# Characterization of a novel B-CLL candidate gene – *DLEU7* – located in the 13q14 tumor suppressor locus

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**Abstract Deletion of chromosome 13q14 is the most frequent genetic aberration in B-cell chronic lymphocytic leukemia (CLL), found in more than 50% of cases, indicating that this region contains a gene(s) involved in the development of CLL. However, the pathogenic gene in the critical 13q14 region has not yet been defined. Here, we have cloned and characterized a novel gene, *DLEU7*, located adjacent to the consensus deleted region, and overlapping the 3′ end of *DLEU1* tail to tail. Human *DLEU7* encodes a putative 221 amino acid protein, with significant conservation in rodents. Mutational and expression analysis in primary CLL samples failed to demonstrate any specific mutations in *DLEU7*, but no *DLEU7* expression could be detected in CLL cells. Methylation of a CpG island in the promoter region of *DLEU7* was further analyzed as a possible mechanism for the absence of *DLEU7* expression, and the promoter was found to be methylated in the majority of the CLL samples investigated.**


*Key words:* 13q14; Chronic lymphocytic leukemia; DLEU7; Deletion; Candidate gene

## 1. Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is the most frequent leukemia in the Western world [1]. The molecular pathogenesis of this disease has remained largely unknown, but previous studies have indicated the presence of a putative tumor suppressor gene on chromosome 13q14, a region deleted heterozygously in 30–60% and homozygously in 10–20% of CLL patients [2–7]. Recently it has become apparent that loss of the same region of 13q is frequent not only in other hematological malignancies such as myeloma [8,9], mantle cell lymphoma [10], diffuse large cell lymphoma [11], and myelofibrosis [12], but also in solid tumors including head and neck tumors [13], oral tumors [14] and prostate carcinoma [15].

Surprisingly, no specific inactivating mutations have been found in any gene mapped to the region. There is, however, some discrepancy concerning the location of the critical 13q deleted region (CDR), with some studies suggesting a slightly more telomeric area being important [3,16]. The lack of mutations of candidate genes, despite the high instances of genomic loss, suggests that the pathomechanism involving 13q14 may involve haploinsufficiency or is of an epigenetic nature. Epigenetic silencing of tumor suppressor genes by hypermethylation has been detected in a variety of malignancies [17,18]. However, no aberrant promoter methylation pattern could be demonstrated in the genes located in the consensus 13q14 CDR, *DLEU1*, *DLEU2* and *RFP2/LEU5* [19].

In the present study we have cloned and characterized a novel gene, *DLEU7*, located adjacent to the critical 13q14 CLL deletion region and with overlapping 3′ ends with *DLEU1*. We have analyzed the deletion status of *DLEU7* and performed mutation screening of the gene on leukemic DNA from a number of B-CLL patients. The methylation pattern of the *DLEU7* promoter has been investigated in B-CLL patient material, in addition to the expression status of the gene.

## 2. Materials and methods

### 2.1. Computer programs

Genomic DNA sequences were obtained from the Human and Mouse assemblies available through the UCSC genome browser home page (http://genome.ucsc.edu/). These sequences were aligned using the *needle* program of the EMBOSS package [20], and analyzed for the presence of conserved transcription factor binding sites using the web resource ConSite (http://www.phylofoot.org/consite/) [21]. Putative transcription start points were predicted by the computer program Eponine (http://www.sanger.ac.uk/Software/analysis/eponine/). The NCBI site (http://www.ncbi.nlm.nih.gov/) was used for BLAST homology searches and peptide sequence retrieval. Sequence alignments were performed with the ClustalW program [22].

### 2.2. Polymerase chain reaction (PCR), rapid amplification of cDNA ends (RACE), real-time PCR and single-stranded conformational polymorphism (SSCP)

RT-PCR was performed as described previously [23] on a cDNA panel of human tissues (Clontech) and on cDNA from CLL patient samples that had been flow sorted to >99% purity on the basis of CD19 and CD5 positivity. All basic molecular biology procedures (isolation of DNA, growing of bacteria, cloning etc.) were performed according to standard procedures [24]. Primers used for amplification of the *DLEU7* gene are shown in Table 1. 3′ RACE-PCR analysis was carried out as previously described [23] on cDNAs from various human tissues as template. Differentially spliced 3′ RACE products were subcloned using the TOPA-TA cloning kit (pCR.2.1; Invitrogen) and sequenced.

RNA was extracted from CD19+ purified B-cells of healthy donors (CD19 Dynabeads, Dynal Biotech, Norway) with Trizol Reagent (Life Technologies). The RNA was reverse transcribed with First-Strand cDNA synthesis using Superscript II (Invitrogen, Life Tech-

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Table 1
Primer sequences

| Primer name | Sequence 5′→3′ |
|---|---|
| Ex1F2 | CAGGTGAATGGGAAAGGACCGCTC |
| Ex1F1 | CTGCACGCCAGAGGGGATGGC |
| Ex1R1 | GCGGGTTAGAAAATGTGAC |
| Ex1R2 | CGCTGCTGCTCCACCTGCAC |
| Ex1R3 | CGGAGCTGGTCAGCGTGGAGC |
| Ex1R4 | GCAGGAGCGGTCCTTTCCCATTCAC |
| Ex2F1 | GCCACTGGTCAGACTGCTCCAC |
| Ex2R1 | CCAGAGTGCTGTCACCAGAAGAGA |
| Ex2R2 | CCTTCCTAATTGAGTCAGCAGAC |
| Ex2R3 | GGAGCAGTCTGACCAGTGGCATC |
| Ex5F1 | GCCAGCAGGTGAGAAGCTAGAC |
| Ex5F2 | GACAGAGCAGCCGGTGGCAGC |
| Ex6F1 | GCTCCTGATGGTCCTCACACTCC |
| Ex6F2 | CTACAGTGACCTTCTTCACTCATTAGC |
| Ex6R1 | CTGGCTAATGAGTGAAGAAGG |
| Ex6R2 | GAGTGTGAGGACCATCAGGAGCTTCA |
| Methyl-F1 | TTGGAGGATGGGAGGTTATTTTGTA |
| Methyl-F2 | TTGTTGGAGGGGTTTTAGTA |
| Methyl-R1 | AACTCTACAAACCTTACAACTACTACAAC |
| Methyl-R2 | AACTCCCCAAAAAAAAAAATAATAC |
| RT-LEU7F | GATCTGTCTCAAGGAAGCACA |
| RT-LEU7R | TCCCATTCACCTGAAGGATAG |
| RT-B2MF | CAAGCAGAGAATGGAAAGTC |
| RT-B2MR | GATGCTGCTTACATGTCTCG |

nologies). Real-time PCR was carried out in an Bio-Rad iCycler by using the SYBR green PCR kit (Bio-Rad Laboratories, Hercules, CA, USA). Primer sequences for *DLEU7* and $\beta_2$-microglobulin (B2M) used as control are shown in Table 1.

The primers used for SSCP analysis are listed in Table 1. For SSCP, the resulting PCR products were further digested by *Alu*I prior to the analysis by polyacrylamide gel electrophoresis as previously described [25].

### 2.3. Northern analysis

Total RNA was isolated using Trizol reagent according to the manufacturer's protocol (Life Technologies). 20 μg total RNA was separated on a 1% agarose formaldehyde gel and blotted onto Zeta-Probe membranes (Amersham). All Northern blots were hybridized with PCR-derived probes for the coding region of *DLEU7*, according to the manufacturer's recommendations (MTN blots, Clontech). Labeling of all probes was performed as previously described [23].

### 2.4. Bisulfite treatment and sequencing

A DNA sample of 1 μg was bisulfite-modified according to standard protocols of Issa and Frommer (http://www3.mdanderson.org/leukemia/methylation/protocols.html). 50 ng of each sample was subsequently used in each methylation-specific PCR reaction. Primers Methyl-F1 plus Methyl-R1 and nested primers Methyl-F2 plus Methyl-R2 were used to amplify a 190 bp fragment from the bisulfite-treated DNA samples. Restriction digestion of the resulting products and size fractionation on 2% agarose gels allowed the determination of the methylation status of *DLEU7*. Methylated samples were identified by the presence of the restriction enzyme sites *Taq*I or *Hha*I that were absent in unmethylated fragments. Some PCR products were further analyzed by bi-directional sequencing, as previously described [26].

## 3. Results

### 3.1. Cloning and definition of the genomic structure of a novel B-CLL candidate gene – DLEU7

Our group previously analyzed 1.3 Mb of genomic sequence covering the commonly deleted 13q14 region in CLL (unpublished data) and identified an EST, termed *DLEU7*, located 150 kb telomeric to the marker D13S25. In the present investigation, the full length *DLEU7* was isolated by RT-PCR on a mixture of normal cDNAs, using primers designed from surrounding ESTs putatively associated with *DLEU7* (Table 1). This revealed that *DLEU7* is alternatively spliced, extends over 130 kb of genomic sequence and is composed of at least six exons (Fig. 1). All intron–exon junctions matched the consensus AG and GT nucleotides at the intron acceptor and donor sites, respectively. In mouse several homologous ESTs from the syntenous region were found that extend further 5′ as compared to the ESTs in human. By RT-PCR, combining primers located in the corresponding human genomic sequence with primers located in downstream exons, followed by sequencing of the PCR products, the existence of a longer 5′ end of *DLEU7* also in human was confirmed.

An in silico analysis of the region covering the first exon and the 1 kb upstream sequence revealed the existence of a CpG island covering the first exon, a putative transcription start site with high probability (threshold of 0.99) as well as the presence of binding sites for several transcriptions factors, such as basic helix-loop-helix transcription factors that bind to E-box motifs (Myf, Tal1 and Myc-Max) and others involved in cell proliferation and differentiation (AML-1, E2F and cFOS (bZIP)) (Fig. 2). All of the above-mentioned transcription factor binding sites are conserved in mouse (Fig. 2), increasing the likelihood that these elements are involved in transcriptional regulation of *DLEU7*.

The *DLEU7* cDNA found to date is 1137 bp, with a 5′ untranslated region (UTR) of 65 bp and an in-frame first ATG codon within a Kozak context for ribosome binding [27]. A putative open reading frame (ORF) of 663 bp extends

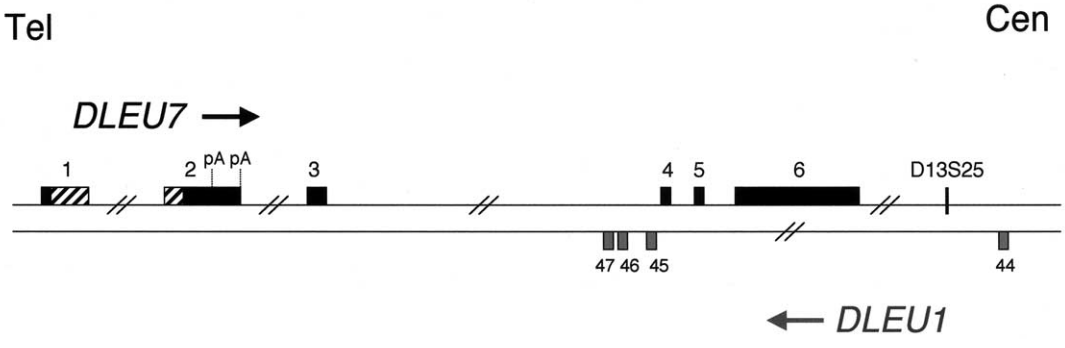


Fig. 1. The genomic structure of the *DLEU7* gene and its relation to *DLEU1*. The individual exons for *DLEU7* and *DLEU1* are marked as black and gray boxes, respectively. The putative *DLEU7* ORF is shown as dashed boxes. Polyadenylation signals are denoted as pA. The directions of transcription are indicated by the arrows. 'Cen' and 'Tel' show the positions of the centromere and telomere, respectively.

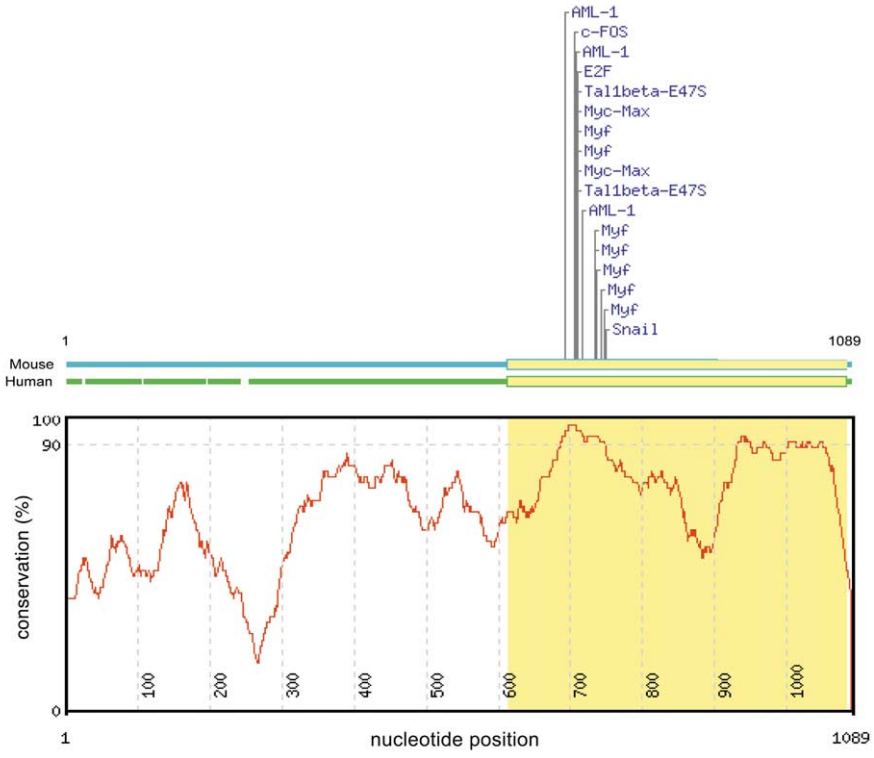


Fig. 2. Conservation within the promoter region of *DLEU7* between human and mouse. Nucleotide 1 is located 613 bp upstream of the putative transcriptional start site (TSS). The yellow area corresponds to exon 1. Potential conserved transcription factor binding sites are indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

from exon 1 to exon 2 with the possibility to encode a 221 amino acid protein (Fig. 3). To identify the 3′ end of the *DLEU7* cDNA, 3′ RACE was carried out, revealing that the *DLEU7* 3′ UTR can have at least three alternative lengths. Putative polyadenylation signals (ATTAAA) could be identified in two of the 3′ ends, 15–18 bp upstream of the poly(A) tail, resulting in one 3′ UTR region of 409 bp and one 3′ UTR of 690 bp. The *DLEU7* cDNA sequence has been submitted to GenBank under accession number AY357595. Interestingly, *DLEU7* was found to be located tail to tail with *DLEU1*, with exons 4, 5 and 6 of *DLEU7* located within intron 44 of *DLEU1* (Fig. 1).

### 3.2. DLEU7 *encodes an evolutionarily conserved protein*

The predicted coding sequence for the dleu7 protein (submitted to GenBank under accession number AY357595) was used to search the GenBank/EMBL protein sequence databases for homologous sequences. Highly conserved dleu7 pro-

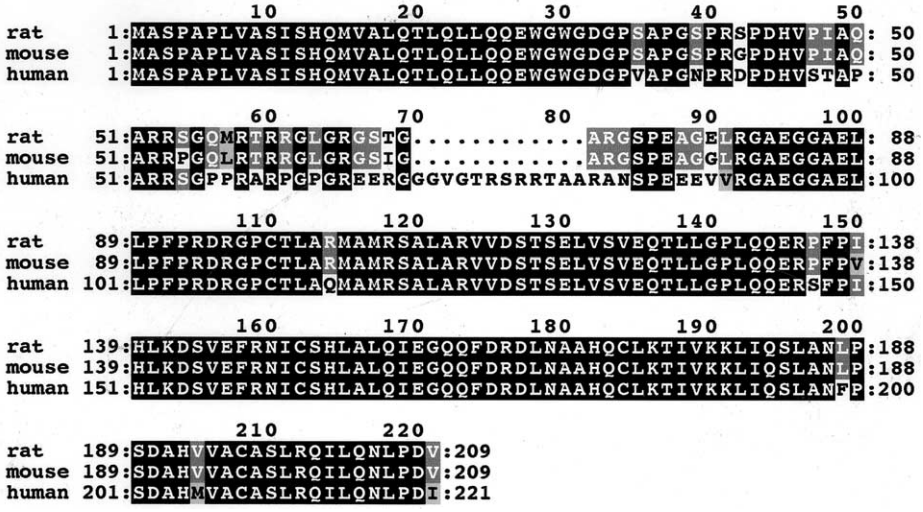


Fig. 3. The dleu7 protein is conserved between human, rat and mouse. A ClustalW alignment of the human dleu7 protein and orthologs from mouse and rat. Columns colored black highlight identical amino acids between the proteins, whereas shaded and white boxes represent conservative and non-conservative amino acid substitutions respectively. The % identity and similarity between the dleu7 in human, mouse and rat are: human–mouse: 82.8% and 86%; human–rat: 83.7% and 86.0%; mouse–rat: 97.1% and 98.1%, respectively.

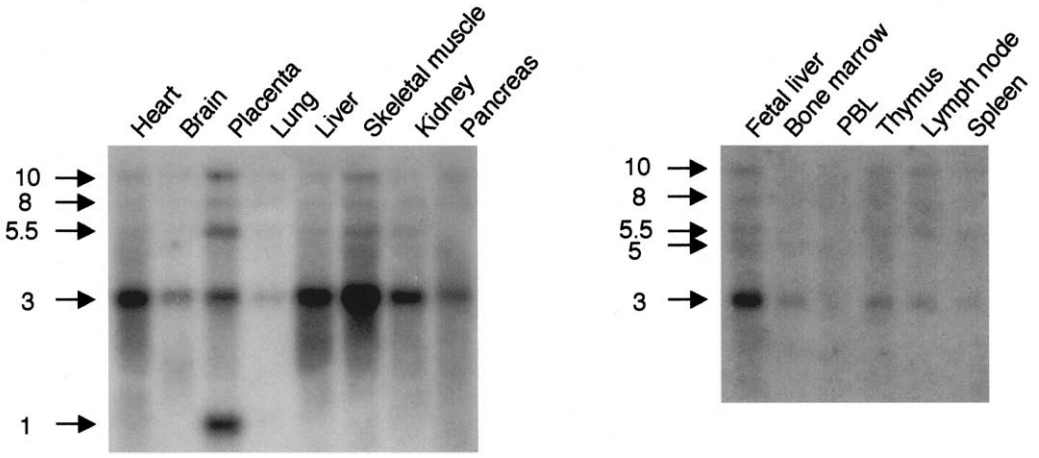


Fig. 4. *DLEU7* expression in various human tissues. Northern blots, containing mRNA from various human tissues, were hybridized with a cDNA probe covering the *DLEU7* coding sequence. The positions of the *DLEU7* transcripts are indicated by the arrows. Hybridization with a β-actin control probe was performed to confirm equal loading (not shown).

teins were found in both mouse and rat (GenBank accession numbers XP_214218 and NP_775595, respectively) (Fig. 3). The dleu7 protein was not found to contain any known functional domains.

### 3.3. *Genomic status of the* DLEU7 *gene in B-CLL*

To investigate genomic abnormalities of the *DLEU7* gene in CLL cells, Southern blot analysis was performed on leukemic cell DNA from CLL patients with a cDNA probe covering the coding part of the gene. These leukemic samples have been analyzed in previous investigations with probes covering the CDR [4], including a probe for the adjacent marker D13S25 located only 40 kb centromeric of *DLEU7*. Consistent with previous studies, the *DLEU7* region clearly is less frequently deleted (51/152 (34%) deleted) compared to our previously defined CDR (93/229 (41%) deleted).

### 3.4. DLEU7 *is not mutated in B-CLL*

In an effort to identify possible point mutations or small nucleotide sequence aberrations in the *DLEU7* gene, PCR-based SSCP analysis was performed across the protein-encoding part of the *DLEU7* gene (exon 1 and exon 2) on DNA from 45 B-CLL patients. Out of these samples, 24 were previously found to have hemizygous loss of the CDR at 13q14 and 21 samples had no detectable deletion of 13q14. No alterations were, however, found in any of the patient samples analyzed (data not shown).

### 3.5. *Expression analysis of the* DLEU7 *gene in normal tissues and B-CLL cells*

Poly(A)$^+$ RNA from various normal human tissues (Clontech) was hybridized with a cDNA probe covering the coding region of the *DLEU7* gene. Five transcripts of 1 kb, 3 kb, 5.5 kb, 8 kb and 10 kb could be detected (Fig. 4). The four larger transcripts were found to be ubiquitously expressed at various levels in all human tissues tested, whereas the 1 kb transcript could only be detected in placenta (Fig. 4).

*DLEU7* expression was further analyzed in total RNA isolated from purified mononuclear cells from B-CLL patients, consisting of $>95\%$ leukemic cells. Samples from nine CLL patients with either homozygous, hemizygous deletions or no 13q deletions as well as two CLL cell lines with homozygous 13q deletions were included in the analysis. None of the *DLEU7* transcripts could be detected in any of the CLL samples analyzed (data not shown).

Expression of *DLEU7* was also analyzed by real-time PCR using cDNA derived from purified CD19+ B-cells from healthy donors, as well as various tissues such as fetal liver, kidney and brain. None of the CD19+ B-cell samples showed detectable levels of *DLEU7* in contrast to the other tissues analyzed, where *DLEU7* expression could clearly be detected.

### 3.6. *The* DLEU7 *promoter is methylated in B-CLL cells*

The lack of *DLEU7* expression, despite the presence of unmutated *DLEU7* alleles in B-CLL cells, could be due to epigenetic changes, such as methylation. To test this hypothesis the promoter region of *DLEU7* was analyzed for methylation in CLL cells.

Sequence analysis of bisulfite-modified DNA from six purified CLL cases within the *DLEU7* 5′ genomic region allowed us to determine that the CpG dinucleotides most frequently methylated in these samples occurred at the sites of *Taq*I or *Hha*I restriction enzymes. These restriction enzymes, which contain CpG dinucleotides within their cutting sites, will thus only cut methylated samples that have retained these dinucleotides following bisulfite treatment. A total of 157 purified CLL cell DNA samples were then analyzed by PCR amplification using bisulfite-specific *DLEU7* primers (Table

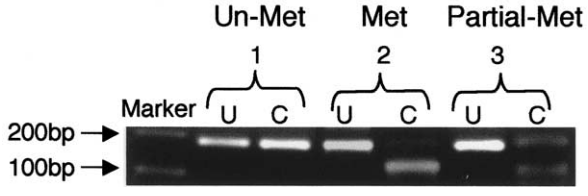


Fig. 5. Analysis of the methylation status of the *DLEU7* promoter in leukemic DNA from CLL patients. U (uncut) shows a PCR fragment of the *DLEU7* promoter prior to digestion with restriction enzyme (*Hha*I). C (cut) shows the same PCR fragment following digestion with restriction enzyme. Sample 1 could not be cut by *Hha*I, indicating an unmethylated status of the *DLEU7* promoter. Sample 2 is digested by *Hha*I indicating a methylated status of the promoter and sample 3 is partially cleaved by *Hha*I indicating a partial methylation of the promoter.

Table 2
Methylation status of the *DLEU7* promoter in primary CLL samples

| Methylation status of the *DLEU7* promoter | CLL patients (%) |
|---|---|
| Methylated | 95 (61) |
| Partial methylation | 22 (14) |
| Unmethylated | 40 (25) |
| Total number of CLL patients | 157 |

1) and restriction enzyme digestion. Of these samples the majority, 61% (95/157), resulted in the digestion of the majority of the PCR product with either *Taq*I or *Hha*I, indicating the presence of methylated CpG dinucleotides within the target sequence (Fig. 5 and Table 2). Approximately 25% (40/157) of the samples did not cut following *Taq*I or *Hha*I digestion, indicating that the region was not methylated in these cases, while the remaining 14% (22/157) of cases resulted in a ~50% digestion of the PCR product, possibly showing evidence of partial methylation of this region or that *DLEU7* methylation did not occur throughout every cell in the purified sample (Fig. 5 and Table 2). There was no significant difference between the methylation in heterozygous 13q deleted compared to non-13q deletion CLL cases.

Methylation analysis of samples from another B-cell malignancy with frequent 13q deletions was also carried out, with 25 cases of mantle cell lymphoma showing methylation in 30% (8/25) of the cases and 20% (5/25) being unmethylated at this locus. The remaining 12 samples (45%) gave a mixed pattern.

Interestingly, two B-cell samples purified by flow sorting from healthy donors gave differing results compared to the CLL samples. One sample (CD5+ CD27−) showed evidence of hypomethylation while another sample containing just CD19+ sorted B-cells showed a mixed methylation result. A sample derived from a pre-B-acute lymphocytic leukemia case on the other hand was methylated at this locus. Nine B-cell malignancy-derived cell lines, mainly lymphoma-derived cell lines, including NCEB-1, Karpas231 and WI33, were also screened for *DLEU7* methylation, all which were found to be methylated at this locus.

## 4. Discussion

The high frequency of both heterozygous and homozygous loss of chromosome 13q14 in B-CLL patients strongly indicates that this region contains a gene(s) involved in the development of this malignancy [7]. None of the three genes *DLEU1*, *DLEU2* and *RFP2*/*LEU5* located in the consensus CDR at 13q14 have, however, been found mutated or methylated in hemizygous loss CLL cases, which would fit with the classical two-hit model for tumor suppressor genes [23,28,29]. The absence of somatic mutations was also described for a series of centromerically located genes, *CLLD6*, *CLLD7* and *CLLD8* [30]. These results either indicate that the classical tumor suppressor mechanism involving hemizygous deletion and mutation of the remaining allele may not underlie the alterations found at this locus, or may simply reflect the fact that the true pathogenic gene at 13q14 has not yet been identified. Some studies pointing towards a slightly more distal region in B-CLL [3,16] indicate that an adjacent telomeric gene could be of importance.

In the present study we have cloned and characterized a novel candidate gene, *DLEU7*, located just outside the consensus deleted CLL region. The *DLEU7* gene is transcribed in a telomeric to centromeric direction with its 3′ end overlapping the 3′ end of the *DLEU1* gene, which is located within the minimal deleted region at 13q14. Both *DLEU1* and the centromeric *DLEU2* lack any convincing ORF, and these genes have been proposed to act at the RNA rather than the protein level. Indeed, we have recently shown that the *DLEU2* transcript (GenBank accession number AY455757) can act as an antisense for the adjacent bicistronic protein encoding *LEU5*/*RFP2* gene (GenBank accession number AY455758) (unpublished data). The fact that *DLEU1* and *DLEU7* have overlapping 3′ ends may indicate that these genes could be functionally related as well, although no directly overlapping exons could be demonstrated.

Northern blot analysis has shown that *DLEU7* is alternatively spliced with five major transcripts. The larger transcripts indicate the possibility that additional exons exist, not yet cloned. These exons might be located towards the CDR, since it is known that a longer 3′ UTR is the most common reason for significantly larger isoforms [31]. *DLEU7* was found to have the capability to encode a 221 amino acid protein, with high conservation in mouse and rat (Fig. 3). The dleu7 protein does not contain any known functional domains that could indicate a possible function of the protein. However, small portions of an ATPase domain and a CRM1 β-importin domain were identified. The functional significance of the partial motifs is unclear, but dleu7 may function as a nuclear protein.

To investigate the role of this novel candidate gene in B-CLL, the mutational and the expression status was examined. However, no specific alterations were discovered in any tumor sample. Despite ubiquitous *DLEU7* expression in human tissues, *DLEU7* was not detectably expressed in any of the CLL patient samples analyzed, regardless of the presence or absence of chromosome 13q14 deletions. Since the exact normal counterpart of B-CLL is unclear [1], it is difficult to address if the lack of expression of *DLEU7* in B-CLL cells just reflects the lack of transcription of this gene in the normal counterpart of B-CLL. The expression level of *DLEU7* has however been analyzed in CD19+ B-cells purified from healthy donors. These cells did not show any detectable expression of *DLEU7*. The lack of *DLEU7* expression in CLL cells could be associated with this finding. Further studies should aim at analyzing the transcription status of *DLEU7* in cells that might be the normal counterpart of B-CLL cells, such as normal naive or memory B-lymphocytes.

The hypermethylation of promoter regions of tumor suppressor genes is a frequent finding and has often been found to be associated with transcriptional non-expression of these genes. For example, CpG island methylation has been described for RB1, VHL, BRCA1, E-cadherin, p15INK4A and p73 [18,32]. In the case of *DLEU7*, the gene contains a clear CpG island overlapping exon 1. The conservation of this region in mouse and rat is significant (Fig. 1), strengthening the notion that this area functions as a true promoter for the *DLEU7* gene. We found that this region was hypermethylated or showed a mixed methylation pattern in 75% of cases (Fig. 5 and Table 2), a finding consistent with the lack of *DLEU7* expression seen in the CLL cases tested. Methylation at CpG islands tends to be a stochastic process and occurs in silenced chromatin. Partial methylation is also frequently seen, since

individual sites have a variable probability of methylation within an overall methylated CpG island [33]. Although our methylation-specific analysis was based on initial studies evaluating the frequently methylated nucleotides across the *DLEU7* promoter by sequencing of bisulfite-treated DNA, the final analysis of a large set of CLL patients only reflects the methylation status at certain residues in the CpG island analyzed. It is possible that samples showing lack of methylation-specific digestion might be methylated at other sites not analyzed in detail in the larger set of CLL patients. Alternatively, the lack of expression of *DLEU7* also seen in cases where no promoter methylation could be detected may be correlated with histone modifications rather than changes at the DNA level. Future studies determining the histone status of this region could thus be of interest.

It is interesting to note that conserved binding sites for a number of transcription factors can be found in the *DLEU7* promoter, several of which have obvious roles in cell proliferation and differentiation as well as tissue-specific expression of genes. It is also notable that conserved binding sites exist for transcription factors whose regulation is altered in hematological malignancies, such as Tal1 and AML-1 [34,35]. It is thus conceivable that *DLEU7* regulation may be altered in these malignancies.

In summary, the present investigation describes the cloning and characterization of a novel conserved protein-encoding gene in the 13q14 deletion region. This candidate gene was found not to be expressed in primary CLL cells, which were also frequently found to have a hypermethylated *DLEU7* promoter. These studies form a basis for the further evaluation of the role of *DLEU7* in B-CLL and other malignancies.

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