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Matters Arising

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 SCCP 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 cells lines, 577M-F and

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

Tumor Type	No. Studied	SSCP Variants		Position of Sequence Variation in cDNA (amino acid)
Germ cell tumor	59	Exon 1–Intron 1	15 (25.5%)	703 G/T (Ala/Ser) 714 G/C (Leu/Leu) Intron 1, 58 G/C
		Exon 3	5 (8.5%)	1328 G/A (Gly/Glu)
Non-Hodgkin's Lymphoma	30	Exon 1–Intron 1	10 (33.3%)	703 G/T (Ala/Ser) 714 G/C (Leu/Leu) Intron 1, 58 G/C
		Exon 3	6 (20.0%)	1328 G/A (Gly/Glu)

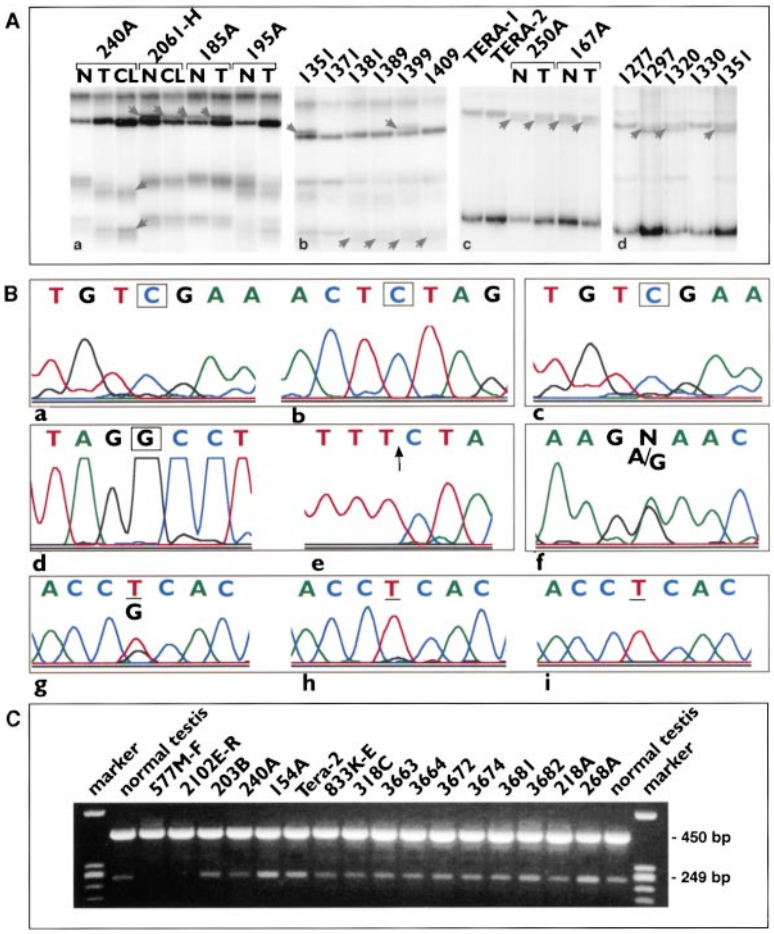

Figure 1. Genetic Analysis of *BCL10* in Germ Cell Tumors (GCTs) and B Cell Lymphomas

(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′-GGACCCGGAAGAAGCGCCATC TCC-3′ and 5′-AAGTAGTCTAACAATTTTCCA GCCC-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.

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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),

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) of *BCL10*. 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

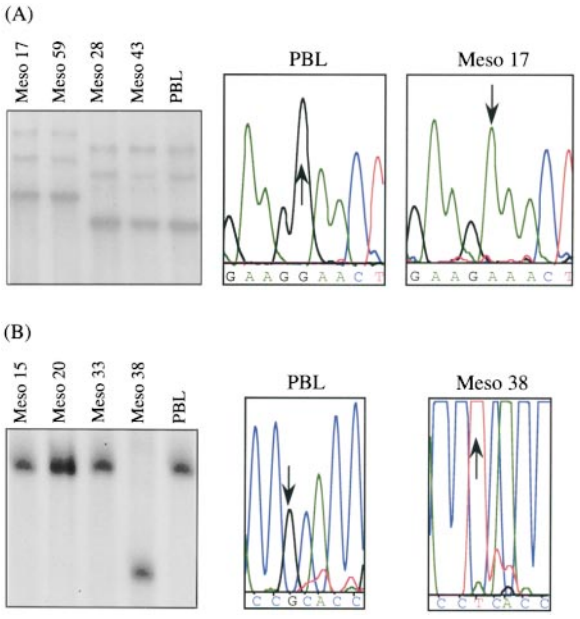

Figure 1. SSCP and Sequence Analyses of *BCL10* in MM Cell Lines

SSCP analysis was performed on genomic DNA. The entire coding sequence of *BCL10* was amplified with AmpliTaq (Perkin Elmer) and previously described primers (Willis et al., 1999), using standard PCR conditions. Autoradiographs at left show PCR products generated by primers corresponding to exon 3.2 (A) or exon 1 (B) analyzed on 10% nondenaturing acrylamide gels. Lanes 1–4: MM cell lines, as indicated; lane 5: PBL DNA from a normal individual (PBL). Templates for sequencing were amplified from genomic DNA by PCR with AmpliTaq and primers flanking each exon of *BCL10*. The chromatograms at right show sequence differences between Meso 17 and PBL (A) or between Meso 38 and PBL (B). Arrows indicate altered nucleotides.

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

Table 1. DNA Sequence Alteration of *BCL10* Detected in MM

Meso Cell Line	Exon	DNA Alteration	Amino Acid Alteration	Allele Frequency[a]
3, 5, 6, 8, 13, 15 17, 23, 25, 28, 33, 36, 38, 42, 43, 44, 52, 59	1	24 G→C	Lys 8 Lys	ND[b]
38	1	13 G→T	Ala 5 Ser	3%
17, 59	3	638 G→A	Gly 213 Glu	9%

[a] Percentage shown represents allele frequency in a general population.
[b] 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 MMs (Table 1) was assumed to be a polymorphism, because it was observed in numerous MMs 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 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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In Response to Fakruddin et al. and Apostolou 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

Table 1. Summary of Tera-2 *BCL10* cDNA Data

Clone #	Point Mutations (codon)		Homopolymeric Runs	Splice Variations	Predicted Protein (amino acids)
(A) Normal Clone					
1	nil		nil	nil	233 wild type
(B) Clones with Insertions/Deletions within the Homopolymeric Runs Alone					
2	nil		499 ins TT	nil	172
3	nil		499 ins T	nil	168
4	nil		136 del A		
			499 ins T	nil	68
(C) Mutated Clones +/− Other Abnormalities					
5	N93S	AAC→AGC	nil	nil	233
6	N93S	AAC→AGC			
	del A codon 149		nil	nil	171
7	H40H	CAT→CAC silent			
	K45Q	AAA→CAA	nil	nil	233
8	M153V	ATG→GTG			
	L177L	CTA→CTG silent			
	A200A	GCT→GCC silent	nil	nil	233
9	R58Q	CGA→CAA			
	S218F	TCT→TTT	nil	nil	233
10	R58Q	CGA→CAA			
	T168T	ACT→ACC silent			
	S218F	TCT→TTT	nil	nil	233
11	H40R	CAT→CGT			
	L41L	CTA→CTT silent			
	N217S	AAC→AGC	499 ins T	nil	168
12	A20T	GCC→ACC			
	V26V	GTA→GTG silent			
	Q92stop	CAG→TAG	nil	nil	91
13	A20T	GCC→ACC			
	V26V	GTA→GTG silent			
	Q92stop	CAG→TAG			
	R226K	AGA→AAA	nil	16 bp	91

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

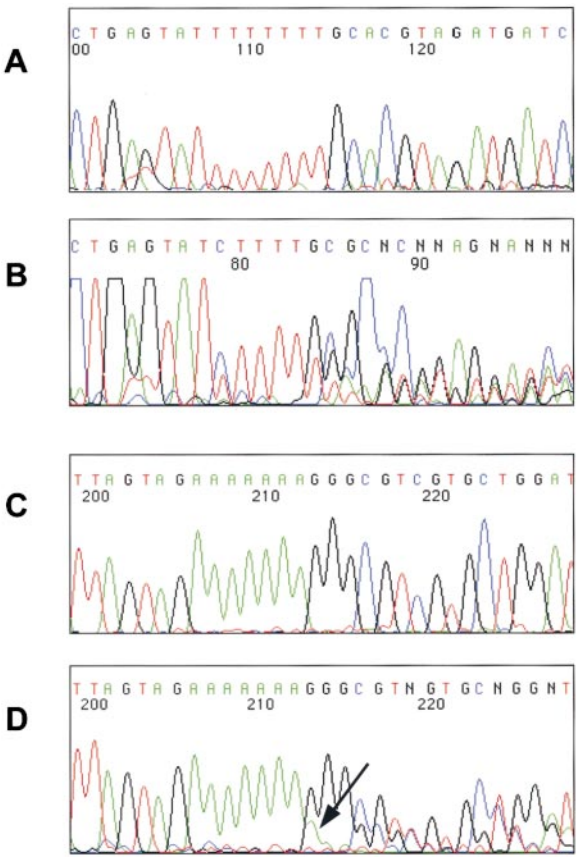

Figure 1. Reverse *BCL10* cDNA Sequences Showing Presence of Alterations within both Homopolymeric Runs

(A) Clone from normal lymphocytes showing 8 A's.

(B) Direct sequencing of cDNA from patient with B cell precursor acute leukemia showing complex aberrations within the 8 A's including deletion of two adenines and A→G point mutation.

(C) Direct sequencing of cDNA from myeloma cell line showing normal run of 7 T's.

(D) Direct sequence of cDNA from normal human fetal brain mRNA (Clontech) showing presence of additional thymidine in about 50% of the transcripts (arrow).

Note loss of readable sequence in lanes B and D subsequent to inserted or deleted nucleotides. RT and PCR reactions performed as described (Willis et al., 1999). Poly(A)$^+$ RNA was reverse transcribed using Superscript II (GIBCO–BRL) or rTth (Amersham) and random hexamers and the *BCL10* open reading frame amplified using either Taq or Pfu polymerase with forward primer: 5′-CCTCCTCTCCTTCT TCCCCATTACC-3′ and reverse primer 5′-GCAATAAAGTGTCATTGT CTGGAAACAGT-3′. RT-PCR products were either sequenced directly or cloned into pCR2.1 (Invitrogen). Clones were sequenced in both directions using either Licor (MWG-Biotech, Germany) or ABI-377 (Applied Biosystems) automated sequencers.

no genomic PCR-SSCP abnormalities, showed (Table 1): (1) all clones had different sequences; (2) multiple abnormalities in some clones; (3) no obvious clonal relationship between all clones; and (4) abnormalities within the homopolymeric runs in 4/13 clones.

We have now sequenced either RT-PCR products and/or multiple cDNA clones from 18 malignant cell lines, 48 primary lymphoid tumors, and peripheral blood lymphocytes from 7 normal individuals, as well as cDNAs from 5 other normal tissues, and found complex abnormalities in all instances. To summarize the three 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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