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Frequent mutation of bcl-6 proto-oncogene in high grade, but not low grade, MALT lymphomas of the gastrointestinal tract

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ABSTRACT

Background and Objective. **Knowledge regarding the molecular pathogenesis and histogenesis of gastrointestinal mucosa-associated lymphoid tissue non-Hodgkin's lymphomas (MALT-NHL) is limited. Mutations of *BCL-6*, a zinc finger transcription factor implicated in lymphoid development, occur frequently in lymphomas and represent a histogenetic marker of B-cell transit through the germinal center. The distribution of *BCL-6* mutations in gastrointestinal MALT-NHL was analyzed in this study.**

Design and Methods. **This study was based on 26 gastrointestinal MALT-NHL, including 16 cases of low grade histology and 10 cases of high grade histology. Mutations of *BCL-6* were investigated by a combination of polymerase chain reaction-single strand conformation polymorphism and DNA direct sequencing analysis.**

Results. **Mutations of *BCL-6* occurred in 6/10 high grade MALT-NHL, whereas they were absent from all low grade cases tested (n = 16; $p = 0.001$). MALT-NHL harboring *BCL-6* mutations included 5 cases of gastric MALT-NHL and 1 case of jejunal MALT-NHL. Mutations were predominantly represented by single nucleotide substitutions which were multiple in most cases. All sequence alterations were unique to individual cases of gastrointestinal MALT-NHL.**

Interpretation and Conclusions. **Mutations of *BCL-6* occur frequently in high grade gastrointestinal MALT-NHL and display characteristics similar to those of *BCL-6* mutations harbored by other B-cell lymphomas. The association of high grade MALT-NHL with *BCL-6* mutations corroborates their histogenetic derivation from germinal center-related B-cells and may be of potential pathogenetic relevance for these disorders.**

Keywords: mucosa associated lymphoid tissue, lymphoma, BCL-6, proto-oncogene, mutation

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Lymphomas of mucosa-associated lymphoid tissue (MALT) represent a distinct category of B-cell non-Hodgkin's lymphomas (NHL) arising in extranodal sites. The gastrointestinal tract, particularly the stomach, is the preferential site of origin of MALT-derived non-Hodgkin's lymphomas (MALT-NHL), which account for most lymphoproliferative disorders targeting primarily the alimentary tract.[1-6] Gastrointestinal MALT-NHL consistently display a B-cell phenotype and can be histologically classified into two groups: low grade and high grade.[1-7] Low grade MALT-NHL are constituted by centrocyte-like small cells and are characterized by the presence of lymphoepithelial lesions.[1-7] Low grade MALT-NHL may undergo histologic transformation to a high grade pattern with variable proportions of residual foci of small cells mingled with the large cell population.[1-7]

The pathogenesis of B-cell NHL involves host factors as well as genetic alterations intrinsic to the tumor clone.[8,9] Host factors implicated in the pathogenesis of gastrointestinal MALT-NHL include infection by *Helicobacter pylori* and stimulation and selection by antigen.[10-16] Knowledge of the molecular alterations of cancer related genes implicated in gastrointestinal MALT-NHL is limited. A fraction of high grade cases associates with inactivation of the p53 and p16 tumor suppressor genes, whereas both low grade and high grade MALT-NHL are generally devoid of other molecular lesions associated with systemic B-cell NHL.[8,17-21]

Recently, it has been shown that molecular alterations of BCL-6, a proto-oncogene coding for a zinc finger transcription factor implicated in lymphoid organ development, are frequent genetic alterations of B-cell NHL that are histogenetically related to germinal center (GC) B-cells.[22-29] Because gastrointestinal MALT-NHL have been proposed to be related to B-cells which have transited through the GC,[14-16] it is possible that BCL-6 alterations may also be involved in this category of lymphomas. Two types of molecular alterations of BCL-6 are known in systemic B-cell NHL, namely chromosomal translocations and mutations.[22-26,28] Chromosomal translocations cluster with

B-lineage diffuse large cell lymphoma throughout the pathologic spectrum of B-cell NHL.[22-24,30] Mutations affect the 5' non-coding sequences of BCL-6 located in the proximity of the BCL-6 promoter and are generally regarded as a B-cell histogenetic marker denoting transit through the GC.[25,26,28,31,32] This notion is supported by the association of BCL-6 mutations with B-cells displaying a GC or post-GC phenotype, such as centroblasts and memory B-cells, whereas mutations are absent in pre-GC B-cells such as virgin B-cells.[25,26,28,31,32]

This study was aimed at investigating the distribution of BCL-6 mutations throughout the clinicopathologic spectrum of gastrointestinal MALT-NHL. We report that mutations of BCL-6 are a common genetic lesion among high grade gastrointestinal MALT-NHL, whereas are consistently absent in cases with a low grade histology.

Design and Methods

Tumor samples and DNA extraction

Biopsy samples of involved organs from 26 patients with MALT-NHL of the gastrointestinal tract were collected during the course of standard diagnostic procedures. All bioptic specimens were collected at diagnosis, prior to therapy for the lymphoproliferative disease. Diagnosis was based on analysis of histopathology, immunophenotypic analysis of cell surface markers, and immunogenotypic analysis of immunoglobulin gene rearrangements, as previously reported.[20] Low grade MALT-NHL were pathologically defined based on previously reported morphologic criteria.[7] High grade MALT-NHL were pathologically defined based on the size of the predominant cell population and the presence of a diffuse architecture.[7,20] Notably, with respect to MALT-NHL cases classified as high grade, our study included only samples in which one or more foci of a low grade MALT-NHL component could be specifically identified. On these grounds, sixteen cases were classified as low grade MALT-NHL and 10 cases as high grade MALT-NHL. The site of the MALT-NHL was the stomach in 23 cases, the jejunum in 2 cases and the colon in 1 case. In most samples, the fraction of the malignant cells in the pathologic specimen was greater than 50 % (and in all cases greater than 30 %), as determined by tissue section immunohistochemical analysis of cell surface markers and by immunoglobulin gene rearrangement analysis. DNA was purified by digestion with proteinase K, *salting out* extraction, and precipitation by ethanol.[33]

Oligonucleotides

All the oligonucleotides used in this study were synthesized by the solid phase triester method. The sequence of oligonucleotides used as primers for the mutational analysis of BCL-6 5' non-coding regions (PCR fragments E1.10, E1.11 and E1.12) was as follows: E1.21B, 5'-CTCTTGCCAAATGCTTTG-3', and E1.24, 5'-TAATTCCCCTCCTTCCTC-3' (for fragment E1.10); E1.23, 5'-AGGAAGGAGGGGAATTAG-3', and IP1.6, 5'-AAGCAGTTTGCAAGCGAG-3' (for fragment E1.11); IP1.7, 5'-TTCTCGCTTGCAAACTGC-3', and E1.26, 5'-CACGATACTTCATCTCATC-3' (for fragment E1.12).[25]

Mutational analysis of BCL-6 5' non-coding regions

Mutations of BCL-6 5' non-coding regions were assessed by two independent methods, namely polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and PCR DNA direct sequencing. PCR-SSCP analysis of BCL-6 5' non-coding regions was performed on three partially overlapping PCR fragments (E1.10, E1.11, E1.12) corresponding to the 739 bp region amplified by primers E1.21B and E1.26. This 739 bp fragment is located downstream of the first BCL-6 non-coding exon and has been shown to harbor > 95 % of BCL-6 5' mutations detected in B-cell NHL.[25,26,28] PCR-SSCP was performed as previously reported.[25,28] Based on the results of reconstruction experiments, the sensitivity of the PCR-SSCP assay in our laboratory allows the detection of a monoallelic mutation harbored by 5-10% of cells in a mixed cell population.

For DNA direct sequencing of BCL-6 5' non-coding regions, a unique PCR product encompassing fragments E1.10, E1.11 and E1.12 and spanning 739 bp (nucleotides +404 to +1142) was amplified by primers E1.21B and E1.26, as previously described.[28] The DNA PCR product was purified by using a commercially available kit (QIAquick gel extraction kit, Qiagen, CA). Subsequently, DNA direct sequencing was performed with either external (E1.21B and E1.26) or internal (E1.23, E1.24, IP1.6, IP1.7) primers as appropriate, using a commercially available kit (Thermosequenase, Amersham Life Sciences, UK). [α-^{33}P]-labeled terminator dideoxynucleotides (purchased from Amersham Life Sciences) were included in the sequencing mixture. For each DNA fragment analyzed, sequencing of both strands was performed on independent PCR reactions.

Analysis of BCL-6 rearrangements

The gross configuration of the BCL-6 locus was investigated by Southern blot analysis.[20,34] Six to ten µg of genomic DNA were digested with the appropriate restriction enzyme, electrophoresed in a 0.8-1 % agarose gel, denatured, neutralized, transferred to Hybond C$^+$ filters (Amersham, Amersham Place, U.K.), and hybridized to probes which had been ^{32}P-labeled by the random priming extension method.[35] Filters were washed in 0.2x SSC (NaCl/Na citrate/ 0.5% sodium dodecyl sulfate) for 2 hours at 60°C and then autoradiographed using intensifying screens. Southern blot analysis was performed using a probe (Sac 4.0) and restriction enzymes (*Bam*HI and *Xba*I) that, in combination, explore a region of 15.2 Kb containing the 5' portion of the BCL-6

gene.[22] This same region was previously shown to contain the cluster of chromosomal breakpoints detected in NHL.[22,24] Cases showing an abnormally migrating band in only one digest were further studied by hybridizing *Bam*HI and *Xba*I digests to a second probe (Sac0.8) derived from the BCL-6 first intron, which, being located 3' of the breakpoint cluster, explores the reciprocal chromosome 3.[36] Only cases showing abnormally migrating bands with two restriction enzymes and/or two probes were scored as rearranged.

Analysis of BCL-6 protein expression

The BCL-6 protein was detected by using the PG-B6 monoclonal antibody that is directed against the aminoterminal portion of the human BCL-6 gene product.[37] Immunostaining for BCL-6 was performed on frozen sections by the APAAP method, as previously described.[38]

Statistical methods

Differences in the distribution of BCL-6 mutations throughout the clinico-pathologic spectrum of gastrointestinal MALT-NHL were assessed by means of Fisher's exact test.

Results

A panel of 26 cases of MALT-NHL of the gastrointestinal tract was included in this study. Cases were representative of the clinico-pathologic spectrum of the disease, including low grade MALT-NHL (n = 16) and high grade MALT-NHL (n = 10). With respect to MALT-NHL cases classified as high grade, our study included only samples in which one or more foci of a low grade MALT-NHL component could be specifically identified. All cases of MALT-NHL displayed a major monoclonal B-cell population based on immunogenotypic analysis and/or light chain restriction immunohistochemical studies (data not shown).

Distribution of BCL-6 mutations in MALT-NHL of the gastrointestinal tract

All 26 samples of MALT-NHL were subjected to PCR-SSCP analysis of 3 partially overlapping PCR fragments (fragments E1.10, E1.11, E1.12) encompassing a 739 bp region of the BCL-6 gene. The selection of these three PCR fragments for the mutational analysis of BCL-6 in MALT-NHL of the gastrointestinal tract was based on evidence derived from systemic B-cell NHL showing that these sequences are consistently mutated in all cases carrying mutations of the BCL-6 gene.[25,26,28]

Cases of gastrointestinal MALT-NHL were scored positive for mutation when one or more PCR-SSCP fragments displayed a variant pattern which could not be attributed to a population polymorphism (see Figure 1 for representative results). Mutations of 5' non-coding regions of BCL-6 were absent in all low grade gastrointestinal MALT-NHL tested (n = 16). Conversely, mutations of 5' non-coding regions of BCL-6 were detected in 6/10 (60%) high grade MALT-NHL, including 5 cases of MALT-NHL of the stomach and 1 case of MALT-NHL of the jejunum (Table 1). The difference in the frequency of BCL-6 mutations in low grade versus high grade gastrointestinal MALT-NHL was statistically significant (p = 0.001; Fisher exact test).

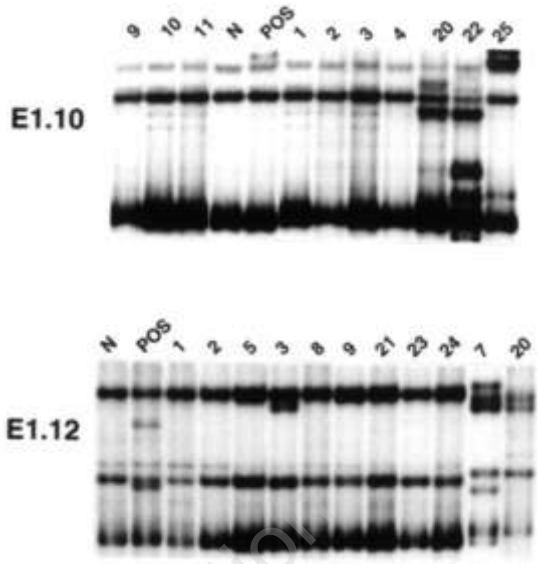

Figure 1. PCR-SSCP analysis of mutations of the 5' non-coding regions of BCL-6 in gastrointestinal MALT-NHL. Representative results obtained for PCR products E1.10 (upper panel) and E1.12 (lower panel) are shown. Samples of gastrointestinal MALT-NHL are indicated at the top of each lane by a numbered code. A positive control (POS), represented by a tumor sample known to harbor BCL-6 5' mutations, as well as a normal (N) sample, represented by a lymphoblastoid cell line, are also included for each PCR-SSCP fragment shown. Samples were scored positive when their migration pattern differed from the normal control (N) and the migrations abnormalities could not be ascribed to population polymorphisms. Among the gastrointestinal MALT-NHL samples shown, cases scored as positive included cases 20, 22, 25 (for PCR product E1.10) and cases 3, 7 and 22 (for PCR product E1.12).

Table 1. Frequency of molecular alterations of the BCL-6 proto-oncogene among gastrointestinal MALT-NHL.

Histology	*BCL-6 mutations**	*BCL-6 rearrangements**
Low grade	0/16	0/12
High grade	6/10°	1/10

**Positive/tested; °excess of mutations in high grade gastrointestinal MALT-NHL, as compared to low grade gastrointestinal MALT-NHL, was statistically significant (Fisher's exact test, p = 0.001).*

Sequence analysis of BCL-6 mutations in MALT-NHL of the gastrointestinal tract

To confirm and characterize the mutations affecting the BCL-6 gene in gastrointestinal MALT-NHL, we performed a sequence analysis of all cases scored positive by the PCR-SSCP assay. In all cases tested, the sequence analysis involved all the fragments displaying an abnormally variant pattern by PCR-SSCP. For each tumor case studied, all abnormal PCR-SSCP fragments were found to contain ≥ 1 mutation upon DNA sequencing.

The characteristics of the mutations detected in gastrointestinal MALT-NHL are reported in Table 2. Representative examples of mutations are shown in Figure 2. A total of 14 alterations was scored in the tumor panel studied. All sequence alterations were unique to individual cases of gastrointestinal MALT-NHL. The mutations observed included single base-pair substitutions (n = 13) and insertions (n = 1). All mutations detected in gastrointestinal MALT-NHL occurred in the presence of the germline sequence of the BCL-6 gene. It could not, however, be determined whether mutations were truly heterozygous or whether the germline sequence was contributed by reactive normal cells which frequently contaminate the tissue biopsies of these lymphomas.

Relationship between BCL-6 mutations and rearrangements

The presence of rearrangements of BCL-6 and their relationship to mutations in the 5' non-coding regions of the gene were investigated further by Southern blot analysis of selected cases for which sufficient DNA was available (n = 22). With one exception, all cases of gastrointestinal MALT-NHL tested were scored negative for rearrangements of BCL-6 (Table 1). The only case which scored positive for BCL-6 rearrangement was a high grade MALT-NHL of the stomach which also harbored mutations of BCL-6 5' non-coding regions (not shown). Overall, these data indicate that BCL-6 mutations in gastrointestinal MALT-NHL can occur independently of the concomitant presence of BCL-6 rearrangements.

Expression of BCL-6 protein

Expression of the BCL-6 protein was analyzed in selected MALT-NHL (n = 8). Expression of BCL-6 scored positive in 3/5 high grade MALT-NHL, whereas it was negative in all low grade cases tested (n = 3) (data not shown). In positive cases, 60-80% of cells expressed the BCL-6 protein. High grade MALT-NHL expressing the BCL-6 protein harbored BCL-6 mutations, whereas cases scored negative for BCL-6 expression were devoid of BCL-6 mutations.

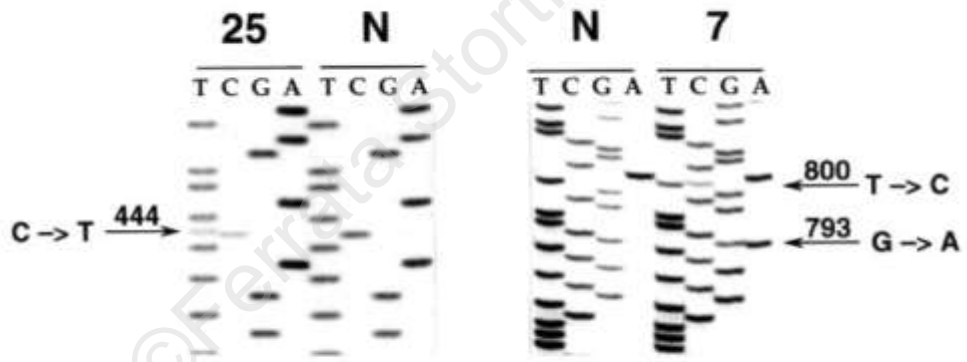

Figure 2. Nucleotide sequencing analysis of mutations in the 5' non-coding regions of BCL-6 in representative cases of gastrointestinal MALT-NHL (cases 25 and 7). The sequence of each case is matched to the sequence of a normal control (N) displaying germline BCL-6 alleles. The position of mutations is indicated by the nucleotide number of the corresponding BCL-6 germline sequence (the first nucleotide of the BCL-6 cDNA was arbitrarily chosen as position +1).

Table 2. Characteristics of mutations of BCL-6 5' non-coding regions in gastrointestinal MALT-NHL.

Sample	*Histology*	*Site*	*Nucleotide substitution(s)**	*Mutation status*
3	high grade	stomach	C→A (967), T→C (1099)	heterozygous
4	high grade	stomach	G→A (684), G→A (733)	heterozygous
7	high grade	jejunum	G→A (793), T→C (800), C→G (871)	heterozygous
20	high grade	stomach	+C (458), A→G (555), G→C (874)	heterozygous
22	high grade	stomach	T→G (542), T→C (554), G→T (651)	heterozygous
25	high grade	stomach	C→T (444)	heterozygous

**The position of the mutated nucleotide is indicated in brackets (the first nucleotide of the BCL-6 cDNA was arbitrarily chosen as position +1); +, insertion.*

Discussion

This study reports that mutations of the 5' non-coding regions of the BCL-6 proto-oncogene are frequent molecular lesions associated with gastrointestinal MALT-NHL. Mutations of BCL-6 are not randomly distributed throughout the clinico-pathologic spectrum of the disease, but rather occur frequently in gastrointestinal MALT-NHL of high grade histology, whereas they are absent in low grade cases. These observations may be of potential relevance for the biology of these lymphomas.

The putative pathogenicity of BCL-6 mutations in gastrointestinal MALT-NHL displaying a high grade histology is suggested by several observations. First, the frequency of BCL-6 mutations detected in high grade gastrointestinal MALT-NHL is superimposable to that of systemic B-lineage diffuse large cell lymphoma, which is the B-cell NHL type known to harbor the highest frequency of these genetic alterations.[25] Second, mutations of BCL-6 affect sequences of the gene that are located in the proximity of the BCL-6 promoter and that contain regulatory elements presumably implicated in the control of BCL-6 expression, suggesting that mutations may perturb the normal pattern of BCL-6 expression.[25] This notion is supported by transient transfection experiments in normal B-cells showing that mutated BCL-6 alleles may deregulate BCL-6 expression.[39] The recent observation that BCL-6 mutations may be detectable in normal B-cells does not contradict the potential pathogenicity of these mutations.[31,32] In fact, it is possible that B-cells harboring specific BCL-6 mutations may be rendered prone to transformation because of the pathogenicity of mutations.

The detection of BCL-6 mutations has histogenetic implications for gastrointestinal MALT-NHL, since these mutations are regarded as a genetic marker indicating GC transit of a given B-cell.[25,31,32] Although MALT-NHL share many phenotypic similarities with marginal zone B cells, it has been proposed that these lymphomas may be histogenetically related to a B-cell subset which has undergone a GC-like reaction.[14-16] This notion is substantiated by the association of both low and high grade MALT-NHL with mutations of immunoglobulin hypervariable (IgV) genes, classical markers of the GC reaction.[14-16] On these bases, the occurrence of BCL-6 mutations in high grade gastrointestinal MALT-NHL corroborates the hypothesis that these tumors are histogenetically related to a B-cell which has undergone a GC-like reaction.

Low grade MALT-NHL are devoid of BCL-6 mutations but score positive for mutations of IgV genes (this study and refs. #14-16). This finding is apparently unexpected, because of the proposed identity between the mutational mechanisms of BCL-6 and the mutational mechanism of IgV genes.[31,32] Several hypothesis may be envisioned to explain the absence of BCL-6 mutations in low grade gastrointestinal MALT-NHL. First, the mutational process of BCL-6 may be hampered by the lack of BCL-6 expression in low grade MALT-NHL (ref. #40 and this study). In fact, by analogy with the IgV hypermutation mechanism, it is assumed that the mutational process of BCL-6 requires expression of the target gene.[31,32] Alternatively, the molecular machinery introducing mutations in the BCL-6 and IgV genes may operate with different efficiency in low and high grade MALT-NHL. Because the mutational process appears to mutate BCL-6 with a lower efficiency than IgV genes,[31,32] it is possible that, in low grade MALT NHL, the efficiency of the mutational mechanism is sufficient for mutation of IgV genes, but not of BCL-6.

At present, the precise stage at which gastrointestinal MALT-NHL accumulate BCL-6 mutations cannot be defined. Since high grade MALT-NHL frequently derive from low grade lymphomas,[7] it is possible that BCL-6 mutations are acquired at the time of histologic transformation. This hypothesis is consistent with the notion that histologic transformation from low grade to high grade gastrointestinal MALT-NHL is also accompanied by the accumulation of novel mutations in the IgV genes.[16] Alternatively, it is possible that BCL-6 mutations may already occur as a subclonal event in those low grade MALT-NHL which will eventually undergo histologic transformation. The subclonal nature of the mutation would prevent its detection by conventional methods of mutational analysis, such as those employed in the current study.

Finally, the frequency and specificity of BCL-6 mutations among high grade gastrointestinal MALT-NHL suggest that these genetic lesions may prove useful as clonal markers for monitoring minimal residual disease in the clinical course of these lymphomas.

Contributions and Acknowledgments

GG, GS and AC conceived and designed the study and wrote the manuscript. DC, AG, LF, DV, AM designed the experimental strategy, performed the molecular (DC, LF, DV) and immunohistochemical (AG) analyses and interpreted the results together with the other authors. CA took part in the assessment and selection of patients.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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