PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Point mutation of 5′ noncoding region of *BCL-6* gene in primary gastric lymphomas

Da-Liu Min, Xiao-Yan Zhou, Wen-Tao Yang, Hong-Fen Lu, Tai-Ming Zhang, Ai-Hua Zhen, Pei-Zheng Cao, Da-Ren Shi

Da-Liu Min, Xiao-Yan Zhou, Wen-Tao Yang, Hong-Fen Lu, Tai-Ming Zhang, Ai-Hua Zhen, Pei-Zheng Cao, Da-Ren Shi, Laboratory of Molecular Pathology, Cancer Hospital of Fudan University, Shanghai 200032, China
Supported by National Natural Science Foundation of China, No. 39900065
Correspondence to: Dr. Xiao-Yan Zhou, Laboratory of Molecular Pathology, Cancer Hospital of Fudan University, Shanghai 200032, China. xyzhou100@yahoo.com
Telephone: +86-21-64175590-3646
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Abstract

AIM: To investigate the mutations of the 5′ noncoding region of *BCL-6* gene in Chinese patients with primary gastric lymphomas.

METHODS: PCR and direct DNA sequencing were used to identify BCL-6 gene mutations in the 5′ noncoding region in 29 cases of gastric diffuse large B-cell lymphoma (DLBCL) and 18 cases of gastric mucosa-associated lymphoid tissue (MALT) lymphoma as well as 10 cases of reactive hyperplasia of lymph node (LRH).

RESULTS: Six of 29 gastric DLBCLs (20.7%), 4 of 18 gastric MALT lymphomas (22.2%) and 1 of 10 LRHs(10%) were found to have mutations. All mutations were single-base substitutions and the frequency of single-base changes was 0.20×10^{-2} -1.02×10^{-2} per bp.

CONCLUSION: Point mutations in the 5′ noncoding region of *BCL-6* gene are found in Chinese patients with primary gastric DLBCLs and MALT lymphomas, suggesting that they may, in some extent, participate in the pathogenesis of primary gastric DLBCLs and MALT lymphomas.

Key words: Gastric lymphomas; BCL-6 gene; 5′ noncoding region; Point mutation

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INTRODUCTION

BCL-6 protooncogene, which is located at chromosome 3q27 encoding a POZ/zinc finger sequence-specific transcription repressor, is one of the three genes most commonly implicated in non Hodgkin's lymphoma (the other two are *BCL-2* and *c-myc* genes)[1-7]. Clonal *BCL-6* gene rearrangements are observed in 30% to 40% of nodal DLBCLs and 5% to 10% of nodal follicular lymphomas (FLs)[3,4]. These rearrangements are clustered within a highly conserved 4.0-kb regulatory region spanning the promoter, resulting in BCL-6 expression deregulation by a heterologous promoter from the partner chromosomes[8-10]. It is believed that the deregulation of *BCL-6* gene expression contributes to lymphomagenesis. Recent studies[11-14] also indicate that *BCL-6* gene may be alterd by somatic mutations clustered within the 5′ noncoding regions of this gene. These mutations have been found in cases displaying either normal or rearranged BCL-6 alleles, indicating their independence of chromosomal translocations. The sequences affected by these mutations are adjacent to the *BCL-6* promotor region and overlapped with MBR. The mutation frequency is more than 70% in nodal DLBCL, which is much higher than that of rearrangement, and the high frequency, tumor specificity and location in the proximity of BCL-6 regulatory regions of these mutations suggest that these genetic alterations may play a role in lymphomagenesis[15-21]. However, most of *BCL-6* mutations are focused on lymphomas originated from lymph node; lymphomas originated from extranodal site were less investigated. This study was aimed to investigate mutations of the 5′ noncoding region of *BCL-6* gene in Chinese patients with primary gastric lymphomas.

MATERIALS AND METHODS

Specimens

A total of 47 cases of paraffin-embedded primary gastric lymphomas, including 29 cases of DLBCL and 18 cases of MALT lymphoma were collected from the Department of Pathology, Cancer Hospital of Fudan University. In addition, 10 paraffin-embedded LRH specimens were included for control. Mean patient age was 56 years, male/female ratio was 1.2:1. In all instances, specimens were collected at diagnosis before specific therapy. Diagnosis was based on histopathological and immunophenotypic analysis of cell surface markers and immunogenotypic analysis of antigen receptor gene rearrangements. All lymphoma specimens were classified according to the new World Health Organization (WHO) classification of lymphoid neoplasms proposed in 1997[22]. The samples which were diagnosed before the advent of the new WHO lymphoma classification were reinvestigated after proper immunohistochemical studies to meet the criteria of the new classification.

Tissue microdissection and DNA extraction

Six μm thick sections from paraffin blocks were dewaxed in xylene, rinsed in ethanol, stained with hematoxylin and air-dried. The desired tumor areas were obtained by microdissection using scalpels under an upside-down light microscope. In most cases, the fraction of malignant cells was ≥85%. Genomic DNA was extracted from collected cells, which were subjected to lysis in 0.5-1.0 mL cell lysis buffer containing 100 mmol/L Tris-Cl pH8.5, 20 mmol/L EDTA, 20 mmol/L NaCl and 2.0% SDS, 0.5-2.0 mg/mL proteinase K and then to conventional phenol/chloroform extraction and ethanol precipitation.

DNA synthesis and polymerase chain reaction

Two PCR products encompassing fragments E1.11 and E1.12 and spanning 490 bp were amplified by primer 5'-AGG AAG GAG GGG AAT TAG-3' (sense), 5'-AAG CAG TTT GCA AGC GAG-3' (antisense) (for E1.11) and primer 5'-TTC TCG CTT GCA AAC TGC-3' (sense), 5'-CAC GAT ACT TCA TCT CAT C-3' (antisense) (for E1.12) respectively. The choice of these fragments was based on the fact that >95% of BCL-6 mutations detected in DLBCL were within these regions[11]. The first nucleotide of the amplified *BCL-6* gene region corresponding to the first nucleotide of the sense primer of E1.11 fragment was arbitrarily defined as position +1 (GenBank accession number AF191831). PCR was performed in a final volume of 25 μL containing 10 pmoL of each primer, 10 mmol/L Tris-Cl (pH8.5), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L of each dNTP, 1.5 units of Taq polymerase (Promega,USA) and 1 000 ng of genomic DNA. The PCR conditions were as follows: denaturation at 94 ℃ for 5 min, 35 cycles were performed, each consisting of denaturation at 94 ℃ for 1 min, annealing at 57 ℃ (for E1.11) or at 52 ℃ (for E1.12) for 30 s, extension at 72 ℃ for 45 s, followed by a final extension at 72 ℃ for 7 min. PCR was performed in a Perkin Elmer 9 700 GeneAmp PCR system. For each PCR, a control with no added template was used to check for contamination. The amplified fragments were checked using 2% agarose gel electrophoresis.

DNA purification and direct sequencing

PCR product of 20 μL was purified with a QIA quick spin column according to the manufacturer's instructions. The purified DNA fragments were directly sequenced on an ABI PRISM 310 DNA sequencer, using ABI PRISM big dye terminator kit as recommended by the manufacturer. Both the sense and antisense strands were sequenced and each fragment with suspected mutations was sequenced at least twice. Controls were also sequenced to ensure the fidelity and reliability of the sequencing results. Sequence was aligned with the BCL-6 germline sequence (GenBank accession number AF191831) by the Internet blast programme (http://www.ncbi.nih.gov/blast). The frequency of mutation was calculated by the detected length of gene fragment (490 bp) divided by the number of mutations.

RESULTS

Checking PCR products

The amplified E1.11 and E1.12 fragments of *BCL-6* gene were shown by 2% agarose gel electrophoresis (Figure 1).

DNA sequencing

Six of 29 gastric DLBCLs (20.7%), 4 of 18 gastric MALT lymphomas (22.2%) and 1 of 10 LRHs (10%) were found to have mutations (Table 1). All mutations were single-base substitutions and the frequency of single-base changes in the 5' noncoding region was as high as 0.20×10^{-2}/bp to 1.02×10^{-2}/bp, similar to those of IgV gene hypermutation during antigen-stimulated clonal selection[23,24]. Single-base changes in the *BCL-6* gene 5' noncoding region and some of the DNA sequencing results from the positive cases are shown in Table 2 and Figure 2.

Table 1 BCL-6 mutation in primary gastric lymphoma and LRH

Sample	Mutated cases (%)
DLBCL	6/29 (20.7)
MALTL	4/18 (22.2)
LRH	1/10 (10.0)

Table 2 Characteristics of changes in the 5' noncoding region of *BCL-6* gene in primary gastric lymphoma

Sample	Diagnosis	Substitution mutations
1	DLBCL	G→C (397), G→A (403)
2	DLBCL	G→C (397), C→T (419)
3	DLBCL	C→A (624)
4	DLBCL	T→A (346), G→A (391), G→A (402), G→A (694), G→A (669)
5	DLBCL	G→C (397)
6	DLBCL	G→A (322), T→C (444)
7	MALTL	G→T (321), G→C (397)
8	MALTL	C→A (665)
9	MALTL	G→C (397), C→G (419)
10	MALTL	C→T(123), G→C (397), T→C (484)
11	LRH	G→C (397)

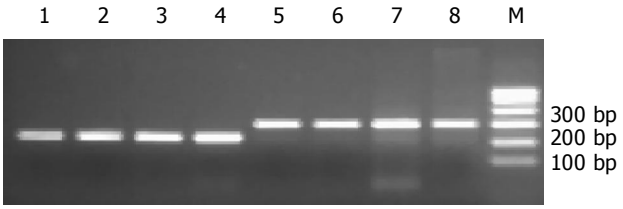

Figure 1 Amplified E1.11 (lanes 1-4) (215 bp) and E1.12 (lanes 5-8) (295 bp) fragments of *BCL-6* gene shown by 2% agarose gel electrophoresis.

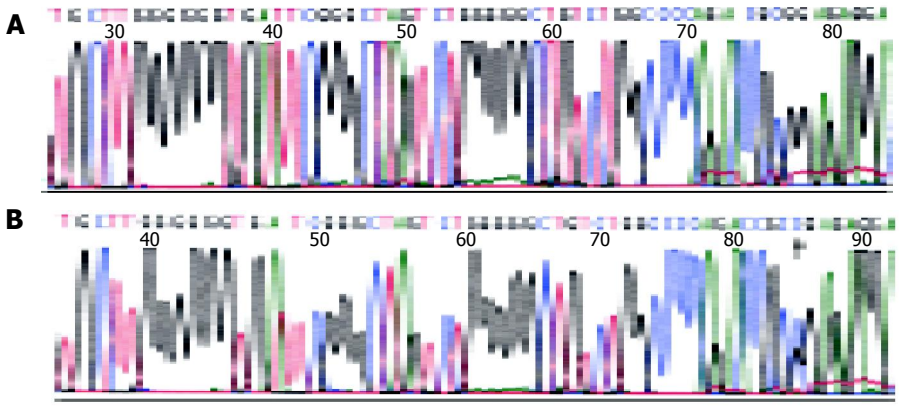

Figure 2 Representative results of direct DNA sequencing of PCR product E1.11 in primary gastric DLBCL. A: Wild-type *BCL-6* gene; B: Mutated-type *BCL-6* gene, arrow points to point mutation (G→C).

DISCUSSION

The commonest site of extranodal lymphomas is located in the gastrointestinal (GI) tract, particularly in the stomach[25-27]. It is uncertain whether primary lymphoma of the stomach is pathogenetically different from that of its nodal counterpart. This study was to analyze mutations in the 5' noncoding region of *BCL-6* gene in Chinese patients with primary gastric lymphoma.

BCL-6 gene was originally identified by virtue of its involvement in chromosomal translocations affecting band 3q27 in NHL[1,2]. *BCL-6* gene contains 10 exons and encodes for a 3.8-kb mRNA that is translated into a 706-amino acid nuclear phosphoprotein characterized by six Kruppel-type C-terminal zinc-finger motifs that have been shown to recognize specific DNA sequences. BCL-6 protein has been identified as a potent transcriptional repressor of promoters linked to its DNA target sequences, and is down-regulated during B cell differentiation to plasma cells. *BCL-6* gene has been shown to be a multifunctional gene, regulating important genes involved in B-cell differentiation (blimp-1, IP-10, and others) and cell-cycle control (such as c-myc, $p27^{KIP1}$, and cyclinD2)[20,28-36].

The 5' noncoding region of *BCL-6* gene contains regulatory elements for its expression. Ohashi *et al*[37] found that the 1.5-kb promoter region of BCL-6 was characterized by a TATA box and a number of potential regulatory elements such as CACCC, E-box and GATA-1 sites, which may be responsible for the low expression of the gene in normal lymphoid tissues and non-germinal-center derived lymphoid malignancies. Previous studies[11-15] suggest that *BCL-6* mutations might have functional significance, based on their frequency and clustering in the proximity of the *BCL-6* promoter. This is supported by *in vitro* studies showing that mutations might alter the transcriptional activity of *BCL-6*. In most tumor cases, mutations are somatic, multiple, often biallelic. And clusteres in the 5' regulatory sequences at frequencies of 7×10^{-4} to 1.6×10^{-2}/bp are comparable with those of *IgV* genes in B cells[23]. Hypermutations of the 5' noncoding region in *BCL-6* gene may cause disordered regulatory cascades of gene expression, thus leading to the destruction of germinal centers and the maintenance of immature B cell status, which could play a key role in the development of germinal center-derived DLBCL[38-40]. Gaidano *et al*[12] demonstrated that one single mutation of *BCL-6* gene 5' regulatory region was able to alter its transcriptional activity, suggesting a pivotal role in the tumorigenesis of germinal center-derived lymphomas[33-39]. *In vitro* studies aimed at transfecting normal B cells with mutated BCL-6 alleles can clarify the precise pathogenetic implications of these mutations.

BCL-6 mutations represent a marker of germinal center (GC) or post-GC cells because in normal lymphoid tissues, they occur in approximately 30 -50% of GC and memory B cells, whereas they are absent in pre-GC and virgin B cells[12-16]. Thus, *BCL-6* mutations are proposed as a genetic marker for defining the histogenesis of B-cell lymphoproliferations, and accumulation of BCL-6 mutations might result from ectopic activity of the *IgV* gene hypermutation mechanism involving sequences displaying no homology with antigen receptor loci[23,24].

It has been reported that the 5' non-coding region point mutation of *BCL-6* gene occur in 73% nodal DLBCLs and 45% FLs in Western populations[11]. But in this study, we found that the mutational incidence of the 5' noncoding region of *BCL-6* gene was 20.7% in Chinese patients with primary gastric DLBCLs which is significantly lower than that in Western populations. This result is in accordance with our previous reports[41] in nodal DLBCL which showed that the mutational incidence of the 5' noncoding region of *BCL-6* gene was 18.4%. The differences of mutational incidence between our study and Western reports in DLBCL might be related to the differences in the screened regions, the distinct aspects of races and social-economic environments and even the different molecular pathologenesis of DLBCL[42].

Gastric MALT lymphoma is of B-cell origin and has a very strong association with *Helicobacter pylori* infection[43-45]. It has been found that eradication of the infection with antibiotics may lead to regression of gastric MALT lymphoma. In gastric MALT lymphoma, the results of investigations on BCL-6 mutations are variable; BCL-6 mutations were found in 2 out of 4 cases in Liang's study[26], but mutations were absent in all 16 cases tested in Gaidano's study[27]. In this study, mutations of the 5' noncoding region of *BCL-6* gene were detected in 4 of 18 gastric MALT lymphomas (22.2%). Because MALT lymphoma has been traditionally viewed as proliferation of marginal region cells, the occurrence of BCL-6 mutations in a fraction of MALT lymphomas suggests that the histogenesis of MALT lymphoma might be more heterogenerous than previously thought[46-50]. This is consistent with the hypothesis that the fraction of MALT lymphoma with BCL-6 somatic mutation might be derived from germinal center-related B cells. Kwon *et al*[42] investigated that tissues obtained from the marginal zone of Peyer's patch by microdissection revealed no *BCL-6* mutations by PCR-SSCP analysis, whereas tissues from gastric MALT lymphomas were shown to have *BCL-6* mutations in 11 of 13 (86.4%). They believe that the acquisition process of *BCL-6* mutations by marginal zone cells might be involved in the lymphomagenesis of gastric MALT lymphoma.

Lossos *et al*[19] demonstrated that mutations occurred in the 5' regulatory region of *BCL-6* gene were ongoing. It is possible that as a result of ongoing *BCL-6* gene somatic mutations, lymphoma cells become heterogeneous, and a mutational variant having a selective growth advantage because of BCL-6 overexpression gives rise to the higher-grade of NHL lymphoma cells.

In the present study, seven recurrent mutations (G→C) were identified at position 397, suggesting that this position may be a mutational hot spot. Several other studies[13,20,41] also reported mutations at this position, but Lossos *et al*[13] thought that the mutation at position 397 might be a polymorphism, because it was also observed in two samples of T cells from patients. Further studies are needed to determine whether the position 397 (G→C) is a real mutation or just a polymorphism.

In conclusion, point mutations of the 5' noncoding region of *BCL-6* gene suggest that they may, in some extent, participate in the pathogenesis of primary gastric DLBCLs and MALT lymphomas.

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