that of the BCL6-low cases (median OS of BCL6-high and BCL6-low cases was 55 and 14 months, respectively; \( p<0.05 \)) (Table 2 and Figure 3C). However, the equality of the survival distributions for other categorical variables showed no significant correlation with the survival value of the gastric lymphoma patients (Table 2).

**BCL6 expression and genetic alterations in GCB and non-GCB subgroups of gastric DLBCL**

32 gastric DLBCL cases were subclassified into GCB or non-GCB subgroup according to Hans et al.\textsuperscript{12} The expression of CD10 was observed in 6/32 cases (18.8%), and MUM1 in 16/32 cases (50%). Overall, 32 DLBCL cases were comprised of 10 GCB (31.2%) and 22 non-GCB phenotypes (68.8%). All 6 cases detected with ongoing \( BCL6 \) SHM were found with the GCB phenotype. The OS of the GCB subtype was significantly better than that of the non-GCB subtype (\( p=0.034 \)) (Figure 3D). In the GCB subgroup, high BCL6 expression was found in all 10 cases, so no comparison could be made between the BCL6 expression level and \( BCL6 \) genetic alterations or OS. However, in the non-GCB subgroup, 9/22 cases (40.9%) had low BCL6 expression while 13/22 cases (59.1%) with high BCL6 expression. 10/13 of the cases (76.9%) with high BCL6 expression were found to have \( BCL6 \) translocations and/or deregulating mutations. All non-GCB cases with \( BCL6 \) deregulating mutations were found to have high BCL6 expression. Within the non-GCB subgroup, no significant correlation was found between OS and BCL6 expression level or genetic alterations.

**Discussion**
Most of the primary gastric lymphomas in Hong Kong Chinese are DLBCL while MALT lymphomas and DLCLML occur rarely. DLBCL is a heterogeneous group of tumours, varying in clinical features, immunophenotype, and cytogenetics. It is uncertain whether gastric DLBCL is pathogenetically different from its nodal counterpart. For example, BCL2 rearrangements occur in about 30% of nodal DLBCL, but are lacking in gastric DLBCL. A high incidence of c-myc (MYC) rearrangements is observed in gastric DLBCL, but involvement of MYC is rare in nodal DLBCL.

In this study 36.4% of gastric DLBCL were identified with BCL6 translocations, and more than half of these BCL6 translocations involved Ig as the translocation partner. The frequency and pattern of BCL6 translocations in gastric DLBCL is largely similar to those found in DLBCL. BCL6 translocations have been reported in about 30% to 40% of DLBCL and chromosomal translocations between BCL6 and Ig genes, especially involving IgH, are the commonest.

No BCL6 translocations were found in MALT lymphoma cases, but BCL6 translocations were identified in half of the gastric DLCLML cases. Two of these translocations involved IgH Sγ3 and IgH Eμ regions, suggesting that these IgH/BCL6 translocations occurred during isotype class switching, similar to gastric DLBCL. BCL6 rearrangements similar to these have been previously described in DLCLML. It is interesting that a proportion of IgM+ gastric MALT lymphoma undergoes aberrant isotype switch recombination. This ability might be directly involved in the progression of malignancy to DLCLML, i.e. the presence of many reactive lymphoid follicles in acquired MALT lymphoma may provide a presetting for genetic alterations of BCL6, resulting in high-grade transformation to DLCLML. However, these preliminary data on BCL6 chromosomal alterations in half of gastric DLCLML cases will need to be
substantiated in a larger series of DLCLML cases before implicating the speculation that BCL6 alterations may be involved in high-grade transformation of gastric MALT lymphoma.

Transgenic mouse models that mimic the common BCL6 translocation, t(3;14), provide evidence that BCL6 translocation may promote lymphomas or predispose the B cell for transformation into a tumour cell.\textsuperscript{50,51} The genes that we observed to rearrange with BCL6 display a broader pattern of expression. Transcriptional regulation of BCL6 by substituted promoters from rearranging genes may lead to its persistent expression beyond the GC stage (Figure 4). However, the BCL6 translocations involving the two novel partner genes, 28S rRNA and DMRT1, suggest total abrogation of BCL6 expression from the rearranged allele, since no translation mechanism would normally work on 28S rRNA, and DMRT1 is expressed specifically in testis.\textsuperscript{52} The unexpected observation of high BCL6 protein expression in the case with DMRT1/BCL6 translocation (Figure 3A), in which BCL6 fusion mRNA expression was not detected, can be explained by its expression from the non-rearranged allele in which mutation in BCL6 binding site BSE1B was detected. It has been widely observed that not all DLBCL tumours express BCL6;\textsuperscript{27,53,54} the detection of 28S rRNA/BCL6 translocation may provide clues to some of these DLBCL cases detected with no BCL6 expression.

Identification of a breakpoint within BCL6 intron 2 in 28S rRNA/BCL6 by 5’ RACE and a new breakpoint cluster\textsuperscript{55} (entitled alternative breakpoint region (ABR)) mapping from 245 to 285 kb 5’ to BCL6 by long-range PCR suggest that BCL6 translocation may be more frequent, as it could be underestimated using traditional detection methods. For identifying the cases with BCL6 gene rearrangement, Southern blot hybridization has been traditionally used on tumour DNA using BCL6 gene arrangement probes to detect
BCL6 translocations involving intron 1. More recently, interphase florescence in situ hybridisation (FISH) has been used on tissue sections using a dual color break apart BCL6 rearrangement probe.\textsuperscript{56} For the cloning of translocation partners of BCL6, LDI-PCR on genomic DNA has been used to amplify BCL6 translocations involving intron 1 and 5’ RACE on mRNA to detect the presence of BCL6 chimeric transcripts involving heterologous partners. The benefits of the LDI-PCR and 5’ RACE methods is that the cloning can be performed in the absence of cytogenetic data when only small amounts of archival tumor materials are available for the study. However, interphase FISH using the BCL6 split probe is recommended in identifying novel BCL6 translocations, in parallel with LDI-PCR and 5’ RACE. Interphase FISH can detect BCL6 translocations with novel breakpoints distant from the known breakpoint cluster,\textsuperscript{50} not usually targeted by LDI-PCR and 5’ RACE.

The frequency and pattern of SHM of BCL6 in DLBCL are largely similar to that of Ig. Ig SHM accumulates at a rate of $10^{-4}$ to $10^{-3}$ per bp and extend 1.5 kb-2kb downstream of the transcription initiation site, with preference for certain hotspots.\textsuperscript{57} Ig SHM favors transition over transversion and displays strand polarity, as inferred from G over C bias. It has been recently shown that SHM in Ig and BCL6 are driven by the same mechanism.\textsuperscript{58-60} BCL6 SHM identified in gastric DLBCL displays characteristics similar to those harbored by other B-cell neoplasms with GC origin;\textsuperscript{42} six cases of gastric DLBCL were detected with BCL6 ongoing somatic mutations which normally occur almost exclusively in the GC. BCL6 SHM were also identified in half of the gastric MALT lymphoma and DLCLML cases, thus suggesting GC origin of these gastric lymphomas.

The results of this study and those of previous reports shed light on the mechanisms
mediating BCL6 translocations. For the IgH/BCL6 translocations, as the breakpoints were clustered at $\gamma$3 and $\mu$ regions, the translocations might be the results of physiological error during Ig isotype class switching in the centrocytes of GC. On the other hand, BCL6 translocations involving non-Ig partners are likely to be also driven by a definite mechanism(s), according to the following observations: (1) Although BCL6 translocations involve heterologous promoters (Figure 5), the translocations are not random. BCL6 translocations involving $\alpha$-NAC, CIITA, GAPD, H4 histone, HSP89$\alpha$, IKAROS, IL-21R, L-plastin, PIM-1, TFRC, and TTF have been frequently detected. (2) Translocation breakpoints of the BCL6 non-Ig fusion partners are localized in a single exon or intron. For GAPD/BCL6 translocations, the breakpoint on GAPD identified in gastric lymphoma is 52 bp 5’ upstream the breakpoint detected in DLBCL of the central nervous system. For TFRC/BCL6 translocation, the breakpoint on TFRC identified in gastric lymphoma is 288 bp 3’ downstream of the breakpoint detected by Yoshida et al (1999). For HSP89$\alpha$/BCL6 translocations, the breakpoint on HSP89$\alpha$ identified in gastric DLCLML case is 109 bp and 258 bp 5’ upstream of the breakpoint detected in DLBCL by Akasaka et al (2000) and in DLBCL of the central nervous system by Montesinos-Rongen et al (2003). Similarly, BCL6 translocation breakpoints in IL-21R, L-Plastin and H4 histone were also localized in a single intron or exon. These findings suggest the BCL6 non-Ig fusion partners possess special structural feature(s) that make a region vulnerable to breakage. The most probable mechanism that drives the non-Ig/BCL6 translocations in gastric lymphoma may be a consequence of the aberrant SHM machinery targeting these non-Ig partner genes. This mechanism is suggested by the remarkable detection of single nucleotide substitutions, deletions and insertions in the HSP89$\alpha$, GAPD, and TFRC sequences at the breakpoints, and a 55-bp deletion of TFRC.
sequence at the TFRC/BCL6 genomic junction (results not shown). The deletions or insertions of variable numbers of nucleotides at the breakpoints are comparable with those observed in IgV. A similar type of mutation was also observed in the H4 sequence in four H4/BCL6 translocations in DLBCL. Moreover, SHM has been shown to act on PIM1, PAX5, TTF and PAX5 in DLBCL, and these four hypermutable genes are also susceptible to chromosomal translocations in the same region, consistent with a role for hypermutation in generating translocations by DNA double stand breaks. These observations suggest that the SHM machinery targets certain non-Ig genes in DLBCL, predisposing the mutated region to translocation with BCL6.

In the present study, the BCL6 translocation breakpoints were mainly located in the MTC region, and approximately half of them were found within the MMC region. Most of the breakpoints in these translocations were located within the SHM hotspot motif RGYW/RGY or its inversion, and there was no difference in the location of the BCL6 breakpoints between the Ig/BCL6 and non-Ig/BCL6 translocations. These findings support a hypothesis that SHM predisposes the mutated region to the subsequent development of translocation.

In this study, somatic mutations were detected at a much higher frequency in the BCL6 sequence adjacent to the translocation junctions in the Ig/BCL6 rearranged allele, compared to the corresponding non-rearranged alleles and to the non-Ig/BCL6 rearranged alleles. Similar phenomena were previously observed for MYC and BCL2 when translocated to Ig loci in lymphoma. In both cases, the mutations were attributed to the IgV gene SHM mechanism acting on sequences that had become linked to the Ig locus following chromosomal translocation. Therefore, it is likely that the same mechanism may also act on the BCL6 sequence of an Ig/BCL6 rearranged allele. It has
been shown experimentally that SHM is not Ig-specific, as non-Ig sequences are mutated when in the context of Ig with the Ig enhancer permitting mutation.68-70

The prognostic significance of BCL6 translocation and expression has been previously analyzed in different series of non-gastric DLBCL and the level of BCL6 expression was reported to depend on two main factors: the presence or absence of BCL6 rearrangement and the specific partners (Ig or non-Ig) involved in BCL6 translocations; these factors have been described as independent markers of favorable clinical outcome in DLBCL and predictive of survival.43-46 However, in this study on gastric lymphoma, we showed that neither BCL6 translocation status nor translocation partners correlated with either BCL6 protein expression or OS in gastric lymphoma. Similarly, in a case with t(3;16)(q27;p11), leading to the fusion of BCL6 with the IL-21R, the level of BCL6 mRNA was unexpectedly low.71 We found that the BCL6 deregulating mutations correlated with its high expression in gastric DLBCL. Although, we also found that high BCL6 expression predicted longer OS, independent of BCL6 translocation status, translocation partner, or BCL6 deregulating mutations, in gastric DLBCL and DLCLML. However, when gastric DLBCL cases were subclassified immunohistochemically into GCB and non-GCB subtypes;12 all cases in the GCB subgroup had high BCL6 expression independent of BCL6 alterations at the genetic level and had significantly better OS than the non-GCB subgroup. On the other hand, in non-GCB subgroup, heterogeneous BCL6 expression (low and high) was detected and BCL6 deregulating mutations correlated significantly with high BCL6 expression, but no significant difference in OS was found between the BCL6 expression level or genetic alterations within the subgroup, which had overall poorer prognosis. The results on the gastric DLBCL GCB/non-GCB comparison seemed to indicate that BCL6 expression level was not a significant determinant of OS
and that the apparent correlation was not causal but due to the association of GCB with high BCL6 expression. However, these results on the BCL6 expression level and genetic alterations and its correlation with prognosis will need to be substantiated in a larger series of cases of each subgroup (GCB and non-GCB) of gastric DLBCL.

Acknowledgement

We thank Kai Yau Wong for his expert technical advice throughout this study.
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Captions for figures

Figure 1. Detection of BCL6 translocations in gastric lymphoma. (A) Ethidium-bromide-stained gel showing BCL6 translocation products detected by various methods in all cases with non-Ig and examples of Ig as BCL6 fusion partner: i) 5' RACE on mRNA, ii) inverse-PCR and iii) long-range PCR on genomic DNA. The case number is shown above each lane. cDNA: purified control cDNA was amplified with control forward and reverse primers. dA: poly dA-tailed control cDNA was amplified with oligo-dT anchor primer and the control reverse primer. (B) Top panel: Restriction map of the BCL6 gene, and distribution of the different breakpoints of BCL6 translocations identified in this study. Small vertical lines below the horizontal line indicate the positions of different translocation breakpoints. Restriction sites: E, EcoRI; B, BamHI; H, HindIII; X, XbaI; S, SacI; Xh, Xhol. Lower panel: The magnified description of top panel showing the region with different translocation breakpoints. The fusion partners of each BCL6 translocations are indicated. (C) Nucleotide sequences of the chromosomal junctions and breakpoints of Ig/BCL6 translocations. The overlapping nucleotides of the junction sequences of BCL6 and the fusion partners are doubly underlined. Variable numbers of nucleotides of unknown origin inserted at the break points are shown in lowercase letters and are not underlined. The number within each bracket indicates the distance between the two nucleotides. (D) Schematic representation of the non-Ig/BCL6 tanslocation detected in gastric lymphoma. Closed and opened boxes represent exons from BCL6 and the fusion partners respectively. Vertical arrows indicate the chromosomal junctions and breakpoints of non-Ig/BCL6 fusion genes. The overlapping nucleotides of the junction sequences of BCL6 and the fusion partner genes are underlined. Opened-arrowheads indicate coding initiation start sites. For (A) to (D), the DLCLML cases are marked by ‘*’.

Figure 2. Mutational analysis of the BCL6 first intron and non-coding exon 1 in gastric lymphoma. (A) Distribution of somatic mutations in the BCL6 hypermutation region (nucleotides +358 to +1148 corresponding to the first transcription start site) covering MMC located at 5' of the first intron detected in 24 cases of gastric lymphoma.
The abscissa represents the position along the MMC region, starting from the first nucleotide of BCL6 non-coding exon 1. The ordinates correspond to the number of cases with somatic mutations at each position. Black bars indicate the number of cases with somatic mutations. Constitutional single-nucleotide polymorphisms (SNPs) were not counted. BCL6 translocation breakpoints within this region are indicated below the graph. Black circles indicate the translocation breakpoints overlapping with somatic mutations detected while opened circles indicate the translocation breakpoints not overlapping with somatic mutations detected. (B and C) Diagrammatic illustration showing frequency of BCL6 somatic mutations detected in Ig/BCL6 (B) and non-Ig/BCL6 (C) rearranged alleles and their corresponding non-rearranged allele in each case. Straight horizontal lines represent non-rearranged alleles while rectangle boxes represent the fusion partner genes. Small vertical bars represent the mutations detected. (D) BCL6 deregulating mutations in the two BCL6 binding motifs (BSE1A and BSE1B) located within the non-coding exon 1 of the gene detected in 8 cases of gastric lymphoma. The positions of the two BCL6 binding motifs are indicated above the case #1. The mutated nucleotides are underlined with the wild type sequences shown below. DLCLML cases are marked by ‘*’.

**Figure 3. BCL6 protein expression in gastric lymphoma.** (A) The results of immunostaining for five gastric lymphoma cases with non-Ig/BCL6 translocations, two representative examples of cases with Ig/BCL6 translocations, and one example of cases with BSE1 mutation in the BCL6 binding motif are shown. Normal lymphoid tissue (tonsil) was used as a control for BCL6 immunostaining. DLCLML case is marked by ‘*’. (B) The distribution of BCL6 protein expression in tumour cells of the 38 cases of gastric lymphoma. Two-tailed $\chi^2$-test was used for statistical comparison. DLBCL cases are shown by black circles and DLCLML cases by empty circles. (C) The Kaplan Meier curve showing BCL6 protein expression vs. overall survival (OS) of gastric DLBCL and DLCLML cases. (D) In gastric DLBCL, the Kaplan Meier curve showing the OS of the GCB subtype vs. the non-GCB subtype.

**Figure 4. Expression of BCL6 and its non-Ig translocation partner genes in a**
series of B-cell lines at different stages of differentiation by semi-quantitative RT-PCR. β-actin mRNA levels were used as control for the normalization of the amount of cDNA used for each sample.

Figure 5. Chromosomal locations of all the BCL6 translocation partners (annotated genes only) described in B-cell NHL. BCL6 fusion partner genes identified by us in gastric lymphoma are underlined.
Table 1. Features of somatic mutations in the BCL6 major mutation cluster (MMC) region (858 bp sequence at 5’ of BCL6 first intron was analyzed) in gastric lymphoma.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Frequency x 10⁻³/bp (range)</th>
</tr>
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<tbody>
<tr>
<td>Single bp substitutions</td>
<td>170</td>
</tr>
<tr>
<td>Deletions</td>
<td>12</td>
</tr>
<tr>
<td>Insertions</td>
<td>10</td>
</tr>
<tr>
<td>Transitions/transversions †</td>
<td>1.63</td>
</tr>
<tr>
<td>Strand polarity ‡, ±</td>
<td>1.57</td>
</tr>
<tr>
<td>RGYW/WRCY bias §</td>
<td>1.43</td>
</tr>
</tbody>
</table>

* The overall frequency of mutations in the BCL6 MMC region in gastric lymphoma is extrapolated from the frequency of mutated cases occurring in this group (24/43 cases; 55.8%).
† The frequency of substitutions affecting each base was corrected for the base composition of the region analyzed.
‡ Values designate the ratio of A → N vs. T → N substitutions.
§ Normalized frequency of mutated G bases occurring in the context of an RGYW (A/G G C/T A/T) and C bases in its inverse compliment WRCY (A/T A/G C C/T) motif.
**Table 2.** Log rank statistics to test the equality of the survival distributions for the different categorical variables in 39 cases of gastric DLBCL and DLCLML

<table>
<thead>
<tr>
<th>Variable Description</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Translocation (n=15) vs. no translocation cases (n=24)</td>
<td>0.3328</td>
</tr>
<tr>
<td>Ig/BCL6 (n=10) vs. non-Ig/BCL6 translocation cases (n=5)</td>
<td>0.3602</td>
</tr>
<tr>
<td>Deregulating mutations (n=8) vs. no deregulating mutations cases (n=31)</td>
<td>0.7839</td>
</tr>
<tr>
<td>Translocations/deregulating mutations (n=22) vs. no translocations/deregulating mutations cases (n=17)</td>
<td>0.3150</td>
</tr>
<tr>
<td>Ongoing BCL6 somatic mutations in MMC† [GCB]† (n=6) vs. acquired but not active ongoing BCL6 somatic mutations in MMC [post-GCB] cases (n=16)</td>
<td>0.7342</td>
</tr>
<tr>
<td>High BCL6 expression (n=26) vs. low BCL6 expression cases (n=12)*</td>
<td>0.0163</td>
</tr>
</tbody>
</table>

† MMC: Major mutation cluster located at 5’ of the first intron; GCB: Germinal centre B cell-like
* BCL6 protein expression was assessed by a 4-point system based on the proportion of positive cells with nuclear staining and was scored as low (− and +) or high (++ and +++). BCL6 expression could not be studied in one case due to the unavailability of paraffin block for the case.
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<table>
<thead>
<tr>
<th>MW</th>
<th>697</th>
<th>NALM-6</th>
<th>BJAB</th>
<th>Daudi</th>
<th>NAMALWA</th>
<th>Raji</th>
<th>Ramos</th>
<th>Sk-MM-2</th>
<th>U266</th>
</tr>
</thead>
</table>

**BCL6** (255 bp)

**28S rRNA** (298 bp)

**DMRT1** (464 bp)

**GAPD** (395 bp)

**HSP89α** (234 bp)

**TFRC** (221 bp)

**β–Actin** (497 bp)
High BCL6 expression predicts better prognosis, independent of BCL6 translocation status, translocation partner, or BCL6 deregulating mutations, in gastric lymphoma

Yun-Wen Chen, Xiao-Tong Hu, Anthony C Liang, Wing-Yan Au, Chi-Chiu So, Michelle L Wong, Lijun Shen, Qian Tao, Kent-Man Chu, Yok-Lam Kwong, Raymond H Liang and Gopesh Srivastava