

The collagen prolyl hydroxylases are novel transcriptionally silenced genes in lymphoma

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BACKGROUND: Prolyl hydroxylation is a post-translational modification that affects the structure, stability and function of proteins including collagen by catalysing hydroxylation of proline to hydroxyproline through action of collagen prolyl hydroxylases 3 (C-P3H) and 4 (C-P4H). Three C-P3Hs (nomenclature was amended according to approval by the HGNC symbols and names at <http://www.genenames.org/> and Entrez database at <http://www.ncbi.nlm.nih.gov/gene>) leucineproline-enriched proteoglycan (leprecan) 1 (*Leprel1*), leprecan-like 1 (*Leprel1*), leprecan-like 2 (*Leprel2*) and two paralogs Cartilage-Related Protein (*CRTAP*) and leprecan-like 4 (*Leprel4*) are found in humans. The C-P4Hs are tetrameric proteins comprising a variable α subunit, encoded by the *P4HA1*, *P4HA2* and *P4HA3* genes and a constant β subunit encoded by *P4HB*.

METHODS: We used RT-PCR, qPCR, pyrosequencing, methylation-specific PCR, western blotting and immunohistochemistry to investigate expression and regulation of the *C-P3H* and *C-P4H* genes in B lymphomas and normal bone marrow.

RESULTS: C-P3H and C-P4H are downregulated in lymphoma. Down-regulation is associated with methylation in the CpG islands and is detected in almost all common types of B-cell lymphoma, but the CpG islands are unmethylated or methylated at lower levels in DNA isolated from normal bone marrow and lymphoblastoid cell lines. Methylation of multiple *C-P3H* and *C-P4H* genes is present in some lymphomas, particularly Burkitt's lymphoma.

CONCLUSIONS: Methylation of *C-P3H* and *C-P4H* is common in B lymphomas and may have utility in differentiating disease subtypes.

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Non-Hodgkin lymphomas (NHLs) are a heterogeneous group of lymphoid neoplasms that display distinct cytogenetic and molecular genetic features (Piva *et al*, 2010; Gutierrez-Garcia *et al*, 2011; Salaverria and Siebert, 2011; Visco *et al*, 2012). Studies of recurrent chromosome aberrations in NHL have provided significant insight into genetics of lymphomagenesis. Most common translocations are t(14;18) in follicular lymphoma (FL), t(11;14) in mantle cell lymphoma (MCL), t(3;14) in diffuse large B-cell lymphoma (DLBCL) and t(8;14) in Burkitt's lymphoma (BL) (Ong and Le Beau, 1998; Chaganti *et al*, 2000). In addition to structural changes, transcriptional silencing is another major mechanism of tumour suppressor gene inactivation in human cancer, including haematological malignancies (Lee *et al*, 2010; Eberle *et al*, 2011). A number of mechanistically important genes have been identified as targets for methylation-dependent transcriptional silencing in lymphomas, including the cyclin-dependent kinase inhibitors

CDKN2A and *CDKN2B*, the p53 homologue *TP73*, the DNA damage-activated protein kinase *DAPK* and other genes with putative tumour suppressor functions (Belaud-Rotureau *et al*, 2008; Guan *et al*, 2010; Murray *et al*, 2010). Using suppression subtraction PCR, we previously identified the Polo-like kinase *PLK2* to be transcriptionally silenced in NHL, with a particularly high frequency of methylation in BL (Syed *et al*, 2006). However, epigenetic changes in some NHL types, for example FL and MCL remain to be elucidated.

Prolyl hydroxylation is an important post-translational modification that affects the structure and function of proteins. A major substrate of prolyl hydroxylation is collagen and appropriate post-translational processing of collagen requires both C-P3H and C-P4H, respectively. Three C-P3H enzymes have been identified in humans: leucineproline-enriched proteoglycan (leprecan) 1 (*Leprel1*), leprecan-like 1 (*Leprel1*) and leprecan-like 2 (*Leprel2*) (Wassenhove-McCarthy and McCarthy, 1999); Vranka *et al*, 2004). Prolyl 3-hydroxylation typically occurs in the Gly-3Hyp-4Hyp sequence of collagen and P3H modified residues are abundant in basement membrane collagens (Gryder *et al*, 1975). Initially, *Leprel1* was shown to localise predominantly in

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the ER and Golgi, but more recently has been detected in tissues rich in basement membranes and shown to participate in the hydroxylation of type IV collagen (Jarnum *et al*, 2004; Tiainen *et al*, 2008). We have previously shown that *Leprel1* and *Leprel2*, but not *Leprel1*, are subject to methylation-dependent transcriptional silencing in breast cancer. Further, whereas methylation in *Leprel2* was also present in multiple tumour types, methylation in *Leprel1* appeared specific to breast cancer as the CpG island was unmethylated in several other carcinomas (Shah *et al*, 2009). The human genome contains two paralogs of the P3H genes, namely Cartilage-Related Protein (*CRTAP*) and leprecan-like 4 (*Leprel4*). Although *CRTAP* is not enzymatically active, it is required *in vivo* for collagen prolyl 3-hydroxylation (Morello *et al*, 2006). The biological importance of prolyl hydroxylation in the post-translational modification of collagen is demonstrated by the observation that germ-line mutations in *CRTAP* and *Leprel1* cause recessive forms of osteogenesis imperfecta and loss or decrease of type I collagen prolyl 3-hydroxylation (Cabral *et al*, 2007; Baldrige *et al*, 2008) and mutation in *Leprel1* is associated with inherited myopia (Mordechai *et al*, 2011). Prolyl 4-hydroxylation is essential for the biosynthesis of collagens because 4-hydroxyproline residues are critical for stability of the collagen triple helix. The human C-P4Hs are tetrameric isoenzymes composed of two β subunits (encoded by *P4HB*) and two (catalytic) α subunits. There are three different α subunits encoded by *P4HA1*, *P4HA2* and *P4HA3*. The β subunit is identical to the protein disulphideisomerase. In contrast to the C-P3Hs, no disorders of collagen synthesis have thus far been associated with heritable change in genes encoding the C-P4Hs. As well as collagen, both C-P3Hs and C-P4Hs may have additional substrates that contain collagen-like domains.

Here, we have investigated expression and regulation of the collagen prolyl hydroxylase genes in lymphoma.

MATERIALS AND METHODS

Cell lines and tumours

NHL cell lines and EBV-immortalised B lymphoblastoid cell lines (LCL) were maintained in RPMI 1640 (Gibco, Invitrogen, Carlsbad, CA, USA) medium containing 10% fetal bovine serum. The following cell lines were used in our study:

Mantle cell lymphoma cell line: JVM2; Transformed FL cell lines: DoHH-1, DoHH-2 and SU-DHL-4. DLBCL cell lines: CRL, DHL-4, DHL-7, LK6; EBV – ve BL: BL2, BL41, DG75, Louckes and Ramos; EBV + ve BL: AW Ramos, Akata 2000, MAK-I, Rael, Namalwa, Raji, Wewack 1, P3HR1 c16 and Jijoye; EBV – ve BL because of loss of EBV: Akata 31; LCLs from peripheral blood: B301, LCL3, X50-7, LCL-K, BM + Akata, PF + B95-8 and CR + B95-8. LCL from cord blood: C2 + BL16.

The study received approval from the Ethics Committee of Ioannina University Hospital, Ioannina, Greece. Formalin fixed, paraffin-embedded tissue samples were retrieved from the archives of the Pathology Department, University Hospital of Ioannina, Ioannina, Greece. As control tissues, we used bone marrow (BM) aspirates from individuals with non-malignant disease that contained no neoplastic cells. In all cases, the diagnosis was confirmed following review by an expert haematopathologist. Genomic DNA was isolated by digestion in proteinase K following xylene treatment of the tissue sections. Primary breast carcinomas (from the Tayside Tissue Bank) and breast and ovarian carcinoma cell lines were described previously (Shah *et al*, 2009).

Methylation reversal

Exponentially growing cells were treated with azacytidine (AZA; Sigma, St Louis, MO, USA) for 5 days. Cells were split every 2 days with the addition of fresh drug. After drug treatment, mRNA was isolated using the RNeasy kit (Qiagen Ltd., West Sussex, UK).

Analysis of gene expression

Total RNA was extracted from exponentially growing cultured cells using RNazolB or Trizol. In all, 500–1000 ng of total RNA was converted to cDNA and used for analysis of gene expression by RT-PCR. GAPDH was used as a control gene. Primers were designed using Primer3 software. Primer sequences for RT-PCR were

Leprel1:	Forward:	5'-CTGCAGCACACACCTTCTTC-3'
	Reverse:	5'-ACAGCTTCCTGTGGCTGTTTC-3'
Leprel1:	Forward:	5'-AATGTGTGAGGGAACCTGCCAC-3'
	Reverse:	5'-CGGGTCAATGCTATCATCCAGC-3'
Leprel2:	Forward:	5'-ATGCTCAGGTGGGCAATCTGTC-3'
	Reverse:	5'-TCAAGGGCTTTGGCTTTCAGT-3'
CRTAP:	Forward:	5'-GGGTTGAAGGCAAGAGGAGAAAG-3'
	Reverse:	5'-GCTGAAGGAGAAACAGAGGATGTG-3'
Leprel4:	Forward:	5'-AGCACGGAGGACATGGAC-3'
	Reverse:	5'-GCTCAAATTTGGCCTCAGCT-3'
P4HA1:	Forward:	5'-CCCTAAGGCAACTGGATGAAGG-3'
	Reverse:	5'-ATTTTGATACCCTCCACACGGC-3'
P4HA2:	Forward:	5'-GGTGAAGCGGCTAAACACAGAC-3'
	Reverse:	5'-CAGTCATCCACACTCAGCATTGC-3'
P4HA3:	Forward:	5'-TTCAACAACCCCTGTGGCTAAC-3'
	Reverse:	5'-GTCATCCCTGTGAGAGAAAAGAG-3'
P4HB:	Forward:	5'-CAAAACAGTTGGCTCCCATTT-3'
	Reverse:	5'-CCCCGTTGTAATCAATGACC-3'
GAPDH:	Forward:	5'-TGCCTCTGCACCACCAACT-3'
	Reverse:	5'-CGCCTGCTTACCACCTTC-3'

Reactions were resolved on 1.2% agarose gels and visualised on a transilluminator after staining with ethidium bromide. GAPDH was co-amplified as a control gene using primers previously described (Syed *et al*, 2006).

Total RNA was extracted from 10 μ m paraffin sections of archival lymphoma cases using commercial systems (RecoverAll Total Nucleic Acid Isolation kit, Ambion, Carlsbad, CA, USA). For qPCR analysis, 25 μ l PCR reactions were performed using 50 ng of cDNA obtained by reverse transcription. Amplification and analysis were done according to the manufacturer's protocol in 96-well plates in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and the pre-cast 'TaqMan Gene Expression Assays' (Applied Biosystems, <https://products.appliedbiosystems.com/>). Quantification of target transcripts was performed in comparison to the reference transcript β 2 microglobulin (Hs99999907_m1), using the 'delta-delta C_t method for comparing relative expression results in real-time PCR as outlined by PE Applied Biosystems. Western blotting for *Leprel1* and *Leprel2* was done as described previously (Shah *et al*, 2009).

Methylation analysis

CpG islands are located in the 5' end of the *Leprel1*, *Leprel1* and *Leprel2* genes (Shah *et al*, 2009). CpG islands were also identified in the 5' sequences of *Leprel4*, *CRTAP* and the *P4H* genes (<http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html>). Genomic DNA (1 μ g) was modified by sodium bisulphite as described previously (Syed *et al*, 2006; Shah *et al*, 2009). Methylation-specific PCR (MSP) primers for the *Leprel1*, *Leprel1* and *Leprel2* CpG islands were as described previously (Shah *et al*, 2009). Primer sequences for MSP analysis of the remaining genes were as follows:

<i>CRTAP</i> :	
Unmethylated	forward: 5'-TTTTATGGGATGAGTTGATGTTGT-3'
Unmethylated	reverse: 5'-AACTAATCTCCAAATAACCCACACT-3'
Methylated	forward: 5'-TTTACGGGACGAGTTGATGTC-3'
Methylated	reverse: 5'-AACTAATCTCCAAATAACCCACG-3'

<i>Leprel4</i> :	
Unmethylated	forward: 5'-TGGGTATGTTTGGAGTAGTATGA-3'
Unmethylated	reverse: 5'-CAATTAAACATAACAAAAACCTCACT-3'

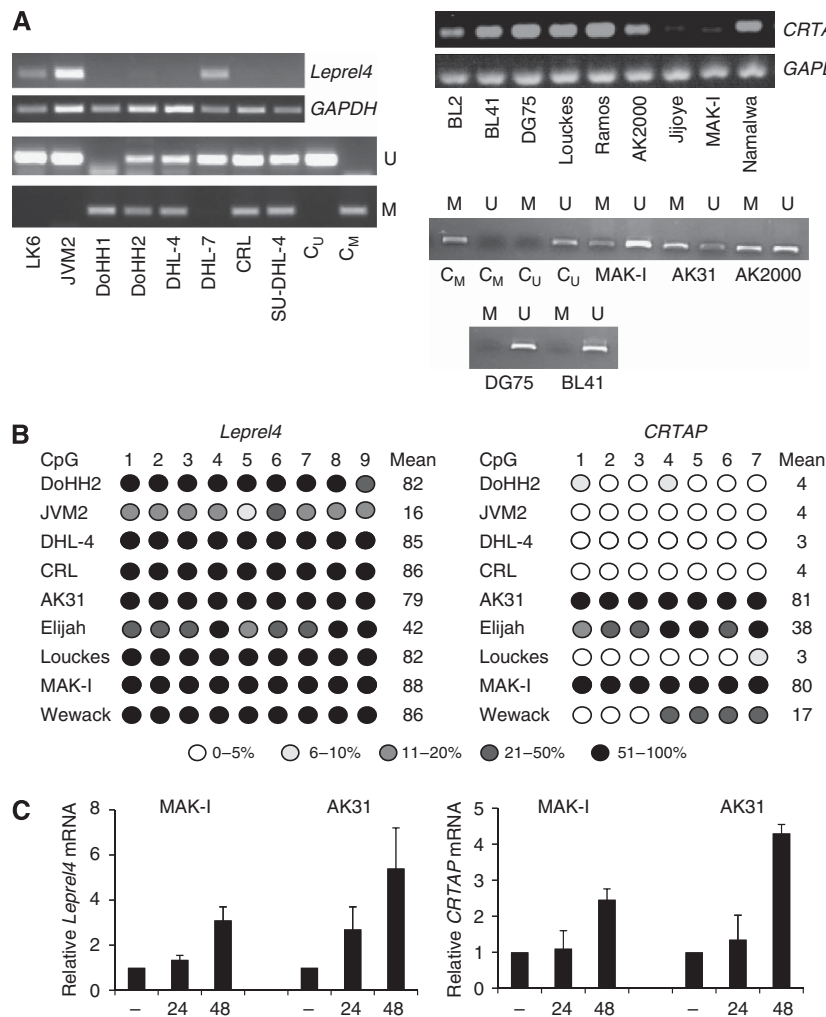


Figure 1 *Leprel4* and *CRTAP* are down-regulated by methylation in NHL. **(A)** RT-PCR and MSP analysis of expression and CpG island methylation of *Leprel4* and *CRTAP* in NHL cell lines. Expression of *Leprel4*, *CRTAP* and *GAPDH* was determined as described in Materials and Methods. The MSP figure shows U and M specific reactions for the indicated NHL cell lines. Also shown is control methylated (C_M) and control unmethylated (C_U) genomic DNA modified in parallel with cell line DNA samples. **(B)** Pyrosequencing analysis of *Leprel4* and *CRTAP* CpG islands in NHL cell lines. The % methylation at each analysed CpG dinucleotide is represented by the intensity of shading in the circles as shown in the figure, together with the mean % methylation for each cell line. **(C)** Demethylation reactivates expression of *Leprel4* and *CRTAP*. MAK-I and AK31 cells (*Leprel4* and *CRTAP* CpG islands methylated) were grown without AZA (—) or in the presence of AZA for 24 or 48 h as indicated and expression of *Leprel4* and *CRTAP* determined by qPCR.

Methylated	forward: 5'-GTACGGGTACGTTTGGAGTAGTAC-3'
Methylated	reverse: 5'-ACAATTAACGTAACAAAAACCTCG-3'

P4HA1:

Unmethylated forward: 5'-GGATTGTTGTAGTTGTTTGAGAAGGTTTAT-3'
 Unmethylated reverse: 5'-ACAATCACCAAAACACTAATATAATCAA-3'
 Methylated forward: 5'-CGTCGTAGTCGTTTCGAGAAGGTTTACG-3'
 Methylated reverse: 5'-GCAATCGCCGAAACGCTAATATAATCG-3'

P4HA2:

Unmethylated forward: 5'-GTTGTAGTTTATTGTATGTAITTTGTGT-3'
 Unmethylated reverse: 5'-CAAATCATAACAACCTCTCCAAACACC-3'
 Methylated forward: 5'-GTTCTAGTTTATCGTACGTATTTGCGGT-3'
 Methylated reverse: 5'-CGAATCGTAACGACCTCTCCAAACG-3'

P4HA3:

Unmethylated forward: 5'-TTGAGGTTTTTGGTGTTTTATTGTG-3'
Unmethylated reverse: 5'-CTCAAAACAACACATTCTCAACACT-3'
Methylated forward: 5'-TTTTTCGGTCGTTTTTATTCGTCGT-3'
Methylated reverse: 5'-GAAACGACACGTTCTCGACGCT-3'

P4HB:

Unmethylated forward: 5'-TTAGGTTTGGTTTCGGATTCGGAAAC-3'
Unmethylated reverse: 5'-AAACAACGAACGCGAAATCCTCGAC-3'

Methylated forward: 5'-TTAGGTTTGGTTTTGGATTGGAATGT-3'
Methylated reverse: 5'-CTAAAAAAAAACAACACAAAATCCTCA-3'

Methylation was also analysed quantitatively by pyrosequencing. Primer sequences were as follows:

Leprel1: Forward: 5'-GGGAGTGGGGAGGATAAG-3'
Reverse: 5'-CCTCCCTAACCTACAACATA-3'

Leprel1: Forward: 5'-GGGGTTGTTTATAGTGG-3'
Reverse: 5'-AAATACCCCAAAACCCCTC-3'

Leprel2: Forward: 5'-GTAGATTGTTGATTAGTG-3'
Reverse: 5'-CCACCTAATAATAAACCCCTC-3'

CRTAP: Forward: 5'-GATGTATAATAATTTATAGGG-3'
Reverse: 5'-CCAACACCTAAAAAATACC-3'

Leprel4: Forward: 5'-CTGGAGGAAGGTGTAGG-3'
Reverse: 5'-TTATCTTCAACCCAAACA-3'

P4HA1: Forward: 5'-GGTGTGTGTGGTAATTTGGG-3'
Reverse: 5'-AACTAAAAACCAATAACAACC-3'

P4HA2: Forward: 5'-GTTTTTTTGTGTAGTTTTTGGG-3'
Reverse: 5'-CTCTCCCTACAACCCC-3'

P4HA3: Forward: 5'-GGTATAGTGTATTTTATTGAG-3'
Reverse: 5'-ACTACTAAACTACTAAAC-3'

PCR conditions were as follows: Initial activation step at 95°C for 10 min, denaturation 95°C for 30 s, annealing 55°C (*Leprel1*)/52°C (*Leprel1*)/54°C (*Leprel2*)/52°C (*Leprel4*)/51°C (*CRTAP*)/55°C (*P4HA1*)/54°C (*P4HA2*)/49°C (*P4HA3*) for 30 s for 30 s, extension 72°C for 40 s and final extension 72°C for 7 min. PCR was performed for 40 cycles after which PCR products were electrophoresed through 2% agarose gel, and visualised using a transilluminator. The PCR products were then analysed by pyrosequencing using the Biotage Sample Prep kit (Diatech, Jesi, Italy). Analysis of percentage methylation at each CpG dinucleotide was performed using Pyromark ID Software (Biotage). Placental DNA (Sigma) was used as negative control for methylation (0% average methylation) and a commercial methylated DNA (Millipore, Billerica, MA, USA) was used as positive control (98% average methylation).

Immunohistochemistry

Expression of *Leprel1* and *Leprel2* was analysed by immunohistochemistry (IHC) performed using standard techniques (with antigen retrieval) on archival lymphoma cases from the archives of the Pathology Department, University Hospital of Ioannina, Ioannina, Greece as described above in cell lines and tumours.

Statistics

We used descriptive statistics and Spearman's rank correlation test to assess the association between methylation and down-regulation of expression. All statistical analyses were performed using Prism 5 (GraphPad software, Inc., La Jolla, CA, USA).

RESULTS

Methylation-dependent transcriptional silencing of *Leprel 4* in NHL

We first analysed expression of *Leprel4* in a panel of NHL cell lines. *Leprel4* transcript was expressed in LK6, JVM2 and DHL-7 and all LCL analysed but was undetectable in the remaining cell lines (Figure 1A and data not shown). Inspection of *Leprel4* revealed the presence of a CpG island in the 5' sequences of the gene (<http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html>), suggesting the

possibility that expression is epigenetically regulated and we analysed the CpG island of *Leprel4* in each cell line using MSP. In the NHL cell lines lacking expression of *Leprel4* mRNA, there was methylation in the CpG island analysed but the CpG island was unmethylated in each cell line which expressed *Leprel4* mRNA (Figure 1). To validate the MSP analysis, we further characterised methylation in the *Leprel4* CpG island using a quantitative technique, pyrosequencing. Representative pyrograms for genes analysed in this study are shown in Supplementary Figures 1 and 2. There was dense methylation in the CpG island in CRL, DoHH-2 and DHL-4 (all cell lines positive by MSP), but only a low level of methylation in JVM2 (negative by MSP) (Figure 1). Next, we analysed methylation in a series of BL cell lines. There was frequent methylation in the *Leprel4* CpG island, with 13/16 cell lines showing dense methylation by pyrosequencing (Figure 1; Tables 1A and b). Exposure to AZA reactivated expression in MAK-I and AK31, consistent with methylation-dependent silencing (Figure 1). Normal BM control DNA was negative for *Leprel4* methylation in 4/4 cases (Tables 1A). *Leprel4* mRNA was expressed and the CpG island uniformly unmethylated in breast and ovarian cancer cell lines and primary breast carcinomas (Supplementary Figure 3).

CRTAP methylation in NHL

These results prompted us to analyse expression of the *Leprel4* homologue *CRTAP* in the same cell line panel. *CRTAP* mRNA was abundantly expressed in LCL and the majority of NHL cell lines analysed, but at lower levels in Jijoye, AK31, AK2000, Elijah, MAK-I and BL2 (Figure 1). Like *Leprel4* the 5' sequences of *CRTAP* contain a CpG island. Methylation-specific PCR analysis of the CpG island revealed dense methylation in five of the seven cell lines with down-regulated expression, but methylation was not detected by MSP in BL2 and Jijoye (Figure 1). To validate the MSP analysis, we further characterised methylation in the *CRTAP* CpG

Table 1B Sensitivity and specificity of analysis of C-P3H and C-P4H genes in diagnosis of BL vs DLBCL

Gene	<i>Leprel1</i>	<i>Leprel2</i>	<i>P4HA2</i>	<i>P4HA3</i>
Sensitivity	0.92	1.0	0.92	1.0
Specificity	0.54	0.93	0.87	0.68

Table 1A Frequency of methylation of CpG islands of C-P3H and C-P4H genes in lymphoma

	<i>Leprel1</i>	<i>Leprel2</i>	<i>Leprel4</i>	<i>CRTAP</i>	<i>P4HB</i>	<i>P4HA1</i>	<i>P4HA2</i>	<i>P4HA3</i>
Cell lines								
Non-BL	0/4	4/4	2/4	1/4	0/4	0/4	0/4	4/4
BL	0/15	16/16	12/16	13/16	4/16	0/15	0/15	15/15
LCL	0/10	0/10	1/10	1/6	0/6	0/10	0/10	2/10
Clinical cases								
DLBCL	2/100	86/100	8/100	10/100	16/100	0/36	0/36	15/100
HIV DLBCL	0/8	6/8	2/8	3/8	2/8	0/8	0/8	3/8
FL	2/11	9/11	0/11	0/11	0/11	0/11	0/11	2/11
MCL	1/10	4/10	1/10	0/10	1/10	0/10	0/10	1/10
MZL	1/28	14/28	0/28	0/28	0/28	0/28	0/28	4/28
BL	1/9	8/9	9/9	4/9	1/9	0/9	0/9	9/9
HIV BL	0/4	4/4	4/4	2/4	1/4	0/4	0/4	4/4
Normal	0/4 ^a	0/4 ^b	0/4 ^b	0/4 ^a	0/4 ^a	0/4 ^a	0/4 ^a	0/4 ^a

Abbreviations: BL = Burkitt's lymphoma; DLBCL = diffuse large B-cell lymphoma; FL = follicular lymphoma; MCL = mantle cell lymphoma; MZL = marginal zone lymphoma; MSP = methylation-specific PCR. ^aDetermined by MSP. ^bDetermined by pyrosequencing.

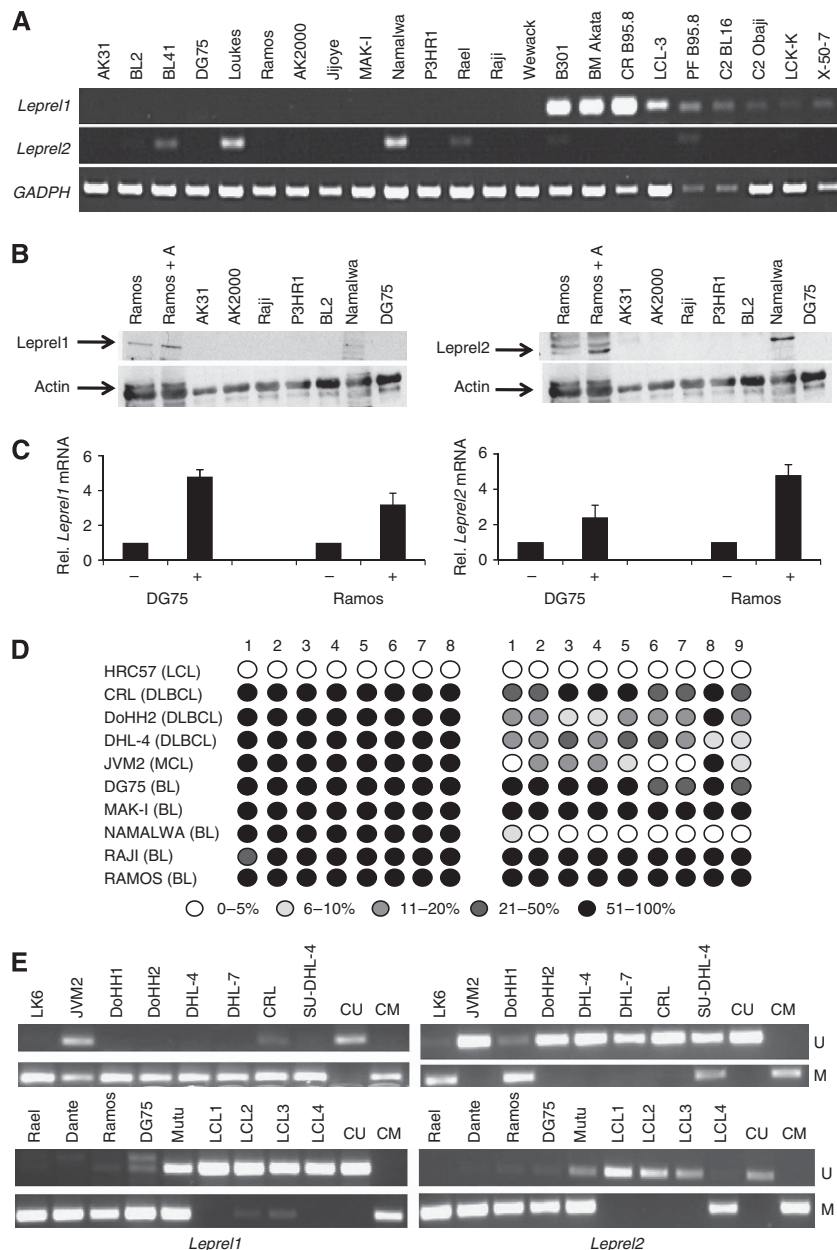


Figure 2 *Leprel1* and *Leprel2* are frequently co-methylated in BL cell lines. **(A)** RT-PCR analysis of *Leprel1* (upper panel), *Leprel2* (middle panel) and *GADPH* (lower panel) in BL cell lines and LCL. **(B)** Western blot analysis of *Leprel1* and *Leprel2* in BL cell lines. Ramos + A indicates Ramos cells treated with AZA. **(C)** Demethylation reactivates expression of *Leprel1* and *Leprel2* in BL cell lines. DG75 and Ramos cell lines were grown without AZA (–) or in the presence of AZA (+) for 48 h and expression of *Leprel1* and *Leprel2* determined by qPCR. **(D)** Quantitative analysis of methylation in *Leprel1* and *Leprel2* CpG islands in NHL cell lines. Cell lines were subjected to pyrosequencing analysis as described in Materials and Methods. The number denotes each individual CG dinucleotide in the amplified fragment analysed by pyrosequencing. Five levels of methylation are represented, the level of methylation increasing with increasing intensity of shading in the circles as indicated. **(E)** MSP analysis of *Leprel1* and *Leprel2* CpG island methylation in NHL cell lines. MSP was performed as described in Materials and Methods. The figure shows U and M specific MSP reactions for the indicated NHL (upper panels) and BL (lower panels) cell lines. Also shown is control unmethylated (CU) and control methylated (CM) genomic DNA modified in parallel with cell line DNA.

island using pyrosequencing. Methylation in LCL was uniformly low, typically 2–5% at each analysed CG (see for example BM Akata, Figure 1). We did not find evidence of methylation in *CRTAP* in the other NHL cell lines (DLBCL, FL and MCL) by either MSP or pyrosequencing (Figure 1; Table 1A). Exposure to AZA reactivated expression in MAK-I and AK31, consistent with methylation-dependent silencing (Figure 1). The *CRTAP* CpG island was unmethylated in breast carcinoma cell lines and ovarian carcinoma cell lines and primary breast carcinomas (Supplementary Figure 3).

Leprel1 and *Leprel2* silencing in NHL cell lines

Leprel4 and *CRTAP* have three paralogs in the human genome *Leprel1*, *Leprel1* and *Leprel2*, each of which has a CpG island (Shah et al, 2009). RT-PCR revealed expression of *Leprel1* mRNA in each LCL, with undetectable expression in all BL analysed (Figure 2) and in each of the four non-BL NHL cell lines (data not shown). Western blotting was consistent with RT-PCR (Figure 2). *Leprel2* mRNA was undetectable by RT-PCR in the majority of BL cell lines (Figure 2) but was detectable in the four non-BL NHL cell

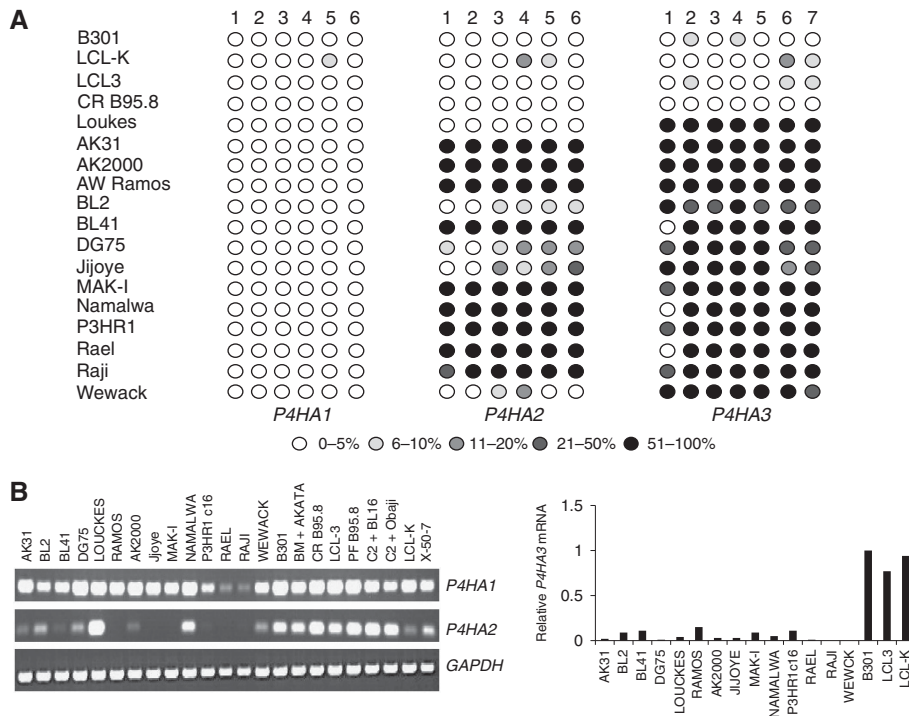


Figure 3 Analysis of *P4H* genes in BL cell lines. **(A)** Pyrosequencing analysis of CpG island methylation in C-*P4H* genes in BL and LCL (B301, LCL-K, LCL3 and CR B95.8). Pyrosequencing was performed as described in Materials and Methods. The degree of methylation at each analysed CG dinucleotide is represented by the intensity of shading in the circles as shown in the figure. **(B)** RT-PCR analysis of *P4H* gene expression in BL cell lines. Expression of *P4H* genes and GAPDH was determined as described in Materials and Methods. **(C)** qPCR analysis of *P4HA3* expression. Expression is shown relative to B301.

lines (data not shown). AZA reactivated expression of *Leprel1* and *Leprel2* mRNA in DG75 and Ramos, consistent with methylation-dependent silencing and western blotting confirmed increased *Leprel2* in Ramos cells treated with AZA (Figure 2). We performed pyrosequencing in the panel of NHL cell lines to analyse methylation in each CpG island (Figure 2). The *Leprel1* CpG island was methylated to a high degree in all NHL cell lines analysed, whereas *Leprel2* CpG island was methylated to a high degree in the majority of BL cell lines but to a lower extent in the non-BL NHL cell lines (Figure 2). Methylation in both CpG islands was low in LCLs (Figure 2). There was an inverse correlation between expression of both *Leprel1* and *Leprel2* and methylation of their CpG islands (Figure 2). As seen with *Leprel4* and *CRTAP*, there was also a good correlation between methylation detected by pyrosequencing and MSP (Figure 2). *Leprel1* was expressed and the CpG island unmethylated in all analysed lymphoma cell lines (Supplementary Figure 4).

Specific C-*P4H* genes are silenced by methylation in NHL cell lines

The frequent methylation of the C-*P3H* genes in NHL prompted us to also analyse expression of the C-*P4H* genes in the same panel of cell lines. The mRNA of *P4HA1*, *P4HA2* and *P4HB* was abundantly expressed in each of the LCL tested (Figure 3). *P4HA1* and *P4HB* were expressed in all BL cell lines at levels comparable to the LCL but the mRNA of *P4HA2* was down-regulated in many of the BL cell lines (Figure 3). *P4HA3* was expressed at low levels in all BL cell lines but was detectable using qPCR and was down-regulated in all BL cell lines analysed (Figure 3). We tested using pyrosequencing whether absence of expression was associated with aberrant methylation in the CpG islands of each C-*P4H* gene. Methylation was low in the CpG island of each gene in LCLs (Figure 3). Consistent with expression results, the *P4HA1* and *P4HB* CpG islands were uniformly unmethylated. The CpG island

of *P4HA2* was methylated to a high degree in all of the BL except DG75 and Loukes and there was a good correlation between methylation of and expression of *P4HA2* (as assessed by RT-PCR) and methylation (Figure 3). The CpG island of *P4HA3* was densely methylated in all cell lines (Figure 3). Together these results imply that expression of *P4HA2* and *P4HA3* is silenced by methylation in BL cell lines. In the non-BL NHL cell lines, the CpG islands of *P4HB*, *P4HA1* and *P4HA2* were unmethylated, but the *P4HA3* CpG island was, as in BL, uniformly methylated (Figure 4).

Methylation of multiple *P3H* and *P4H* genes in clinical cases

Our cell line studies showed that some of the C-*P3H* and C-*P4H* genes are down-regulated by methylation in B lymphomas. We therefore analysed methylation in the CpG island of each gene in a clinical series comprising all common types of B lymphomas, including BL, DLBCL, FL, MCL and MZL (Table 1A). As control tissues, we used BM aspirates from individuals (with non-neoplastic diagnoses), confirmed on histopathology to contain no malignant cells. To fully validate our MSP assays, we initially analysed a small series of cases by both pyrosequencing and MSP and this showed an excellent correlation between the two methodologies (Figure 5A). We also confirmed that methylation is associated with transcriptional down-regulation and this was the case (see Figure 5B for representative scatter plots for *Leprel1* and *Leprel2*).

We then proceeded to analyse the clinical cases using the validated MSP assays (for representative assays see Figure 6; for summary of data see Table 1A and Supplementary Figure 5). Methylation was not detected in any of the nine analysed genes in the normal BM controls. *Leprel4* was methylated exclusively in high-grade lymphomas, the highest frequency being in BL. Similarly, *CRTAP* was methylated predominantly in high-grade

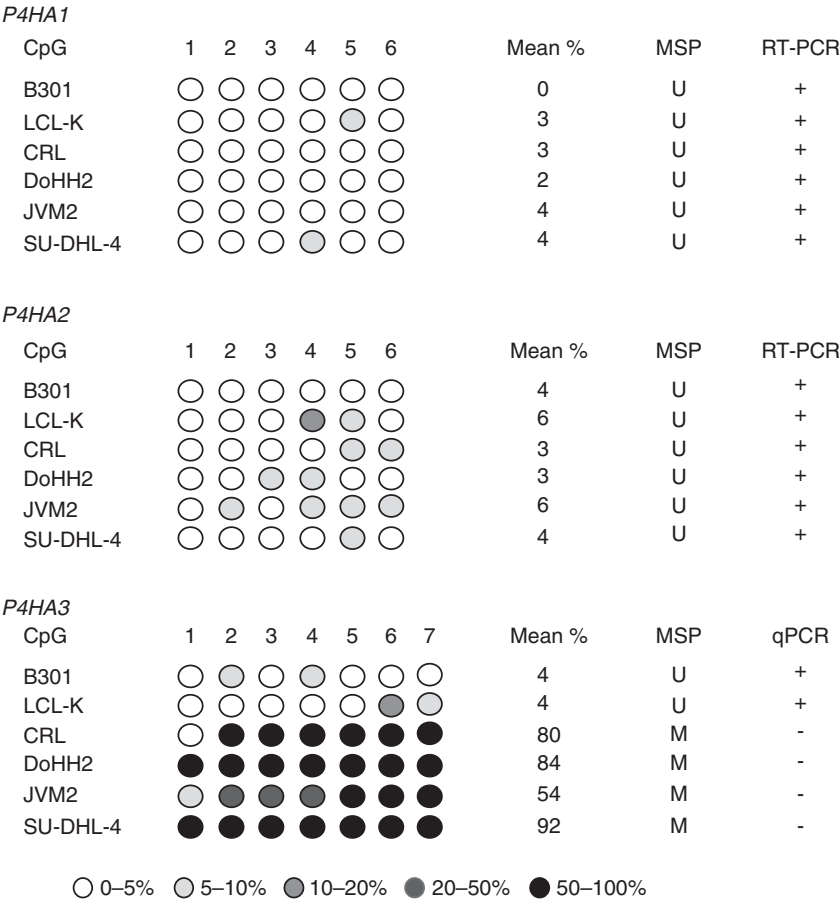


Figure 4 Analysis of methylation in C-*P4H* gene CpG islands in NHL cell lines. The figure shows pyrosequencing analysis of the CpG islands of each *P4H* gene in NHL cell lines and LCL. The degree of methylation at each analysed CG dinucleotide is represented by the intensity of shading in the circles as indicated in the figure. Also shown in the mean % methylation of the analysed fragment and the result of expression analysis (RT-PCR or qPCR).

lymphomas with the highest frequency in DLBCL, although a single case of MCL showed methylation. *Leprel1* was clearly the most frequently methylated of the *C-P3H* genes, with methylation in the *Leprel1* CpG island detected in 86/100 (86%) of DLBCL and 9/11 (82%) of FL. *Leprel2* methylation was infrequent and was detected only in DLBCL (at low frequency) and in BL (at high frequency) but not in other lymphoma types. Although methylation in the CpG island of *Leprel1* was not detected in NHL cell lines, we nonetheless observed methylation in all lymphoma types, albeit at low frequencies, in the clinical series (Table 1A). In the case of the *P4H* genes, we did not detect methylation in *P4HA1* or *P4HB* in any lymphoma analysed, consistent with analysis of cell lines. *P4HA2* was methylated predominantly in BL, whereas *P4HA3* was methylated to a high degree in all types of NHL and BL (Table 1A). To seek evidence that CpG island methylation does indeed result in reduced protein expression, we performed IHC for *Leprel1* and *Leprel2* (Figure 6B). In these studies, we observed down-regulation of both proteins in all common B lymphoma types, consistent with qPCR and methylation analyses, confirming that transcriptional silencing is associated with reduced or absent protein expression in lymphoma.

Analysis of C-*P3H* and C-*P4H* genes discriminates DLBCL from BL

Inspection of the combined data set (Table 1A; Supplementary Figure 5) showed that *Leprel1* and *P4HA3* are methylated at high frequency in the majority of high-grade NHL, both in cell lines and in clinical cases of lymphoma, whereas *P4HA2* and *Leprel2* are

methylated predominantly in BL and less frequently in DLBCL. We constructed ROC curves to address the possibility that methylation analysis of specific *C-P3H* and *C-P4H* genes facilitates discrimination between (i) NHL and LCL (ii) BL and DLBCL. In cell lines, methylation of *Leprel1*, *Leprel2* and *P4HA3* clearly discriminated between NHL and LCL with high sensitivity and specificity but for *P4HA2* the sensitivity was lower, consistent with lower frequency of methylation in non-BL lymphomas (Figure 7). In clinical lymphomas, methylation of *Leprel2* and *P4HA2* was a sensitive and specific discriminator between BL and DLBCL (Figure 7). We further assessed MSP results to determine whether non-quantitative analysis of methylation can also distinguish BL from DLBCL. Using MSP, sensitivity and specificity for both *Leprel2* and *P4HA2* were similar to values obtained from pyrosequencing (Table 1B). Together, these results from two independent techniques suggest that methylation profiling of the CpG islands of *Leprel2* and *P4HA2* BL and DLBCL can be useful in distinguishing between BL and DLBCL. Finally, to further extend these observations, we asked whether immunohistochemical analysis of *Leprel2* discriminates DLBCL from BL. Consistent with methylation analysis, expression of *Leprel2* was absent or greatly down-regulated in BL at a significantly higher frequency than in DLBCL ($P < 0.05$).

DISCUSSION

We show that expression of the C-*P3H* and C-*P4H* collagen prolyl hydroxylases is epigenetically downregulated in NHL cell lines and primary lymphomas. Our data is the first demonstration of

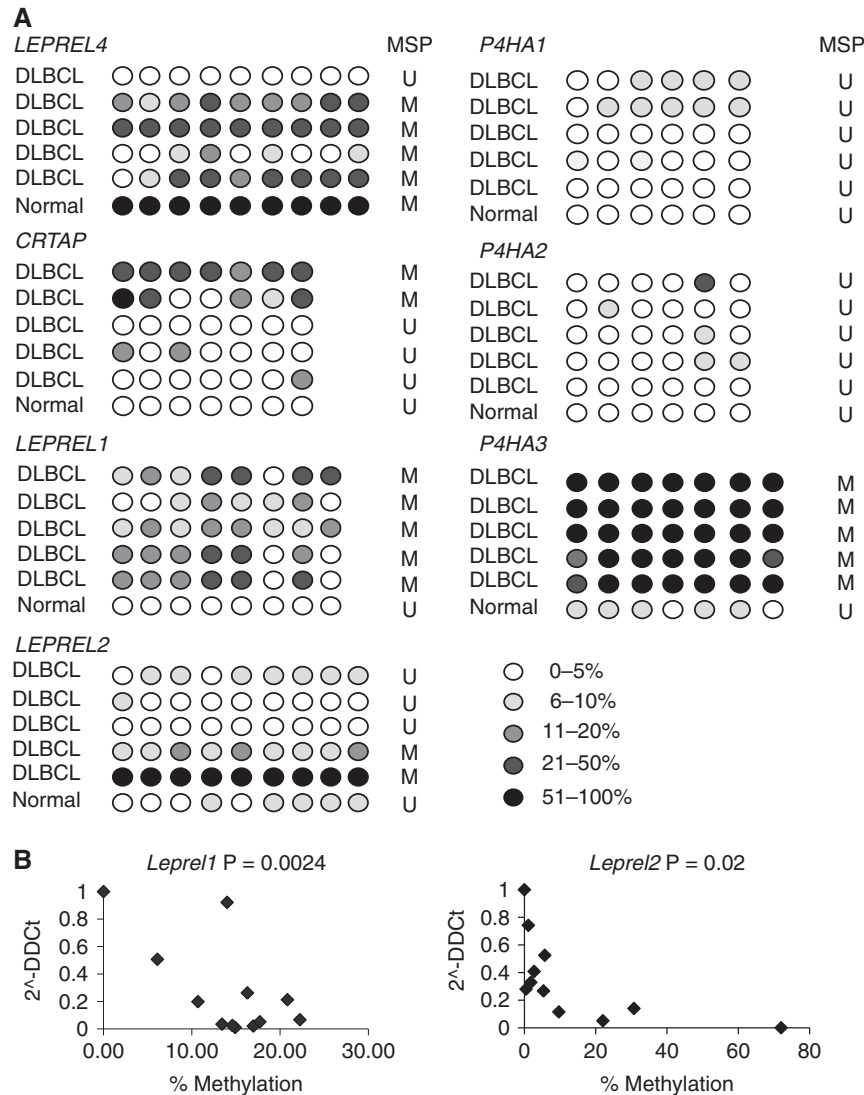


Figure 5 Analysis of methylation in *P3H* and *P4H* genes in primary DLBCL. **(A)** Representative profiles of CpG island methylation for each *C-P3H* and *C-P4H* gene in clinical DLBCL cases. Pyrosequencing was performed as described in Materials and Methods. Five levels of methylation are represented, the level of methylation increasing with increasing intensity of shading in the circles as shown in the figure. The result of simultaneous MSP analysis is indicated. **(B)** CpG island methylation correlates with down-regulation of expression in clinical cases of DLBCL. Scatter plots are shown of mRNA vs mean % CG methylation for *Leprel1* and *Leprel2* in DLBCL.

epigenetic silencing of the *C-P3H* and *C-P4H* genes in lymphoid neoplasia and to the best of our knowledge the first study describing epigenetic changes in the *C-P4H* genes in human cancer. Our study of the *C-P3H* and *C-P4H* genes was prompted by initial identification of *Leprel4* as a novel epigenetically regulated gene in lymphoma. This led us to investigate four paralogous *C-P3H* genes in the human genome (*CRTAP*, *Leprel1*, *Leprel2* and *Leprel4*) and the *C-P4H* genes *P4HB*, *P4HA1*, *P4HA2* and *P4HA3*, all of which encode proteins with functions in collagen biosynthesis. We demonstrate methylation-dependent transcriptional silencing of specific members of each gene family in B-cell lymphoma cell lines and primary lymphomas.

In the case of the *C-P3H* family members, *Leprel1* was silenced at high frequency in all lymphoma types, with lower methylation frequencies in the CpG islands of each of the other *C-P3H* genes aside from BL in which we observed simultaneous silencing of *Leprel1*, *Leprel2*, *Leprel4* and *CRTAP* in a high proportion of cases (both sporadic and HIV-associated). In previous work, we showed that *Leprel2* is silenced in numerous epithelial tumour types, but

silencing of *Leprel1* was restricted to breast cancer with a strong preference for oestrogen receptor positive carcinomas (Shah *et al*, 2009). We did not detect methylation in the CpG islands of *Leprel4* or *CRTAP* in breast and ovarian cancer cell lines or in primary breast cancers, suggesting that methylation in these genes is specific for haematological neoplasia. It is striking therefore that *Leprel1* is transcriptionally silenced in the majority of B lymphomas irrespective of subtype. Immunohistochemistry confirmed down-regulation of *Leprel1* and *Leprel2* (albeit with different frequencies) in clinical lymphoma cases.

In the case of the *C-P4H* family, we detected CpG island methylation only in *P4HA2* and *P4HA3*, methylation never being seen either in *P4HA1* or in *P4HB* (encoding the β subunit of the *C-P4H* tetramer). Of note, whereas the *P4HA3* CpG island was methylated in a high proportion of lymphomas of all types, that of *P4HA2* was methylated predominantly in BL and there was simultaneous methylation of *P4HA2* and *P4HA3* in many cases of BL. This parallels the situation with the *C-P3H* genes with *Leprel1* and *Leprel2*, which are co-silenced at high frequency in BL.

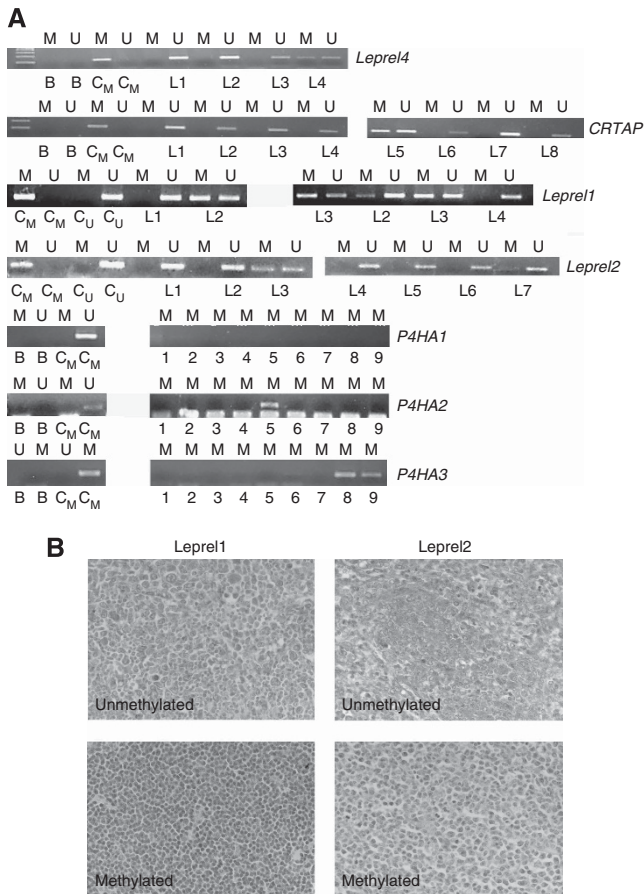


Figure 6 Analysis of the CpG islands in *C-P3H* and *C-P4H* genes in clinical lymphoma cases. **(A)** Examples of MSP analyses for *C-P3H* and *C-P4H* genes. For *Leprel4*, *CRTAP*, *Leprel1* and *Leprel2* reactions with unmethylated (U) and methylated (M) primer pairs are shown. For *P4HA1*, *P4HA2* and *P4HA3* cases are analysed with M primers only. For all genes, specificity and sensitivity of the MSP is demonstrated by the control DNA amplifications. Abbreviations: C_M = control methylated DNA; C_U = control unmethylated DNA. **(B)** Representative immunohistochemical analysis of *Leprel1* and *Leprel2* in B lymphomas. Cases with and without methylation in the respective CpG islands are shown.

It remains to be determined whether this reflects differences between *P4HA2* and *P4HA3* in terms of substrate specificity or affinity.

Previous studies have linked germ-line mutations in *Leprel1* and *CRTAP* to rare forms of the collagen disorder osteogenesis imperfecta (Morello *et al*, 2006; Cabral *et al*, 2007; Baldrige *et al*, 2008), mutations resulting in reduced 3-hydroxylation of the α chain of type I collagen. It is an interesting hypothesis that transcriptional silencing of the *C-P3H* and *C-P4H* genes may contribute to lymphomagenesis as a result of aberrant collagen biosynthesis and/or post-translational modification causing basement membrane malformation or other collagen abnormalities which promote neoplasia. Alternatively, failure of prolyl hydroxylation of one or more of the other identified substrates for these proteins could contribute to neoplasia in cells lacking specific *C-P3H* and *C-P4H*. A third possibility is that *P3H* and *P4H* have direct growth inhibitory properties. This has been directly demonstrated for *Leprel1*, *Leprel2* and *Leprel3* (Kaul *et al*, 2000; Shah *et al*, 2009). Further, mice lacking both *CRTAP* alleles exhibit increased proliferation in some tissues, also consistent with an anti-proliferative function (Baldrige *et al*, 2010). Resolution of these hypotheses will require detailed mechanistic studies.

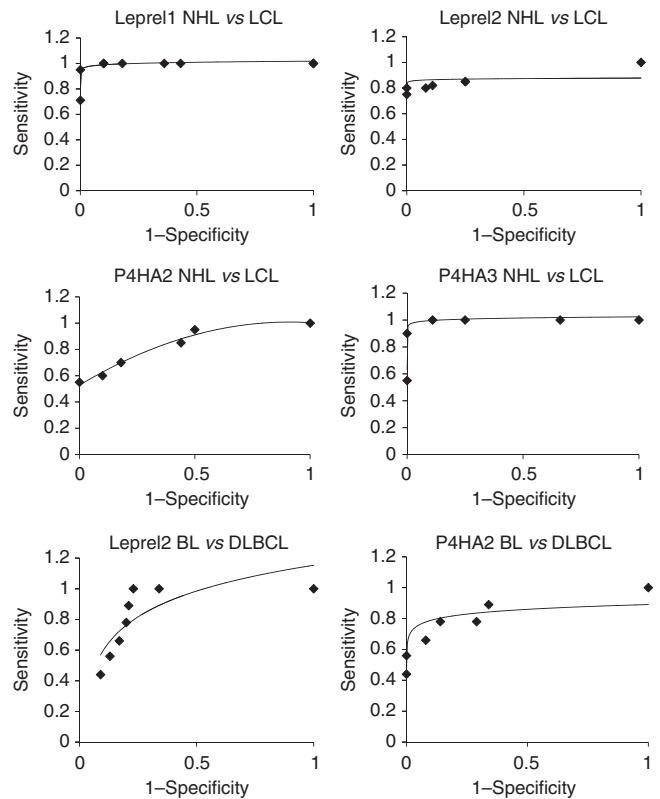


Figure 7 Methylation in *C-P3H* and *C-P4H* CpG islands discriminates NHL from LCL and BL from DLBCL. ROC curves demonstrating utility of analysis of methylation in CpG islands of *C-P3H* and *C-P4H* genes to distinguish BL from DLBCL.

Even with modern histopathology, immunoprofiling and molecular genetics, it is on occasion challenging to differentiate between BL and DLBCL (de Leval and Hasserjian, 2009). This is of major clinical importance because first-line chemotherapy for the two lymphoma subtypes is very different. Differences in methylation frequency between BL and DLBCL, especially in *Leprel2* and *P4HA2*, led us to address the possible utility of these genes as biomarkers to differentiate between the lymphoma types. We show that analysis of CpG island methylation in both *Leprel2* and *P4HA2* discriminates between BL and DLBCL with high sensitivity and specificity. In preliminary studies, we showed using IHC that analysis of *Leprel2* expression in BL and DLBCL may also have biomarker utility. Clearly, larger numbers of cases will require analysis in order to independently verify these observations.

In conclusion, we report the identification of *C-P3H* and *C-P4H* as novel transcriptionally silenced gene families in B lymphomas. Additional studies are required in order to validate the initial findings presented in this report and to further elucidate the possible role of transcriptional silencing of the *C-P3H* and *C-P4H* genes in lymphomagenesis.

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