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The cyclin D1 alternative transcripts [a] and [b] are expressed in normal and malignant lymphocytes and their relative levels are influenced by the polymorphism at codon 241

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Background and Objectives. The cyclin D1 gene, CCND1, is alternatively spliced to produce transcripts [a] and [b] in a manner apparently modulated by a polymorphism (A/G) at codon 241. Studies have indicated that the polymorphism can affect the prognosis of patients with different types of solid tumors. This study aimed to determine the relative levels of transcripts [a] and [b] in normal and malignant peripheral blood mononuclear cells (PBMNC), and to investigate whether these were influenced by the polymorphism. The impact of the polymorphism on the survival of a group of mantle cell lymphoma (MCL) patients was also to be studied.

Design and Methods. The polymorphism was genotyped, using restriction fragment length polymorphism analysis, in 74 patients (42 MCL, 19 chronic lymphocytic leukemia, 13 normal controls) and the relative level of transcripts [a] and [b] determined using a competitive reverse transcription polymerase chain reaction method. Kaplan-Meier survival curves and the log-rank test were used to analyze the survival data.

Results. Of the cases genotyped, 39 were heterozygous for the polymorphism, 24 homozygous G and 11 homozygous A. Both transcripts [a] and [b] were expressed in normal PBMNC and malignant lymphocytes, with the polymorphism affecting their relative levels. Neither the predominant transcript, nor genotype, significantly influenced survival of the MCL patients studied.

Interpretation and Conclusions. Contrary to previous reports, patients who were homozygous A at the polymorphism produced more transcript [a] whilst homozygous G patients had more transcript [b]. In the small cohort studied, the polymorphism did not appear to affect the prognosis of the patients with MCL.

Key words: cyclin D1, alternative transcripts, mantle cell lymphoma, lymphocytes, polymorphism

The *CCND1* gene located at 11q13 encodes the protein cyclin D1. This protein plays a vital role in regulation of the cell cycle through its interactions with the cyclin dependent kinases.[1] Although cyclin D1 is classed as a proto-oncogene,[2] it does require co-operation with other transforming factors, and is therefore regarded as a weak oncogene.[3,4] It is, however, found to be overexpressed in a number of different tumor types including carcinomas of breast, liver and parathyroid, and in mantle cell lymphoma (MCL).[5] Several mechanisms are responsible for the upregulation of expression including chromosome translocations, inversions and gene amplification.[5]

In 1995 a frequent polymorphism (A/G) was identified in the final codon of exon 4 of the gene.[6,7] Although the base change does not alter the amino acid sequence, the base is integral to the splice donor site between exon 4 and intron 4. This finding led to the discovery of an alternatively spliced cyclin D1 transcript, transcript [b].[6] Transcript [b] differs from the previously reported cyclin D1 transcript (now called transcript [a]); exon 5 is missing, there is no splicing at the exon 4 – intron 4 boundary and the mRNA sequence continues downstream into intron 4.[6] Exon 5 encodes for the polypeptide sequence enriched in proline (P), glutamic acid (E), serine (S) and threonine (T) (PEST) which targets proteins for rapid destruction. It was therefore speculated that the protein translated from transcript [b] may have a longer half-life and play an important biological role, especially when overexpressed in tumors. However, in B-cell lines the ratio of transcripts [a]:[b] and the ratio of the resulting proteins have been shown to be the same,[8] leaving the role of the PEST sequence in transcript [a] open to question. The pattern of expression is also unclear; some reports suggested that it could be biased towards either tran-

script [a] or transcript [b],[6,9] whereas others found significant amounts of transcript [b] only in B-cell lymphoma with a t(11;14).[8,10] Studies on normal and malignant lung tissue have indicated that the differential splicing is modulated by the polymorphism.[6]

In this study we amplified transcripts [a] and [b] using a competitive reverse transcription-polymerase chain reaction (RT-PCR) method, and investigated the influence of the polymorphism on their relative levels. Normal peripheral blood mononuclear cells (PBMNC) and malignant lymphocytes, including cases of MCL that overexpress cyclin D1, were studied.

Design and Methods

Patients' details

Peripheral blood (PB) (from various times during the course of the disease) or lymph node samples (at diagnosis) were obtained from 74 patients, with their informed consent. Of these, 42 had MCL, 19 chronic lymphocytic leukemia (CLL) and 13 were age-matched control patients with non-malignant disorders and a white blood cell count within the normal range.

All MCL patients had either a t(11;14) (demonstrated by PCR or cytogenetics) or overexpressed cyclin D1 mRNA by competitive RT-PCR.[11] The median age of the MCL patients was 68 years (range 40-89) and the male:female ratio was 36:6. Only the MCL patients with Ann Arbor stage IV disease who had a significant number of malignant cells in the PB (n=28) were used to study the levels of transcripts [a] and [b]. Of the patients used in the survival analysis 7 are dead (median time to death 3 months, range 1-23 months) and 20 are currently alive (median time from diagnosis 18.5 months, range 1-100 months). These patients were not entered into a specific drug trial but treated according to the protocol of the hospital in which they were managed.

The CLL patients fulfilled the diagnostic criteria defined by the National Cancer Institute.[12] The median age of the CLL patients studied was 64 years (range 30-78) and the male:female ratio was 10:9. Eight patients had Binet stage A disease at the time of study, 8 stage B and 3 stage C disease. All the patients had a lymphocytosis of $>10\times10^6$/mL at the time of study.

DNA and RNA extraction

All the blood samples were initially processed to isolate PBMNC using Lymphoprep (Life Technologies, Paisley, UK).

DNA was extracted from PBMNC or fresh lymph nodes using NucleoSpin columns (Machery-Nagel, Düren, Germany) according to the manufacturer's protocols.

RNA was extracted from 5×10^6 PBMNC from the MCL and the CLL patients using Trizol (Life Technologies) or RNA columns (Machery-Nagel). In the control group of patients the MNC isolated from 5 mL PB were divided into 2 aliquots for RNA and DNA extraction.

PCR primers

Primer sequences and their locations are shown in Table 1. The RT-PCR primer positions were chosen to avoid areas of homology with cyclin D2 and D3.

Genotype of the exon 4-intron 4 boundary polymorphism

DNA samples from all the MCL, CLL and PBMNC controls were genotyped for the exon 4-intron 4 boundary polymorphism using restriction fragment length polymorphism (RFLP) analysis. DNA was amplified using primers 26 and 27 and the following PCR reaction mix; 1 × PCR buffer, 200 μM dNTPs, 100 ng of each primer, 1.5 mM $MgCl_2$, 1 U Platinum Taq (Life Technologies) and 2μL of DNA in a final 25mL volume. Cycling conditions were as follows: 94°C for 1 min followed by 30 cycles of 94°C, 1 min; 55°C, 1 min and 72°C, 1 min. The PCR product was digested by the direct addition of 10 U of Nci I (New England Biolabs, Hitchin, UK) and incubated at 37°C for at least 1 hour. Digested products were run on 3% agarose gels containing ethidium bromide, revealing bands of 145bp and 22bp when a G was present and a single band of 167bp when an A was present (Figure 1).

Competitive RT-PCR amplification of transcripts [a] and [b]

The relative levels of transcripts [a] and [b] were determined in 28 of the 42 MCL patients and all of the CLL and PBMNC samples. All 28 of the MCL patients had sufficient malignant cells in the PB to

Table 1. CCND1 primer sequences.

Primer	*Sequence 5' to 3'*	*Position*	
RT-PCR			
AL	GAACAAACAGATCATCCGCA	nt 663-682 Forward	Exon 3
AR	AGGGACTGTCATGTGGAGCA	nt 1110-1091 Reverse	3' UTR
BR	ATTTCCGTGGCACTAGGTGT	nt 940-921 Reverse	Intron 4*
Genotyping			
26*	GTGAAGTTCATTTCCAATCCGC	nt 721-742 Forward	Exon 4
27*	GGGACATCACCCTCACTTAC	nt 890-871 Reverse	Intron 4*

*CCND1 sequence accession number M64349; *primers and intron 4 sequence from Betticher et al.[6]*

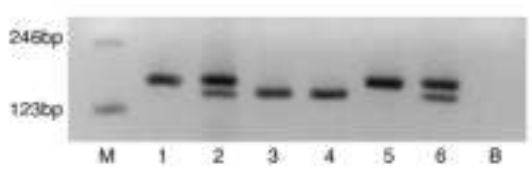

Figure 1. RFLP analysis of polymorphism genotype. M: molecular weight marker; Lane 1: undigested PCR product; Lane 2: heterozygous; Lane 3: homozygous G; Lane 4: homozygous G; Lane 5: homozygous A; Lane 6: heterozygous; B: water control.

cause RT-PCR detectable overexpression of cyclin D1.[11]

cDNA was synthesized from 10 μL of RNA using random hexamers (or oligo dT) and Moloney-Murine Leukemia Virus (M-MLV) reverse transcriptase (Life Technologies).

A competitive multiplex RT-PCR was employed to determine the relative level of transcripts [a] and [b]. A shared upstream primer (AL) was used in conjunction with two downstream primers, one specific to transcript [a] (AR) and the other to transcript [b] (BR). Since primer BR is in intron 4, amplification of both DNA and transcript [b] cDNA would yield PCR products of the same size. Several CLL samples were tested for DNA contamination using mock cDNA (from reverse transcription reactions without M-MLV-reverse transcriptase) as template for the competitive RT-PCR.

After optimization of the PCR the following conditions were used; 1 × PCR buffer, 200 μM dNTPs, 25ng of each primer, 3 mM $MgCl_2$, 1M betaine (Sigma, Poole, UK), 2 μL of cDNA and 1.25U Platinum Taq in a final 25 μL volume. The following cycling conditions were employed: 96°C, 5mins followed by 40 cycles of 95°C, 30sec and 60°C, 30 sec with a final extension step of 72°C for 6 mins.

The PCR products were run on a 2% agarose gel containing ethidium bromide. The expected PCR product sizes for transcripts [a] and [b] were 448bp and 275bp, respectively. Gel pictures were scanned into a computer using a Umax flatbed scanner and VistaScan software (Umax, Willich, Germany). The net intensity of the bands was calculated using QuantiScan (Biosoft, Cambridge, UK) and the proportion of transcript [a] determined using the formula [a]/[a]+[b]. A mean and standard error was then calculated from at least three separate PCR reactions for each patient.

Table 2. Distribution of polymorphism genotypes amongst different subgroups of patients.

	AG	*GG*	*AA*	*Total*
MCL	23 (55%)	13 (31%)	6 (14%)	42
CLL	9 (47%)	7 (37%)	3 (16%)	19
Normal PBMNC	7 (54%)	4 (31%)	2 (15%)	13
Total	n=39	n=24	n=11	n=74

Validation of the competitive RT-PCR

PCR products of transcripts [a] and [b] were cloned using the pGem T-Easy vector system (Promega, Southampton, UK). Mini-prep DNA (Life Technologies) from these clones was used to determine that the two transcripts amplified with equal efficiency in the competitive PCR. The sensitivity of the PCR was also established using these clones.

Statistical analyses

Sufficient follow-up data were available on 27 of the 28 MCL patients used to study the levels of the transcripts [a] and [b]. Kaplan-Meier survival curves and the log-rank test were used to compare the survival of the classified groups.

Results

Genotype of the exon 4- intron 4 boundary polymorphism

The results of the RFLP analysis of the 74 patients in this study are shown in Table 2. A similar distribution of the different genotypes was observed between the patient groups and was comparable to those reported in other studies.[6,13]

Competitive RT-PCR amplification of transcripts [a] and [b]

Competitive RT-PCR of the two transcripts produced PCR products of the expected sizes (Figure 2). The identity of the two transcripts was confirmed by direct sequencing (Cambridge Biosciences, Cambridge, UK) using the shared upstream primer (AL). Any contaminating DNA was demonstrated, by PCR using the mock cDNA, to be insufficient to produce transcript [b] size product in the competitive RT-PCR.

Using the cloned transcripts [a] and [b] it was found that both amplified with equal efficiency and a three-fold excess of one transcript over the other would be detectable (Figure 3).

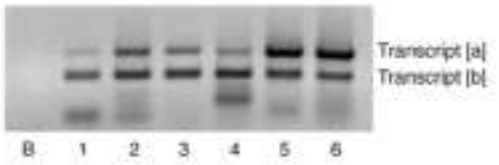

Figure 2. Amplification of transcripts [a] and [b] using the competitive RT-PCR. B water control; Lane 1 PBMNC; Lane 2 CLL 1; Lane 3 CLL 2; Lane 4 MCL 1; Lane 5MCL 2; Lane 6 repeat PCR of MCL 2.

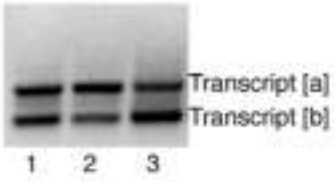

Figure 3. Amplification of the cloned transcripts [a] and [b] using the competitive RT-PCR. Lane 1: amplification of equal amounts of cloned transcript [a] and [b]; Lane 2: amplification of a three-fold excess of transcript [a]; Lane 3: amplification of a three-fold excess of transcript [b].

To check the reproducibility of our PCR, a blood sample from a MCL patient was divided in two aliquots of 5×10^6 cells and processed separately. The level of transcript [a], compared to the total amount of CCND1 transcript, was 0.60 and 0.58 for the two samples. Repeat PCR of each sample from every patient used in this study showed a standard error of <0.02. These experiments demonstrate that this PCR is robust and generates reproducible results.

Relative levels of transcripts [a] and [b] in normal and malignant lymphocytes

Using the competitive RT-PCR described, both transcripts [a] and [b] were expressed in the MCL, CLL and normal PBMNC samples (Figure 4). Different relative levels of the transcripts were seen in all 3 patient groups. Transcript [b] was observed in almost all lymphocyte samples, with a higher level of transcript [b] than transcript [a] in 4/13 of the normal PBMNC controls, 5/19 of the CLL and 13/28 of the MCL cases.

Influence of the exon 4- intron 4 boundary polymorphism on the relative levels of transcripts [a] and [b]

From Figure 4 it can be seen that the genotype of the polymorphism does influence the relative levels of the two transcripts. Of the homozygous G patients, 4/4 of the normal PBMNC controls had more transcript [b] than transcript [a], as did 5/7 of the CLL and 6/9 of the MCL patients. All the homozygous A patients (7/7) had more transcript [a]. Although in the majority of the homozygous cases there was considerable bias towards one transcript, in all but a couple of cases both transcripts were present.

Analysis of the heterozygous patients showed that all the normal and CLL samples had more transcript [a], but 7/17 MCL heterozygotes expressed more transcript [b].

Survival analysis of MCL cases

Clinical data were available on 27/28 of the MCL patients used to study the transcript [a] and [b] levels. Grouping patients according to the polymorphism genotype and comparing GG (n=9) to GA plus AA (n=18), as Betticher *et al.* did,[6] failed to show any difference in survival (p=0.55).

Dividing patients according to their predominant transcript type (transcript [a] n=15, transcript [b] n=12) also failed to show any significant difference in survival (p=0.17).

Median survival has not yet been reached in any of the groups analyzed.

Discussion

In this study we used a competitive multiplex RT-PCR to amplify the cyclin D1 transcripts [a] and [b] and determine their relative levels in PBMNC and malignant lymphocytes. Equal amplification efficiency of transcripts [a] and [b], under the PCR conditions described, was proven using clones of the transcripts. Results obtained were consistent (standard errors of <0.02), probably as a result of using a shared upstream primer. Others have shown that in PCRs using a shared upstream primer the relative amounts of product remain constant through both the log and plateau phases of the PCR.[11] Although the use of a shared upstream primer will exaggerate extremes, the method should give a reliable indication of the predominant transcript present.

Transcripts [a] and [b] were not only detected in the cyclin D1 overexpressing MCL cases, but also in CLL cells and normal PBMNC that do not overexpress cyclin D1. Our findings of the presence of both cyclin D1 transcripts in the normal PBMNC and CLL cells is in contrast to results of a previous study in which no cyclin D1 transcripts were found by PCR in the B-cell line, Raji, or the T-cell leukemia cell line, Jurkat.[8] Transcript [b] mRNA has been detected by PCR in a number of different tissue types and cell lines,[6,10] but its level relative to transcript [a] is unclear. Using Northern blotting and RT-PCR, only minute amounts of

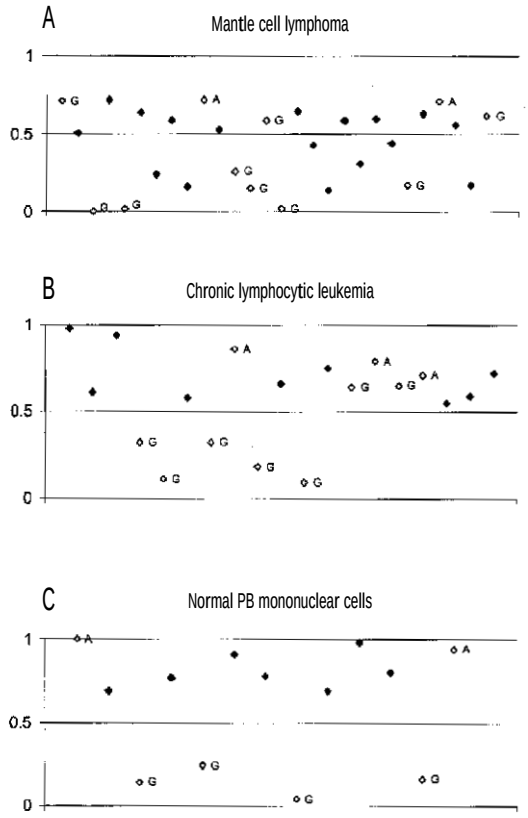

Figure 4. Genotype at the polymorphic base and relative levels of transcripts [a] and [b] for each patient. Open diamonds = homozygous patients; Black diamonds = heterozygous patients. The numbers on the y-axis refer to the relative amounts of transcript [a] and [b] calculated using the formula [a] / [a]+[b].

transcript [b] were found in breast cancer cell lines and primary breast cancer.[10] More significant amounts have been reported in lung tumors,[6] human glioma cell lines[9] and cell lines with a t(11;14).[8] Using our competitive RT-PCR, transcript [b] was observed in almost all lymphocyte samples, with relatively more transcript [b] compared to transcript [a] in a number of the normal PBMNC controls as well as in some of the CLL and MCL cases. We have demonstrated that, if the RT-PCR is sensitive enough, the cyclin D1 mRNA transcripts are detectable, even when the protein is not evident, such as in the CLL and PBMNC samples.

The study by Betticher *et al.*[6] showed that the polymorphism (A/G) at the exon 4 - intron 4 boundary (codon 241) modulates splicing of the cyclin D1 mRNA transcripts [a] and [b]. The results of our study indicate that, in homozygous patients, if the polymorphic bases are 'G' the predominant transcript is [b] and if they are 'A' the predominant transcript is [a], which is contrary to other studies.[6,9] One possible reason for the divergent results might be that different tissue types have been studied. Betticher *et al.* investigated lung tissue[6] whilst the study of Sawa *et al.* was limited to three glioma cell lines and did not include a homozygous G line.[9] Our demonstration that both transcripts [a] and [b] occur in the majority of homozygous patients, albeit with a considerable bias towards one transcript, confirms the finding that both alleles can splice to form both transcripts.[6]

A comparison of the consensus splice donor sequence and the cyclin D1 splice donor site sequence between exon 4 and intron 4 is illustrated in Table 3. The polymorphism in the cyclin D1 gene affects the base at position –1. Replacement of the G at –1 with an A should not prevent splicing in itself since the base at +5 is also important.[14] Three classes of splice site exist; class A in which there is a G at both –1 and +5, class B with a G at –1 only and class C in which there is a G at +5 only. With the cyclin D1 gene intron 4 splice site, even when the nucleotide at –1 is an A, a class C splice site still exists as there is a G at +5. Further comparison of the splice site sequence shows that cyclin D1 also differs from the consensus sequence at –2 with the more common A being replaced by a C. The presence of a C at –2 creates a CG dinucleotide, a known mutation hot spot,[15] and one could speculate that this gave rise to the polymorphism. The divergence from the consensus sequence at this base could also lead to sub-optimal splicing because of the reduced stability of spliceo-

Table 3. Comparison of consensus donor splice site sequence and CCND1 donor splice site sequence between exon 4 and intron 4.

Nucleotide position	*-4*	*-3*	*-2*	*-1*	*+1*	*+2*	*+3*	*+4*	*+5*	*+6*
Consensus sequence *	C_{29}/A_{34}	C_{38}/A_{35}	A_{62}	G_{77}	G_{100}	T_{100}	A_{60}	A_{74}	G_{84}	T_{50}
CCND1 sequence	C	C	C	G/A#	G	T	A	A	G	T

**Sequence from Krawczak et al.[15] #Position of CCND1polymorphism. Subscript numbers refer to percentage frequency of occurrence.*

some binding.[14]

Several studies have demonstrated that the polymorphism has an impact on the histology and prognosis of some tumors,[6,16] as well as on the age of onset of hereditary non-polyposis colorectal cancer.[17] Relapse-free survival was longer in resected non-small cell lung cancer patients who had a GG genotype compared to in those who were AG and AA.[6] In contrast, a GG genotype was associated with a shorter disease-free interval in patients with laryngeal and pharyngeal tumors,[16] suggesting that the tissue type is also relevant. In our study the survival of MCL patients with a GG genotype was almost identical to that in patients with an AG or AA genotype. A recent study also found that the polymorphism had no impact on the survival of patients with colorectal cancer.[13] However, dividing MCL patients according to their genotype is complicated by the presence of the t(11;14) which will upregulate one allele. In heterozygous patients the predominant transcript will be dependent on which of the alleles is affected by the translocation. As a result of the competitive RT-PCR described in this paper, we were able to divide patients according to the predominant transcript. When the survival analysis was repeated using this criterion to categorize patients there was a trend towards shorter survival for the patients with more transcript [b], although this was not significant. This result does suggest that the predominant transcript type, rather than genotype, might be the more influential factor with regard to survival. This might explain some of the differences seen in the various cancers studied to date, since most analyses concentrate solely on genotype[6,16] and use previous work[6] to infer the predominant transcript, rather than determining it for their particular patients.

Given the generally poor prognosis of MCL, it is perhaps not surprising that neither the genotype of the polymorphism, nor predominant transcript type, had a significant influence on the survival of these patients. Data on a larger group of patients with MCL are required. It would also be interesting to repeat the exercise on a larger group of patients with CLL, a more indolent disease.

Contributions and Acknowledgments

Both authors contributed equally to the work described in the paper, and the contributions of others are detailed in these acknowledgments.

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Potential implications for clinical practice

The RT PCR method developed will allow extension of our small study to investigate a wider range of MCL patients for any correlation between CCND1 transcripts [a] and [b] expression and disease progression, which alongside the new therapies emerging for MCL could dictate treatment regimes.

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