Lack of BCL10 Mutations in Germ Cell Tumors and B Cell Lymphomas

The BCL10 gene has recently been cloned from the chromosomal translocation t(1;14)(p22;q32) in a low-grade mucosa-associated lymphoid tissue (MALT) lymphoma, and was implicated in the pathogenesis of this and several other tumor types (Willis et al., 1999). BCL10 is a cellular homolog of the equine herpesvirus-2 E10 gene, which contains an amino-terminal caspase recruitment domain (CARD) and plays a role in apoptosis. Willis et al. (1999) also showed that BCL10 exhibits hypermutations in MALT lymphomas with t(1;14) as well as frequent mutations in 45% of B and T cell lineage lymphomas without the 1p22 chromosomal rearrangements. In addition, they reported BCL10 mutations in cell lines derived from several solid tumor types including three each of male germ cell tumors (GCTs) and mesotheliomas, suggesting that it may be commonly involved in the pathogenesis of many human malignancies. The 1p22 region is affected by frequent deletions in male GCTs (Mathew et al., 1994) and mesotheliomas (Lee et al., 1996), suggesting that inactivation of a critical gene in this region may play a role in the genesis of these tumors.

In order to validate a possible role for BCL10 in male GCT and lymphoma pathogenesis, we performed mutation analysis of its coding region in a panel of 59 GCTs (41 primary tumors and 18 cell lines) (Murty et al., 1994), 15 MALT lymphomas, and 15 follicular lymphomas (Offit et al., 1993). The coding region of BCL10 was amplified as described (Willis et al., 1999) and SSCP analysis was performed by standard methods (Orita et al., 1989). We reasoned that if BCL10 is the target gene deleted, it should exhibit mutations in cases with loss of heterozygosity (LOH), and we included 11 such tumors in the panel of 41 primary GCTs (Mathew et al., 1994). Among the cell lines studied, we also included the Tera-1, Tera-2, and GCT-44 cell lines studied by Willis et al. (1999). Conformational changes were noted with high frequency in both GCTs and lymphomas in exon 1 and in 11 cases in exon 3 (5 GCTs and 6 lymphomas) (Table 1). No variants were found in either tumor in exon 2. All SSCP variants in exon 3 in both tumors were identical while exon 1 variants exhibited a complex pattern (Figure 1A). In order to determine if the SSCP variants represent somatic mutations or genetic polymorphisms, we analyzed paired normal-tumor DNAs by SSCP in 14 of 15 exon 1 variants and all 5 exon 3 variants in GCTs. We found identical SSCP variants in all cases, suggesting that these represent genetic polymorphisms.

To identify the nature of alterations associated with conformation variations, we determined the sequences of 9 exon 3 (paired normal-tumor DNAs in 5 GCTs and 4 lymphomas) variants and 12 exon 1 variants (paired normal-tumor DNAs in 7 GCTs and 4 lymphomas) (Table 1). We found identical sequences in both normal and tumor DNAs in each case. Although we did not find SSCP variants in the 3 GCT cell lines (Tera-1, Tera-2, and GCT-44), we sequenced the exons and found no sequence alterations (Figure 1B). This analysis also identified the sequence variation reflected in SSCP variants. The region amplified for exon 1 exhibited 3 different polymorphisms, 2 in the coding region and one in 5' intron 1. These included G/T in the first base of codon 5, G/C at the third base of codon 8, and a C/G polymorphism in intron 1. The polymorphism in exon 3 was G/A at second base of codon 213. Consistent with the reported high frequency of LOH at the 1p22 region in GCT, the tumor and the cell line from GCT-240A were reduced to homozygosity at all polymorphic loci in exon 1-intron 1 compared to germline heterozygosity (Figure 1B). Thus, these data are at variance with the results reported by Willis et al. (1999). The changes noted by them, mostly truncations or missense mutations supported a tumor suppressor gene (TSG) role for BCL10. Our data contradict this presumption in GCTs and B cell lymphomas, and provide evidence for polymorphisms in BCL10 coding region.

Lack of mutation in the BCL10 coding region may not exclude it as a deleted TSG. Other genetic or epigenetic mechanisms such as alterations in promoter or methylation patterns can silence gene expression (Baylin et al., 1998; Forget, 1998). In order to examine this possibility, we employed a multiplex RT-PCR strategy to analyze BCL10 expression. This analysis revealed detectable levels of expression in 14 of 16 GCTs (10 cell lines and 6 primary tumors) examined. BCL10 message could not be detected in the remaining 2 cell lines, 577M-F and 577M-G.

Table 1. Analysis of BCL10 Sequence Alterations in GCT and NHL

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>No. Studied</th>
<th>SSCP Variants</th>
<th>Position of Sequence Variation in cDNA (amino acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germ cell tumor</td>
<td>59</td>
<td>Exon 1-Intron 1 15 (25.5%)</td>
<td>703 G/T (Ala/Ser)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Exon 3        5 (8.5%)</td>
<td>714 G/C (Leu/Leu)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intron 1      58 G/C</td>
<td>1328 G/A (Gly/Glu)</td>
</tr>
<tr>
<td>Non-Hodgkin's Lymphoma</td>
<td>30</td>
<td>Exon 1-Intron 1 10 (33.3%)</td>
<td>703 G/T (Ala/Ser)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Exon 3        6 (20.0%)</td>
<td>714 G/C (Leu/Leu)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intron 1      58 G/C</td>
<td>1328 G/A (Gly/Glu)</td>
</tr>
</tbody>
</table>
Figure 1. Genetic Analysis of BCL10 in Germ Cell Tumors (GCTs) and B Cell Lymphomas

(A) PCR-SSCP analysis. (a) and (c), GCT; (b) and (d), lymphoma. (a) and (b), exon 1; (c) and (d), exon 3.2. Arrows indicate conformational changes. N, normal; T, tumor; CL, cell line; tumor numbers are indicated on top.

(B) PCR sequence analysis. (a) and (b), Tera-1; (c), GCT-44; (d) and (e), Tera-2; (f), T-243A; (g)-(i), 240A. Panels (a)-(e) show absence of mutations in GCT cell lines in relation to Willis et al. (1999) report, where boxes indicate base change and upward arrow indicates insertion positions. Positions in coding region of cDNA are: (a), 172 bp; (b), 653 bp; (c), 172 bp; (d), 58 bp; (e), insertion at 499 bp. Panel (f), A/G heterozygosity at position 2 of codon 213. Panels (g)-(i) indicate loss of heterozygosity at codon 5 (underlined) in tumor and cell line 240A. Panel (g), normal DNA; panel (h), tumor DNA; panel (i) cell line DNA. The residual peak of $G$ in panel (h) indicates the presence of contaminating normal cells.

(C) Multiplex RT-PCR analysis of BCL10 expression in GCT. Primers from the 5′ coding region (5′-GGACCCGGAAGAAGCGCCATCTCC-3′ and 5′-AAGTAGTCTAACAATTTTCAAGCCC-3′) spanning two different exons were used to amplify a 249 bp PCR product. PCR was performed utilizing AmpliTaq DNA polymerase (PE Applied Biosystems, Foster City, CA). The 450 bp PCR product represents β-actin as control. Marker, PhiX HaeIII; tumor and cell line numbers are shown on top.

2102E-R, after 45 cycles of PCR amplification (Figure 1C). Thus, the present analysis rules out a role for BCL10 as a TSG in the majority of GCTs. On the basis of the data presented, we conclude that BCL10 is not a target TSG at 1p22 in male GCTs or B cell lymphomas.

Absence of BCL10 Mutations in Human Malignant Mesothelioma

It has recently been suggested that a novel gene, BCL10, is mutated in mucosa-associated lymphoid tissue (MALT) B cell lymphoma and multiple tumor types (Willis et al., 1999). This gene was identified through the cloning of a (1;14)(p22;q32) translocation breakpoint in a case of low-grade MALT lymphoma. Mutations of BCL10 were identified not only in MALT lymphomas, but also in other lymphoid tumors of B or T cell lineage, and tumor cell lines derived from malignant mesotheliomas (MMs),

References


Murty, V.V.S., Bosl, G.J., Houldsworth, J., Meyers, M., Mukherjee,

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340A 2001-H 165A 195A
NTCLCLCN T N C
15211 1819 1948 1951
NT NT NT N
13121 1351
NT NT NT N
germ cell neoplasms, and colon carcinomas. Thus, the investigators proposed that alterations of BCL10 may contribute to the pathogenesis of several types of neoplasia.

The observation of most interest to us was that BCL10, which maps to chromosome 1p22, exhibited mutations in all three MM cell lines analyzed. We had previously shown 1p22 to be a frequent site of allelic loss in MM (Lee et al., 1996), which implicated the involvement of a tumor suppressor gene (TSG) at this location. The aim of this letter is to report the results of our studies of BCL10 as a candidate for the 1p22 putative TSG in MM, and to prompt further discussion about the potential role of this gene in human malignancy.

Fifty MM cell lines were examined for loss of heterozygosity (LOH). Since YAC 929E1 contains BCL10 (Willis et al., 1999), two microsatellite markers, D1S2766 and D1S1618, were selected for analysis, as they were determined through a database search (http://www-genome.wi.mit.edu) and PCR analysis to map to this YAC. Overall, 25 of 45 informative cases (55.6%) showed LOH of at least one of these two loci, with the higher frequency of allelic loss being at marker D1S1618 (18 out of 30 informative cases, 60%) (data not shown). However, the percentage of MMs with LOH in the vicinity of BCL10 is less than that previously observed for markers within the minimally deleted 1p22 region in this malignancy (Lee et al., 1996). Furthermore, YAC 929E1 resides distal to this minimally deleted segment.

RT-PCR was performed on MM cell lines that displayed LOH to determine the expression pattern of BCL10. Normal mesothelial cells and MM cell lines not exhibiting LOH at 1p22 were included as controls. All cell lines examined expressed BCL10 at similar levels (data not shown). The housekeeping gene GAPDH was also expressed at uniform levels in all cell lines tested. In summary, BCL10 was demonstrated to map to a region showing frequent LOH in MM, but expression of BCL10 did not appear to be altered in any of the tumor cell lines examined.

To determine whether the BCL10 gene is mutated in MM, SSCP analysis was performed on genomic DNAs from 25 MM cell lines displaying LOH in 1p22, using primers described by Willis et al. (1999). Peripheral blood lymphocyte (PBL) samples from two healthy donors were included as controls. Band shifts were observed in MM PCR products amplified with primers specific for exons 1 and 3.2 (257 bp at the 3′ end of exon 3). Variant exon 1 bands were demonstrated in 18 of 25 MM cell lines. However, these band shifts appeared to be identical in 17 of these cell lines and in one of the normal PBL samples. Products from two MM cell lines amplified with exon 3.2 primers displayed identical banding patterns, which differed from those observed in normal PBL samples. Examples of band shifts are shown in Figure 1. We also carried out SSCP analysis of eight tumor samples matched to MM cell lines displaying variant bands. In each case, identical patterns were observed in the tumor and the corresponding MM cell line. Variant bands were not observed in BCL10 products from exons 2 and 3.1 (262 bp at the 5′ end of exon 3).

Reproducible band shifts in DNA from MM cases were further investigated by direct sequencing of purified PCR products. The sequences derived from the MM samples were compared with the published BCL10 sequence (accession number AJ006288). Nucleotide alterations detected in PCR products from MM samples are

<p>| Table 1. DNA Sequence Alteration of BCL10 Detected in MM |</p>
<table>
<thead>
<tr>
<th>Meso Cell Line</th>
<th>Exon</th>
<th>DNA Alteration</th>
<th>Amino Acid Alteration</th>
<th>Allele Frequency*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meso 3, 5, 6, 8, 13, 15</td>
<td>1</td>
<td>24 G→C</td>
<td>Lys 8 Lys</td>
<td>ND*</td>
</tr>
<tr>
<td>Meso 17, 23, 25, 28, 33, 36, 36, 42, 43, 44, 52, 59</td>
<td>1</td>
<td>13 G→T</td>
<td>Ala 5 Ser</td>
<td>3%</td>
</tr>
<tr>
<td>Meso 38</td>
<td>3</td>
<td>638 G→A</td>
<td>Gly 213 Glu</td>
<td>9%</td>
</tr>
</tbody>
</table>

* Percentage shown represents allele frequency in a general population.

* ND, not determined.
presented in Table 1. Some of these changes were predicted to result in substitutions of amino acids at the corresponding positions in the protein sequence. In each of these cases, individual tumor DNA samples possessed the same sequence as that of the corresponding cell line.

To determine if the divergent sequences represented mutations of the BCL10 gene or polymorphisms, a panel of 50 normal genomic DNA samples from the general population was screened (data not shown). The nucleotide alteration in exon 1 seen in 18 MM (Table 1) was assumed to be a polymorphism, because it was observed in numerous MM and a control sample, and it did not predict an amino acid change. Thus, this variant was not studied further. The nucleotide change in exon 1 (nucleotide 13) of Meso 38 destroys an AciI restriction enzyme site. Restriction enzyme analysis revealed that three of 50 samples from the general population do not possess this AciI site, which corresponds to an allele frequency of 3%. The nucleotide alteration in exon 3 (nucleotide 638) of Meso 17 and Meso 59 does not occur at a restriction enzyme site. Therefore, we performed SSCP analysis on these two MMs and 50 normal DNA cell samples. Nine of 50 DNA samples from normal individuals (allele frequency, 9%) displayed banding patterns identical to those observed in Meso 17 and Meso 59 (Table 1). Thus, we conclude that all of the nucleotide differences detected in our MM cell lines and tumors represent polymorphisms in the general population. We did not observe any of the nucleotide alterations reported by Willis et al. (1999). Therefore, it would be important to determine if any of their alterations are polymorphisms.

Finally, we performed sequence analysis on DNA from PBL samples matched to the three cell lines (Mesos 17, 38, and 59) that exhibited nucleotide changes predicting amino acid substitutions. The identical nucleotide sequence was observed in DNA from both tumor and normal tissues from the same individual, indicating that these base changes are representative of the constitutional (inherited) genome in each case. In conclusion, BCL10 is not mutated or abnormally expressed in MM, and this gene can be ruled out as a candidate for the 1p22 TSG in this malignancy.

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References

In Response to Fakruddin et al.

We agree with many of the above findings, including the frequency and nature of BCL10 polymorphisms. However, it appears that some BCL10 abnormalities may be found only in RNA and not genomic DNA, suggesting that BCL10 may undergo posttranscriptional sequence modification.

Wild-type BCL10 is proapoptotic and behaves as a tumor suppressor gene (Koseki et al., 1999; Thome et al., 1999; Willis et al., 1999; Yan et al., 1999; Zhang et al., 1999). BCL10 sequence abnormalities were first detected in cDNA clones from a MALT lymphoma with t(1;14) and were unusually heterogeneous. An identical spectrum of cDNA abnormalities has been detected in other t(1;14) MALT lymphomas (Zhang et al., 1999), making it unlikely that all these changes represented RT, PCR, or cloning artifacts.

We initially sequenced cDNA clones from 6 malignant cell lines, including Tera-1, Tera-2, and GCT-44 and three mesothelioma lines, and in each, BCL10 abnormalities were detected (Table 2 of Willis et al., 1999). Additional cDNA clones from Tera-2, a cell line that exhibits

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Table 1. Summary of Tera-2 BCL10 cDNA Data

<table>
<thead>
<tr>
<th>Clone #</th>
<th>Point Mutations (codon)</th>
<th>Homopolymeric Runs</th>
<th>Splice Variations</th>
<th>Predicted Protein (amino acids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Normal Clone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>233 wild type</td>
</tr>
<tr>
<td>(B) Clones with Insertions/Deletions within the Homopolymeric Runs Alone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>nil</td>
<td>499 ins TT</td>
<td>nil</td>
<td>172</td>
</tr>
<tr>
<td>3</td>
<td>nil</td>
<td>499 ins T</td>
<td>nil</td>
<td>168</td>
</tr>
<tr>
<td>4</td>
<td>nil</td>
<td>136 del A</td>
<td>499 ins T</td>
<td>68</td>
</tr>
<tr>
<td>(C) Mutated Clones +/− Other Abnormalities</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>N93S AAC→AGC</td>
<td>nil</td>
<td>nil</td>
<td>233</td>
</tr>
<tr>
<td>6</td>
<td>N93S AAC→AGC del A codon 149</td>
<td>nil</td>
<td>nil</td>
<td>171</td>
</tr>
<tr>
<td>7</td>
<td>H40H CAT→CAC silent K45Q AAA→CAA</td>
<td>nil</td>
<td>nil</td>
<td>233</td>
</tr>
<tr>
<td>8</td>
<td>M153V ATG→GTG L177L CTA→CTG silent</td>
<td>nil</td>
<td>nil</td>
<td>233</td>
</tr>
<tr>
<td>9</td>
<td>A200A GCT→GCC silent R58Q CGA→CAA</td>
<td>nil</td>
<td>nil</td>
<td>233</td>
</tr>
<tr>
<td>10</td>
<td>R58Q CGA→CAA S218F TCT→TTT</td>
<td>nil</td>
<td>nil</td>
<td>233</td>
</tr>
<tr>
<td>11</td>
<td>R58Q CGA→CAA T168T ACT→ACC silent</td>
<td>nil</td>
<td>nil</td>
<td>233</td>
</tr>
<tr>
<td>12</td>
<td>H40R CAT→CGT L177L CTA→CTG silent</td>
<td>nil</td>
<td>nil</td>
<td>233</td>
</tr>
<tr>
<td>13</td>
<td>N217S AAC→AGC A20T GCC→ACC</td>
<td>499 ins T</td>
<td>nil</td>
<td>168</td>
</tr>
<tr>
<td>14</td>
<td>A20T GCC→ACC V26V GTA→GTG silent</td>
<td>Q92stop CAG→TAG</td>
<td>nil</td>
<td>91</td>
</tr>
<tr>
<td>15</td>
<td>Q92stop CAG→TAG R226K AGA→AAA</td>
<td>nil</td>
<td>16 bp</td>
<td>91</td>
</tr>
</tbody>
</table>

Summary of cDNA sequence abnormalities observed in 13 clones derived from cell line Tera-2.
Matters Arising

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types of deviation from the wild-type sequence observed in our own and others’ studies (Zhang et al., 1999):

(A) BCL10 utilized three alternative splice sites at the exon 3/4 boundary, two of which result in deletion of either 16 or 33 base pairs from wild-type sequence. These splice variants were detected as different-sized PCR products.

(B) Nucleotide insertions and deletions were common, particularly within the homopolymeric runs of 8 A’s and 7 T’s. The homopolymeric runs were also sites of point mutation (Figure 1B). In some instances, abnormalities within these runs were seen by direct cDNA sequencing, indicating they comprised a common transcript (Figure 1D). These data preclude cloning artifacts. In comparison, from 500 genomic sequences obtained in our laboratories, only 4 cases exhibited deletions or insertions within the two homopolymeric runs. These data suggest that such changes are largely nontemplated and may constitute an unusual form of RNA editing.

(C) Point mutations were multiple and ongoing. Whether all point mutations are present in genomic DNA or whether some are nontemplated is not yet clear. These cDNA abnormalities appeared with comparable frequency irrespective of reverse transcriptase, DNA polymerase, or sequencing method used. A BCL10 pseudogene has not been identified by FISH, Southern, or genomic PCR experiments.

In summary, at least some of the discrepancies between our initial data and those reported above can be ascribed to our use of cDNA rather than genomic DNA and to possible posttranscriptional modification of BCL10. However, our analysis of genomic DNA from primary tumors continues to demonstrate BCL10 mutations in some tumor types (H. P. et al., submitted). The reasons for this discrepancy are not clear but may include the ongoing nature of BCL10 mutations and the presence of individual BCL10 mutations within only a subset of tumor cells.

All sequences will be available via <http://www.icr.ac.uk/haemcyto/bcldata/index.html>.


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References


