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Dysregulated expression of the major telomerase components in leukaemic stem cells

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Telomere loss is rapid during the progression of chronic myeloid leukaemia (CML) and correlates with prognosis. We therefore sought to measure expression of the major telomerase components (hTR and hTERT) in CD34$^+$ cells from CML patients and normal controls, to determine if their altered expression may contribute to telomere attrition *in vivo*. High-purity (median 94.1%) *BCR-ABL*$^+$ CD34$^+$ cells from CML ($n=16$) and non-CML ($n=14$) patients were used. CML samples had a small increase in telomerase activity (TA) compared to normal samples (approximately 1.5-fold, $P=0.004$), which was inversely correlated with the percentage of G_0 cells ($P=0.02$) suggesting TA may not be elevated on a cell-to-cell basis in CML. Consistent with this, hTERT mRNA expression was not significantly elevated; however, altered mRNA splicing appeared to play a significant role in determining overall full length, functional hTERT levels. Interestingly, Q-RT-PCR for hTR demonstrated a mean five-fold reduction in levels in the chronic phase (CP) CML samples ($P=0.002$), raising the possibility that telomere homeostasis is disrupted in CML. In summary, the molecular events regulating telomerase gene expression and telomere maintenance during the CP of CML may influence the disease progression observed in these patients.

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Introduction

Chronic myeloid leukaemia (CML) is a clonal myeloproliferative disorder that arises in a haemopoietic stem cell (HSC). At diagnosis, the leukaemic cells are characterised by the Philadelphia (Ph) chromosome and *BCR-ABL* oncogene.[1] Typically, the disease follows three relatively distinct phases: chronic phase (CP) lasting on average 5 years, accelerated phase (AP) lasting 6–12 months and an aggressive blast phase (BP), which may be myeloid or lymphoid and is invariably fatal. CML, in the CP, is unlike any other form of cancer and represents an excellent model to study disease progression. Early on, *BCR-ABL* confers only subtle alterations to cellular behaviour including an antiapoptotic phenotype and mild proliferative growth advantage.[2] Information derived from atomic bomb survivors demonstrate that it takes many years (on average seven) from the initial causative genetic event in an HSC before the leukaemia presents in patients.[3]

Mature blood cells derived from the Ph$^+$ stem and progenitor cells function normally as red cells, platelets and neutrophils, and patients require minimal therapy with hydroxyurea to curb the expansion of myeloid cells and thus prevent symptoms caused by leucocytosis and splenomegaly. During this CP, and before additional genetic mutations induce disease progression, we might therefore expect molecular pathways involved in HSC homeostasis and turnover to parallel/mirror those of Ph$^-$ normal counterparts, and not to be deregulated to the degree observed in other forms of cancer. In the case of telomere regulation, the subtle increase in proliferation within the Ph$^+$ stem cell compartment is thought to result in enhanced telomere shortening within the Ph$^+$ clone as compared to polyclonal T cells within the same individuals.[4] The degree and rate of telomere shortening appear to offer prognostic information regarding time to progression, and correlate with Hasford risk score.[5] In the latter study, during progression of CP and AP CML, telomere loss in Ph$^+$ peripheral blood leucocytes was measured at a rate some 10–20 times that of normal. Such rapid loss raises questions regarding the integrity and efficiency of telomere maintenance mechanisms in Ph$^+$ HSC during CP, where critical telomere shortening has been postulated to contribute to genomic instability and progressive disease.

Telomerase, the enzyme responsible for telomere extension, has been shown to be upregulated in most forms of human cancer and is thought to play a critical role in tumour cell immortality.[6] It is of particular interest, therefore, to investigate the key components that reconstitute telomerase activity (TA) in the earliest phase of CML at the point where only *BCR-ABL* is known to be present in the abnormal HSC compartment, during which rapid telomere loss occurs and before the disease progresses and becomes similar to other forms of cancer. CP is generally considered to be the therapeutic window during which attempts to cure CML are made. An understanding of the complexities of telomerase gene regulation during CP may therefore offer new therapeutic approaches to the treatment of CML.

The aim of this study was, therefore, to quantitate expression of the major telomerase components hTR and hTERT (and its alternatively spliced mRNA species) in purified *BCR-ABL*-positive and negative CD34$^+$ cells, and to correlate these data with TRAP activity and cell-cycle status of the cells.

Methods

Patient samples and cell lines

All cell samples were obtained from CML patients leucapheresed prior to treatment and at the time of diagnosis ($n=16$), or non-CML patients (ie nonclonal normal CD34$^+$ cells, $n=14$) at the time of peripheral blood stem cell mobilisation and collection (allogeneic peripheral blood stem cell donors $n=2$, non-Hodgkin's lymphoma $n=10$, multiple myeloma $n=2$). Normal PBL ($n=5$) were obtained by venepuncture of healthy volunteer donors with subsequent NH_4Cl red cell lysis. Written

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informed consent was obtained according to an Ethical Committee-approved protocol. Human spleen and testes cDNA were obtained from OriGene Technologies Inc. (Rockville, MD, USA). A series of alternative lengthening of telomeres (ALT) and human leukaemia cell lines were used to establish dynamic ranges for hTERT Q-RT-PCR (SUSM-1, KMST-6, KY01, LAMA84, K562, EM2, KU812, BV173, TOM1 and ALL-MIK).

CD34$^+$-cell selection

From each leucapheresis product, a sample was taken for subsequent enrichment of CD34$^+$ cells. All samples were processed within 24 h of collection. Cell populations, enriched for CD34$^+$ cells, were obtained using either the Isolex® Immunomagnetic Cell Selection System (Baxter Healthcare, Irvine, CA, USA) or the StemSep™ Negative Selection System (StemCell Technologies, Vancouver, BC, Canada). These samples were cryopreserved in 10% DMSO (Quest Biomedical, Solihull, UK)/ALBA (4.5% albumin solution, SNBTS, Edinburgh, UK) solution. Upon thawing, and to routinely obtain samples with >90% purity of CD34$^+$ cells, these pre-enriched samples were further selected using the MACS Direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturers instructions. CD34 purity was assessed on a FACScan flow cytometer (Becton Dickinson, Oxford, UK).

FISH for BCR-ABL

To confirm that the selected populations of CD34$^+$ cells from CML patients were clonal in origin (ie *BCR-ABL* positive), we performed dual colour FISH on an appropriately fixed aliquot. FISH staining was performed using the Vysis LSI *BCR-ABL* Dual Colour Translocation Probe (Vysis, Richmond, UK) and DAPI (Sigma, Poole, Dorset, UK) as a counter stain according to the manufacturers' instructions and imaged on an epi-fluorescence microscope with a 100-watt mercury lamp with appropriate single band pass filters.

Cell-cycle analysis

This was done using the method of Jordan *et al*[7] as previously described. Flow-cytometric analysis was performed with log-linear analysis on FL1 (Ki67, Becton Dickinson) and linear analysis on FL3 (7-AAD, Sigma). To distinguish cells from aggregates and debris, the cell population was gated within FSC *vs* 7-AAD. This gate was analysed in Ki67 *vs* 7-AAD and the relative percentages in each stage of the cell cycle calculated. In all experiments at least 10^4 events were collected.

PCR

Total cellular RNA was extracted using TRIzol reagent (Life Technologies), according to the manufacturer's instructions. cDNA was synthesised from 1 μg of total RNA using the Abgene Reverse-iT™ 1st Strand Synthesis Kit (Abgene, Epsom, UK). The integrity of generated cDNA was assessed by performing PCR for GAPDH as a housekeeping gene with the Taq PCR Core Kit (Qiagen, Crawley, West Sussex, UK) and using the primers 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ (forward) and 5′-CATGTGGGCCATGAGGTCCACCAC-3′ (reverse) to generate a 983 bp product (Clontech, Palo Alto, CA, USA). Settings used were 94°C for 45 s, 60°C for 45 s, 72°C for 2 min (all repeated for 30 cycles) then 72°C for 10 min. Real-time RT-PCR (Q-RT-PCR) using the Light-Cycler and the TeloTAGGG hTERT and TeloTAGGG hTR kits (Roche Diagnostics, IN, USA) were used to quantitate hTERT and hTR mRNA, respectively, from 0.1–0.2 μg total RNA, according to the manufacturer's instructions. Where appropriate, cDNA was used in the assay in place of RNA. The design of the primers and probes in the hTERT kit is such that only full-length hTERT transcripts (ie $+\alpha+\beta$, Figure 1) are detected. Intraexperimental variation in results was extremely low (CV 2.2%, data not shown). Analysis of interexperimental variation gave a CV of 17.5% (data not shown). As the hTR gene lacks introns, the Ambion DNA-*free*™ Kit (Ambion UK, Huntingdon, UK) was used to remove contaminating DNA prior to hTR Q-RT-PCR. Minus-RT controls were incorporated to exclude amplification of contaminating DNA. Only samples that demonstrated <0.1% levels of DNA contamination (as calculated by [−RT control hTR transcript number/+RT hTR transcript number] × 100) were included for analysis. For each sample simultaneous quantitation of the housekeeping gene PBGD was performed, thereby correcting for RNA loading. Results were expressed as a percentage of PBGD expression. Use of the same housekeeping gene and identical PCR conditions in both the hTR and hTERT assays permitted direct comparison of transcript levels of each gene in the same sample. For hTERT splice-variant PCR we used primers designed to generate a 457 bp product containing the A and B reverse transcriptase motifs, and therefore able to detect the α and β deletions when present (Figure 1). Splice variant products were amplified from 1 μl cDNA using the hT2164F (5′-GCCTGAGCTGTACTTTGTCAA-3′) and hT2620R (5′-CGCAAACAGCTTGTTCTCCATGTC-3′) primers. The Taq PCR Core kit was used with Hot-StarTaq reverse transcriptase

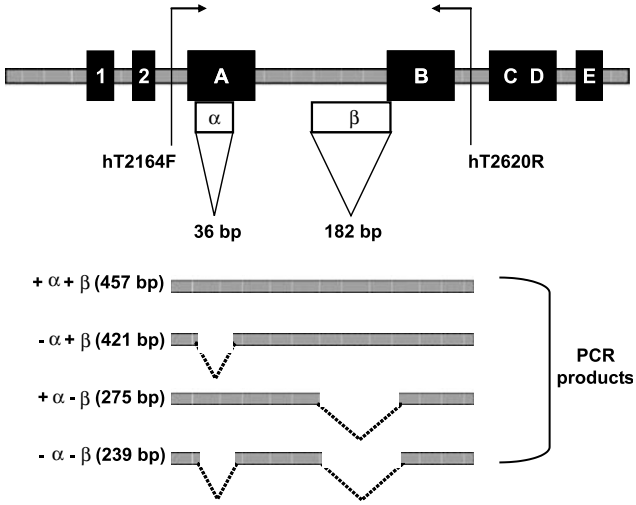

Figure 1 The reverse transcriptase domain of hTERT mRNA with alternative splicing sites and respective PCR products. 1, 2 and A–E represent the conserved reverse transcriptase (RT) motifs. The position of deletions α and β are illustrated (as originally described by Kilian *et al*[33]), flanked by primers hT2164F and hT2620R. The potential PCR products from the alternative splice combinations are shown, with the full-length product ($+\alpha+\beta$) extending from base 2164–2620 (457 bp). Loss of the α domain ($-\alpha$) removes a 36 bp sequence from the A motif, with the translated protein appearing to confer dominant-negative hTERT activity.[34] Splicing of the β site causes a 182-bp deletion, resulting in translation of a nonfunctional truncated protein.[35] Domains 1 and 2 are hTERT specific and A–D are common to all reverse transcriptases.[36]

Table 1 Characterisation of CML *BCR-ABL*$^+$ and normal CD34$^+$ cells

Samples (number)	*% CD34 (median, range)*	*% BCR-ABL pos (median, range)*	*% in G_0 (mean ± s.d.)*	*% in G_1 (mean ± s.d.)*	*% in G_2/S/M (mean ± s.d.)*
CML *(16)*	94.1 (88.9–98.1)	96.3 (86.5–99.4)	5.8 ± 4.4*	79.6 ± 5.4	11.8 ± 4.4**
Non-CML *(15)*	94.1 (88.0–99.0%)	—	15.0 ± 8.1*	74.9 ± 8.7	5.1 ± 4.2**

High-purity CD34$^+$ cells and (in the case of CML samples) primarily *BCR-ABL*$^+$ cells were used for this study. Significant differences in percentages of cells were observed between the G_0 and S/G2/M compartments for the two populations (but not between G_1). * and **, $P<0.05$; s.d., standard deviation; NA, not applicable.

(Qiagen, Crawley, UK). We used the following cycles: 94°C for 15 min, followed by 40 cycles of 95°C for 30 s, 64°C for 45 s, 72°C for 45 s, and finally 72°C for 5 min. PCR products were separated and quantitated on an Agilent 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA, USA) using a DNA 1000 LabChip™ (Agilent). PCR product concentrations were automatically calculated in ng/μl, and sized in bp by the instrument.

Telomeric repeat amplification protocol (TRAP assay)

TA of PBL and CD34$^+$ selected samples was determined using a modification of the TRAP assay described by Kim *et al*.[8] Dry cell pellets (10^4–10^5 cells) were stored at –80°C until required, whereupon they were lysed with NP-40/NaDOC (Calbiochem, Nottingham, UK) lysis buffer. Protein concentrations were measured, and 10 μl of appropriately diluted extracts (containing 1 and 0.1 μg protein extract of each sample) was added to 40 μl reaction buffer (50 μM dNTPs, 1 μg telomerase substrate (TS) primer (5′-AATCCGTCGAGCAGAGTT-3′), 20 mM Tris/HCl pH 8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 1 mM EGTA, 0.1 mg/ml bovine serum albumin, 2 U Taq DNA polymerase (Invitrogen) and 1 μg alternative complementary (ACX) primer (5′-GCGCGGCTTACCCTTACCCTTACCCTAACC-3′)). Samples were incubated at room temperature for 30 min and then heat inactivated for 10 min at 85°C. Telomerase elongated product in samples was amplified: 90 s at 90°C then 31 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 90 s. An aliquot of each sample was heat-treated (10 min, 85°C) before assaying to serve as a negative control. An internal telomerase assay standard (ITAS, 15 attogram) was included in the PCR buffer. All samples were assayed blind and experiments were performed in duplicate. For semiquantitative analyses, 1 μl of PCR product was analysed on the Agilent Bioanalyser, using a DNA 500 Chip. The concentrations (ng/μl) of the first six 6-bp incremental bands were added together (as previously described[9]) and expressed as a percentage of 10 000 GLC4 cell equivalents. The first peak detected was 50 bp in length, and consisted of the primers plus one telomere repeat. This was therefore excluded from analysis and the next six peaks analysed. Limit of detection was 100 GLC4 cell equivalents. All samples were assayed blind.

Statistics

All statistical tests were two-sided. Comparison of groups of continuous variables was performed using the Mann–Whitney *U* test. GB-Stat Statistical Software for Windows (Dynamic Microsystems, Silver Spring, MD, USA) was used for all analyses, and significance was assumed for *P*-values <0.05.

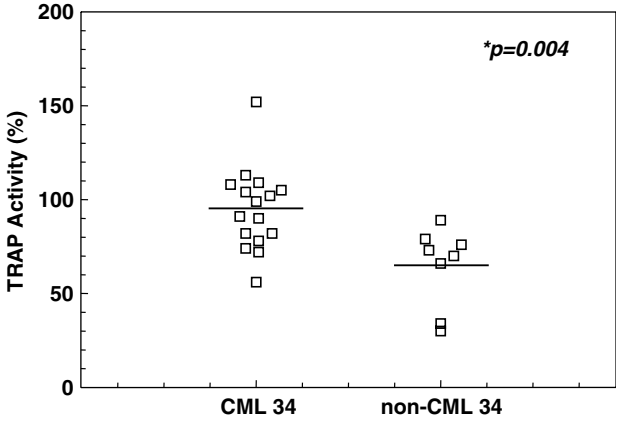

Figure 2 TRAP activity in CML *vs* non-CML CD34$^+$ cells. A small (1.5-fold) but statistically significant increase in TRAP activity was observed for the CML samples. Activity was expressed as a percentage of that seen in 10 000 GLC-4 cells.

Results

Peripheral blood BCR-ABL$^+$ *CD34*$^+$ *cells demonstrate increased cell-cycle activity*

CP CML is associated with marked telomere shortening detectable in PBL. To quantify any increased cell-cycle activity in CML (and allow correlation with TRAP activity), we performed high-resolution cell-cycle analysis on a pure progenitor cell/HSC (CD34$^+$ selected) population. Resolution of G_0 *vs* G_1 and S/G_2/M (data not shown) demonstrated significantly fewer CML as compared to normal CD34$^+$ cells in stage G_0 and correspondingly greater numbers in S/G_2/M (Table 1). This was in keeping with previous studies (reviewed in Holyoake *et al*[10]). Furthermore, these data allowed us to control for any confounding effect on proliferation-dependent telomerase expression from G-CSF administration (for HSC mobilisation).

In vitro *TA, as measured by TRAP, is increased in the* BCR-ABL$^+$ *CD34*$^+$ *compartment and correlates with cell-cycle profile*

It is conventional to assess TA semiquantitatively by using the TRAP assay. All CD34$^+$ samples expressed TRAP activity, with significantly higher levels (some 1.5-fold) detectable in CML ($n=16$) as compared to normal ($n=9$) cell populations (94.8 ± 22.1 *vs* 64.6 ± 21.3%, respectively, mean percentage activity of 10^4 GLC4 cells ± s.d., $P=0.004$) (Figure 2). Importantly, TRAP activity correlated significantly with the cell-cycle

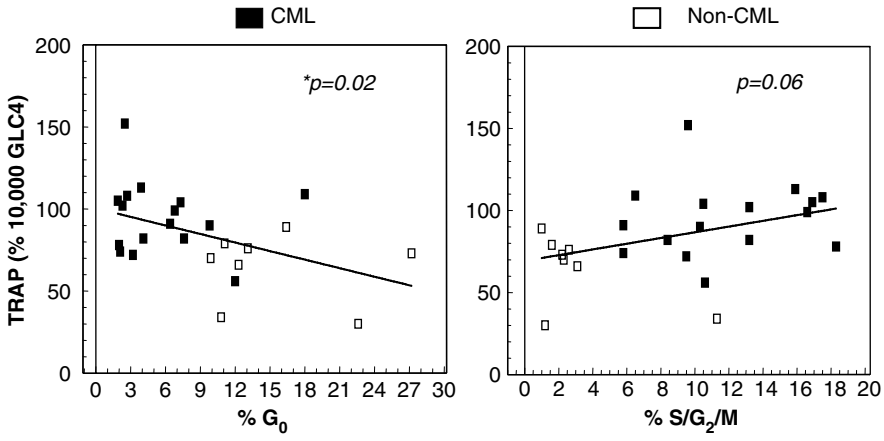

Figure 3 TRAP activity correlates with cell-cycle profile of $CD34^+$ cells. TRAP activity was seen to correlate inversely with the percentage of CD34+ cells in G_0, as might have been expected (adjusted $r^2 = 0.17$, $P = 0.02$). This relationship was only observed when all samples (CML and non-CML) were analysed together, presumably as this increased the dynamic range of measurements. Conversely, a trend towards higher TRAP levels was observed with increasing numbers of cell in $S/G_2/M$ (adjusted $r^2 = 0.11$, $P = 0.06$). No correlation was observed with G_1 (adjusted $r^2 = 0.005$, $P = 0.3$). ■, CML samples; □, non-CML samples.

profile of the samples, with samples containing a greater proportion of G_0 cells (ie normal samples) having the lowest TRAP activity (Figure 3). Conversely, there was a trend towards samples with greater numbers in $S/G_2/M$ (ie primarily CML) having greater TRAP activity. After correction for the proportion of cells in G_0 however (assumed to be negative for TRAP activity) no statistically significant difference in TRAP between the populations was observed (98.5 ± 22.5 *vs* $76.6 \pm 25.7\%$, mean percentage activity of 10^4 GLC4 cells ± s.d., $P = 0.09$, CML and non-CML samples, respectively). This supports the notion that relatively small differences in TRAP between these populations are primarily a reflection of cell-cycle status, and may not reflect a genuine cell-to-cell increase in TA in CML.

Full-length functional (+α+β) hTERT mRNA expression is not increased in BCR-ABL$^+$ *CD34$^+$ cells*

To establish the dynamic range of hTERT gene expression we performed Q-RT-PCR for full-length ($+\alpha+\beta$, see Figure 1) functional hTERT mRNA on a number of primary cells or tissues and cell lines, which exhibit a well-established range of TA (Figure 4a). Full-length ($+\alpha+\beta$) hTERT mRNA was detectable over a 3-log range in these samples. As would be expected, ALT cell lines had almost negligible full-length ($+\alpha+\beta$) hTERT expression while leukaemia cell lines had the greatest. Levels in unfractionated PBL from normal donors were comparable to splenic tissue, and expression was some six-fold higher in testes, a tissue capable of maintaining telomere length. Interestingly, despite their leukaemic origin, increased cycling and TRAP activity, the CML $CD34^+$ cells did not demonstrate a significant increase in full-length ($+\alpha+\beta$) hTERT expression (Figure 4b, $P = 0.08$). A trend towards higher levels in the CML samples was largely the result of two samples, which exhibited markedly higher $+\alpha+\beta$ hTERT expression than the remainder. Comparison of the median level for both groups confirmed this: at 3.6 and 3.2% for CML and non-CML respectively. No correlation (either positive or negative) was observed between $+\alpha+\beta$ hTERT expression and percentage of cells in each of the cell-cycle stages (data not shown). However, Q-RT-PCR on the LightCycler quantitated full-length ($+\alpha+\beta$) functional hTERT mRNA only; it is known that alternative splicing may play a role in regulation of hTERT expression and that other hTERT transcripts may be present, some of which may have a regulatory role.

Accumulation of full-length hTERT transcripts in $CD34^+$ cells involves mRNA splicing

Two possible ways in which an absolute increase of full-length ($+\alpha+\beta$) functional hTERT mRNA may occur are via a proportional increase in the total number of all hTERT transcripts, or via a shift in splicing patterns leading to an increase in the overall proportion of full-length ($+\alpha+\beta$) functional hTERT mRNA. While the former mechanism does not necessitate a shift in splicing patterns, the latter clearly does. To determine the relative expression of alternatively spliced hTERT mRNA products we utilised a single primer set to detect the four common patterns (Figure 1) in a single PCR reaction, in conjunction with GAPDH PCR to control for cDNA quality (Figure 5). Each isoform (full length, β-deleted, α-deleted or $\alpha\,\beta$-deleted) was expressed as a percentage of total transcripts detected (ie all alternatively spliced species) thereby generating a profile of relative expression. By combining these data with quantitative (LightCycler) analysis of full-length ($+\alpha+\beta$) functional hTERT expression, a complete expression profile of the hTERT gene can be derived (as illustrated by histogram height in Figure 6). As can be seen from the height of the histogram in Figure 6, the total transcript levels are similar for both CML and normal $CD34^+$ cells. Interestingly however, within the CML samples, a significantly greater proportion of the total transcript level is derived from full-length hTERT mRNA ($P = 0.001$), and this appears to be at the expense of the $+\alpha-\beta$, which was significantly reduced ($P = 0.004$). When the results were analysed on a sample-to-sample basis, the proportion of full-length transcript as determined by splice variant RT-PCR displayed a highly significant correlation with the absolute levels (as measured by Q-RT-PCR) for both CML and normal samples (Figure 6). This suggests that subtle shifts in splicing patterns determine overall full-length $+\alpha+\beta$ hTERT expression

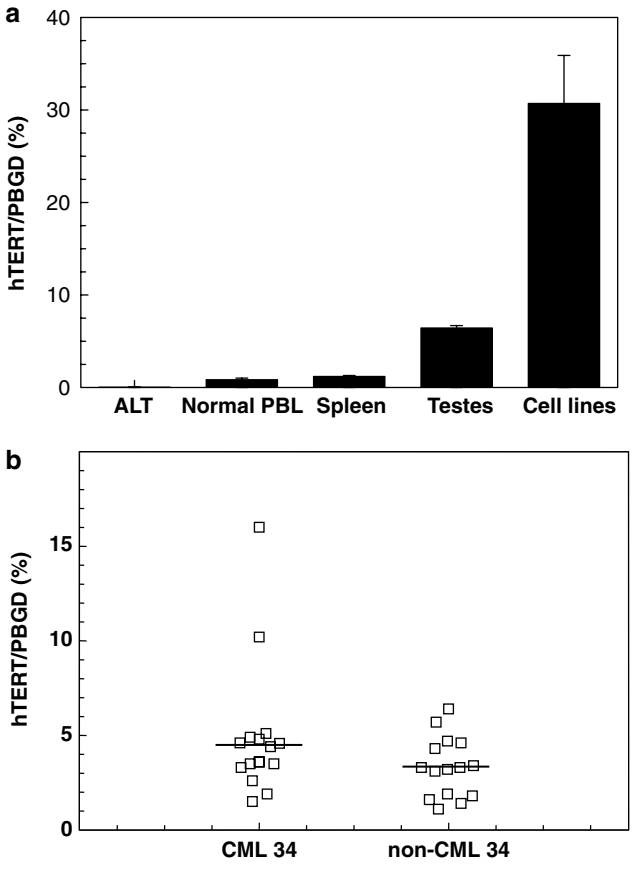

Figure 4 (a and b) $+\alpha+\beta$ hTERT mRNA expression by Q-RT-PCR in a range of normal cells, CML cell-lines and primary $CD34^+$ cells. Total RNA from ALT and CML cell lines and normal PBL, and cDNA from normal spleen and testes was analysed for hTERT expression (a) in comparison to that of the housekeeping gene PBGD (%). Mean values are shown with standard error bars. The values obtained were: alternative lengthening of telomeres (ALT) cell lines (SUSM-1 and KMST-6), 0.04 and 0.06%; normal PBL 1.1, 0.4, 1.0, 0.6 and 1.2%; spleen 1.1 and 1.3%; testes 6.2 and 6.7%; KY01 33.3%, LAMA84 62.7%, K562 21.9%, EM2 22.1%, KU812 17.1%, BV173 34.7%, TOM1 20.2%, ALL-MIK 33.8%. Data from CML *vs* non-CML $CD34^+$ is shown in (b). Although there was a trend towards higher levels of $+\alpha+\beta$ hTERT in the CML samples, this did not reach statistical significance ($P=0.08$) and was skewed by two samples exhibiting high levels; median values for the two groups were similar (see text).

in the $CD34^+$ population, as opposed to simply switching expression on or off.

hTR expression is reduced in BCR-ABL$^+$ *compared to normal* $CD34^+$ *cells*

The other major component of telomerase, hTR, is also tightly regulated in the haemopoietic system, with elevated levels detectable in T-lymphocytes following stimulation.[11] Additional studies have shown it to be upregulated in solid tumours.[12,13] To determine if hTR levels may be limiting for telomere maintenance in CP CML, we performed Q-RT-PCR for hTR in CML *vs* normal $CD34^+$ selected cells (Figure 7). Interestingly, the mean hTR level in CML $CD34^+$ cells was some five-fold lower than that for normal. Furthermore, as hTERT and hTR are the minimal requirements for reconstituting TA *in vitro*,[14] it may therefore be of functional relevance if the ratio between these molecules is altered significantly as a result of altered expression of one or both components. The use of the same housekeeping gene for quantitation as that for hTERT (PBGD, with the same primers, probes and PCR conditions) allowed direct comparison of the relative hTERT:hTR expression ratio. This was $1:10^2$ for hTERT:hTR in *BCR-ABL*$^+$ $CD34^+$ cells and $1:10^3$ for normal cells. An hTERT:hTR ratio of up to $1:10^4$ has previously been described for tumour cell lines,[15] in which telomere maintenance occurs. It is therefore possible that an altered hTR:hTERT ratio, primarily as a result of reduced hTR expression as observed in our samples, adversely affects telomere maintenance.

Discussion

Marked telomere shortening has been observed during the progression of CML. To identify potential mechanisms for this phenomenon we have, for the first time, completely characterised the expression of hTERT (and its splice-variants), hTR and TRAP activity in a purified leukaemic progenitor/HSC population. By incorporating nonmalignant $CD34^+$ cells as normal controls, we have established the threshold expression of telomerase components in equivalent polyclonal cell types with which to compare leukaemic cells at all stages of the disease.

Our data explain, at least in the context of CML, the paradox of increased TRAP activity in association with greatly reduced telomere length: an increased proportion of cycling $CD34^+$ cells in CML results in greater detectable TRAP activity. That peripheral blood CML $CD34^+$ cells display increased cycling activity is in agreement with our previous study[16] and is supportive of a mechanism of replicative telomere loss. However, previous data describing a 2–5 fold increase in TRAP activity in CML $CD34^+$ cells[17] are very likely due to the differing cell-cycle profiles of the CML and non-CML populations. Our correlation of low TRAP activity in G_0-rich samples is in agreement with previous studies demonstrating a clear correlation with TRAP and cell-cycle activity in tumours[18] and with downregulation of activity on entry into a quiescent state.[19] Many other investigators have demonstrated a clear pattern of TRAP activity in proliferating tissues such as oral mucosa[20] and endometrium.[21] In the haemopoietic system a similar relationship between proliferation and TRAP status exists: activity is repressed in $CD34^+CD38^-$ (quiescent) cells, with upregulation upon proliferation and expansion in the $CD34^+CD38^+$ compartment prior to downregulation upon differentiation.[22] Thus, it seems that in normal tissues, primary solid tumours and leukaemias the over-riding determinant of TRAP status may be the percentage of cells in the cycling compartment rather than a cell-specific increase, as recently hypothesised.[23]

TA is dependent on many factors but the expression of the hTERT and hTR genes are essential components of the enzyme. We therefore investigated the expression of hTR and hTERT in the context of CP CML. We showed no significant correlations between cell-cycle status and expression levels of hTERT or hTR, and in addition there was no obvious relationship between these parameters and TRAP activity in quantitative terms. However, only full-length $+\alpha+\beta$ hTERT is associated with TA and, in this regard, we demonstrated a 100% correlation (all samples expressed $+\alpha+\beta$ hTERT and exhibited TRAP activity).

The presence of alternatively spliced mRNA variants of hTERT represents an additional level of gene regulation as only the full-length variant has the ability to make functional hTERT protein. Comparable median levels of full-length $+\alpha+\beta$ hTERT mRNA

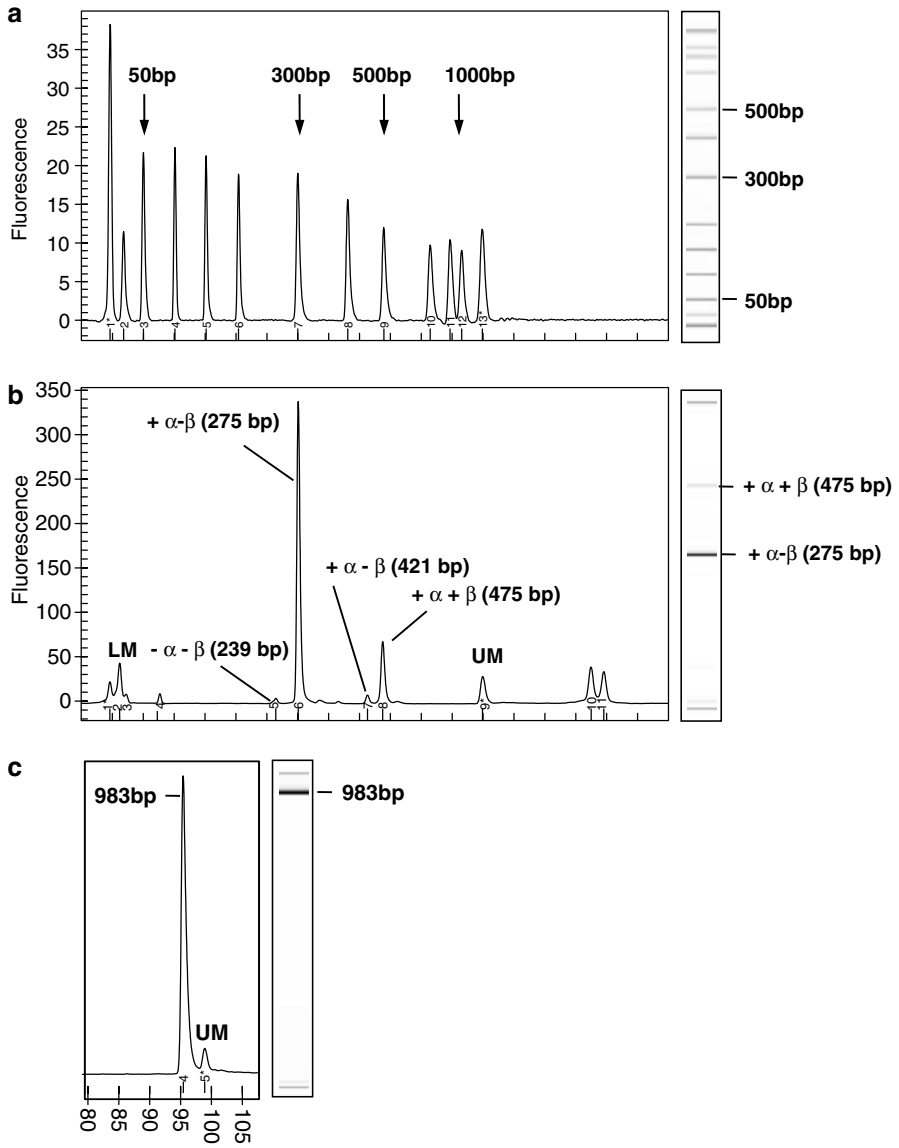

Figure 5 Electropherogram analysis of alternatively spliced hTERT mRNA RT-PCR products. A single set of primers was utilised to amplify the four potential alternatively spliced hTERT mRNA products. These were analysed on the Agilent Bioanalyzer instrument, and quantitated in $\mu g/\mu l$. Incorporation of a DNA ladder (a) facilitates automatic sizing of each peak, the concentration of which is then determined automatically. The relative percentages of each product were then calculated. A representative CML $CD34^+$ selected sample exhibiting all four potential splice products is illustrated (b), with concentrations (and relative percentages of all transcripts) as follows; $+\alpha+\beta$ 4.0 $\mu g/\mu l$ (16.5%), $-\alpha+\beta$ 0.5 $\mu g/\mu l$ (2.1%), $+\alpha-\beta$ 19.3 $\mu g/\mu l$ (79.8%) and $-\alpha-\beta$ 0.4 $\mu g/\mu l$ (1.7%). RT-PCR for the housekeeping gene GAPDH is shown in (c). Sizes (in bp) of each of the relevant peaks are indicated and a 'gel' like image is shown for comparison in each of the right-hand panels. LM, lower marker; UM upper marker.

transcripts were detected in CML $CD34^+$ cells *vs* non-CML. Interestingly, however, we demonstrated a close correlation between absolute full-length $+\alpha+\beta$ transcript levels and the percentage of all hTERT transcripts (by splicing pattern RT-PCR) that these represented. This suggests that subtle shifts in splicing patterns determine overall full-length $+\alpha+\beta$ hTERT expression in the $CD34^+$ population, as opposed to an alternative scenario in which splicing patterns remain static and transcriptional control solely determines absolute hTERT transcript levels. As far as we are aware, this is the first time such a quantitative relationship has been detected. Previously, a qualitative (ie on–off) relationship has been observed in foetal development,[24] with a remarkably consistent splicing pattern observed in tumour cell-lines.[15] However, cell lines are, by nature, a highly selected cell population and patterns of telomerase expression in such samples cannot necessarily be extrapolated to primary material. Unpublished studies by our group show widely different expression patterns of hTERT species in advanced-stage CML and acute leukaemia. Importantly, these data indicate that a single primer set is able to detect differences in relative expression of transcripts, and that the more uniform data from CP cells is not artefactual (eg due to size-dependent competitive amplification).

A primary aim of this study was to determine if there were any CML-related differences in expression levels of hTERT or hTR, which could explain the rapid telomere loss observed during

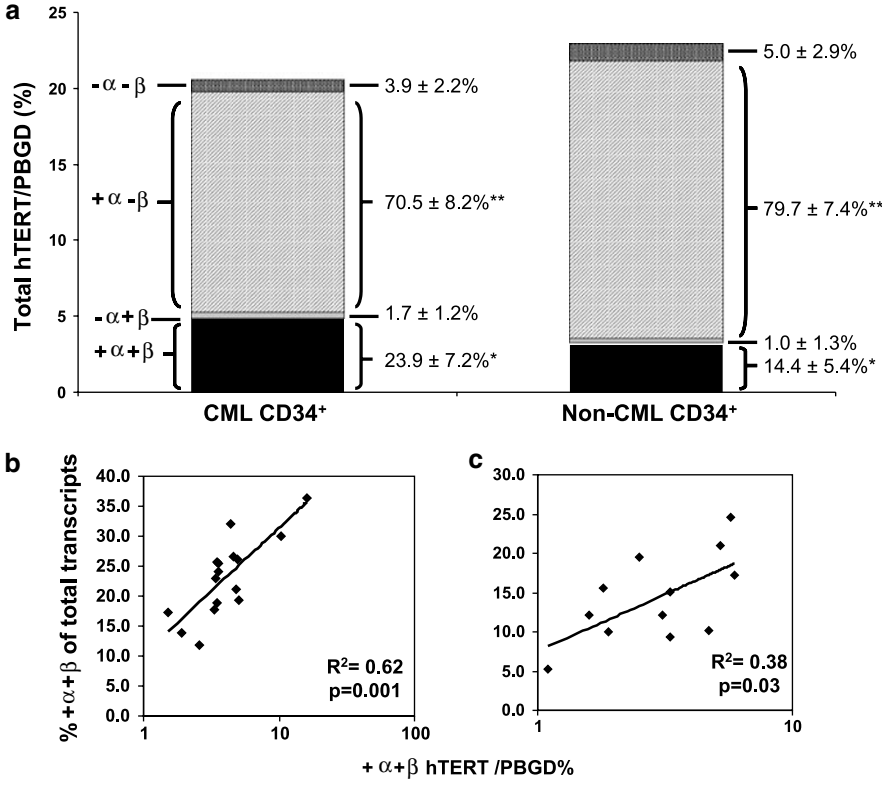

Figure 6 Total hTERT expression in CML and non-CML CD34$^+$ cells. By combining data from $+\alpha+\beta$ hTERT Q-RT-PCR and splice variant RT-PCR (see text), a complete picture of steady-state hTERT expression could be constructed (a). Total transcript levels (as indicated by histogram height) were similar for CML and non-CML CD34$^+$ cells; however, full-length $+\alpha+\beta$ transcripts constituted a significantly greater proportion of these (as indicated by numbers to the right of the bars) in the CML samples ($P=0.001$). When results generated by the two PCR techniques were compared, a significant correlation was observed between $+\alpha+\beta$ hTERT/PBGD and the percentage of $+\alpha+\beta$ hTERT mRNA as determined by splice variant RT-PCR (b and c).

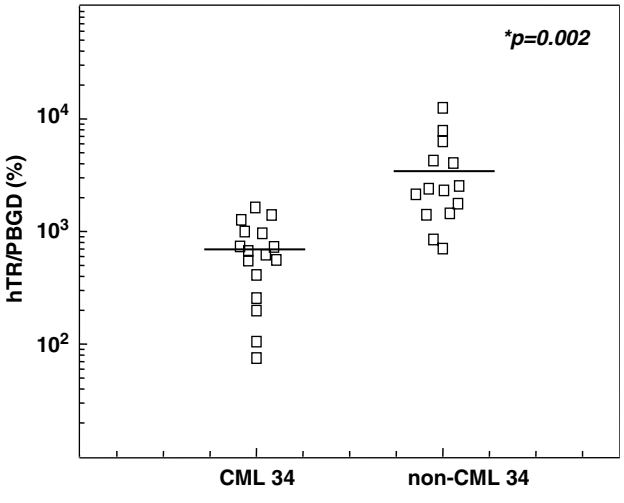

Figure 7 Reduced hTR expression in CML CD34$^+$ cells. Q-RT-PCR was performed for hTR, using identical PCR conditions (and the same housekeeping gene) as for hTERT. A significant ($P=0.002$) reduction in hTR expression was observed for the CML samples.

disease progression. Analysis of hTR expression revealed a five-fold decrease in expression in *BCR-ABL*$^+$ CD34$^+$ cells compared to normal. This is of particular interest given that hTR has now been shown to be limiting for telomere homeostasis *in vivo*,[25] as also seen in the inherited disorder DKC.[26,27] Indeed, that haploinsufficiency of hTR can result in telomere shortening would imply that a normal cell contains just enough hTR for telomere maintenance, as recently suggested.[28] Interestingly, several studies have detailed low hTR levels resulting in telomere shortening with no detectable fall in TRAP activity.[29,30] Such data would support a model in which CML cells expressing subnormal levels of hTR (but not detectably deficient in TRAP activity) undergo progressive telomere shortening as a result. hTR may only become limiting transiently, as a cell requires maximal telomerase function[27] (eg during S phase). Thus, it may have no detectable effect on baseline TRAP activity, or the latter assay may be insufficiently sensitive to detect it. Whether the ratio of hTR to hTERT is significant in this regard is entirely speculative, but is an intriguing possibility. We observed a log-change in the ratio of the major telomerase components in CML CD34$^+$ cells, the first time such an observation has been made in normal and leukaemic HSC. These data may help to explain the telomere erosion observed during CP CML and highlights the value of investigating telomerase gene expression during a key phase of CML progression.

The BCR-ABL tyrosine kinase has myriad downstream effects upon many cellular pathways. It is not unreasonable, therefore, to speculate that TA may be altered as a result of BCR-ABL acquisition. Data to support such a notion have been provided by Kharbanda *et al*,[31] demonstrating c-abl to be a key inhibitor of TRAP activity via phosphorylation of hTERT. In support of

these data, a recent study demonstrated that antisense inhibition of BCR-ABL promoted TA.[32] Furthermore, the phosphorylation status of hTERT appeared crucial to determining its subcellular localisation.[11] In light of our data demonstrating markedly reduced hTR levels in CML HSC, it is tempting to speculate that BCR-ABL-mediated dysregulation and/or altered localisation of key telomerase components (eg hTERT) could compromise telomere homeostasis, perhaps via reduced stability of hTR. Such hypotheses demand further testing *in vitro*.

In summary, we have fully characterised expression, activity and mRNA splicing-mediated regulation of the major telomerase components, and provide evidence to suggest that hTR is dysregulated in CP CML $CD34^+$ cells. This, in conjunction with increased cell-cycle activity, may contribute to the rapid telomere loss seen in the early stages of this disorder and during disease progression.

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References

1 Sawyers CL. Chronic myeloid leukemia. *N Eng J Med* 1999; **340**: 1330–1340.
2 Holyoake T. Recent advances in the molecular and cellular biology of CML: lessons to be learned from the laboratory. *Br J Haematol* 2001; **113**: 11–23.
3 Ichimaru M, Ishimaru T, Mikami M, Yamada Y, Ohkita T. Incidence of leukemia in a fixed cohort of atomic bomb survivors and controls, Hiroshima and Nagasaki, October 1950–December 1978. Technical Report RERF TR 13-81. Hiroshima: Radiation Effects Research Foundation, 1981.
4 Brummendorf TH, Holyoake TL, Rufer N, Barnett MJ, Schulzer M, Eaves CJ *et al*. Prognostic implications of differences in telomere length between normal and malignant cells from patients with chronic myeloid leukemia measured by flow cytometry. *Blood* 2000; **95**: 1883–1890.
5 Drummond M, Lennard A, Bruemmendorf T, Holyoake T. Telomere shortening correlates with prognostic score at diagnosis and proceeds rapidly during progression of chronic myeloid leukemia. *Leuk Lymphoma* 2004; **45**: 1775–1781.
6 Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL *et al*. Specific association of human telomerase activity with immortal cells and cancer. *Science* 1994; **266**: 2011–2015.
7 Jordan CT, Yamasaki G, Minamoto D. High-resolution cell cycle analysis of defined phenotypic subsets within primitive human hematopoietic cell populations. *Exp Hematol* 1996; **24**: 1347–1355.
8 Kim NW, Wu F. Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). *Nucleic Acids Res* 1997; **25**: 2595–2597.
9 Hamad NM, Banik SS, Counter CM. Mutational analysis defines a minimum level of telomerase activity required for tumourigenic growth of human cells. *Oncogene* 2002; **21**: 7121–7125.
10 Holyoake TL, Jiang X, Drummond MW, Eaves AC, Eaves CJ. Elucidating critical mechanisms of deregulated stem cell turnover in the chronic phase of chronic myeloid leukemia. *Leukemia* 2002; **16**: 549–558.
11 Weng N, Levine BL, June CH, Hodes RJ. Regulation of telomerase RNA template expression in human T lymphocyte development and activation. *J Immunol* 1997; **158**: 3215–3220.
12 Soder AI, Hoare SF, Muir S, Going JJ, Parkinson EK, Keith WN. Amplification, increased dosage and *in situ* expression of the telomerase RNA gene in human cancer. *Oncogene* 1997; **14**: 1013–1021.
13 Soder AI, Going JJ, Kaye SB, Keith WN. Tumour specific regulation of telomerase RNA gene expression visualized by *in situ* hybridization. *Oncogene* 1998; **16**: 979–983.
14 Weinrich SL, Pruzan R, Ma L, Ouellette M, Tesmer VM, Holt SE *et al*. Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT. *Nat Genet* 1997; **17**: 498–502.
15 Yi X, Shay JW, Wright WE. Quantitation of telomerase components and hTERT mRNA splicing patterns in immortal human cells. *Nucleic Acids Res* 2001; **29**: 4818–4825.
16 Holyoake T, Jiang X, Eaves C, Eaves A. Isolation of a highly quiescent subpopulation of primitive leukemic cells in chronic myeloid leukemia. *Blood* 1999; **94**: 2056–2064.
17 Engelhardt M, Mackenzie K, Drullinsky P, Silver RT, Moore MA. Telomerase activity and telomere length in acute and chronic leukemia, pre- and post-*ex vivo* culture. *Cancer Res* 2000; **60**: 610–617.
18 Belair CD, Yeager TR, Lopez PM, Reznikoff CA. Telomerase activity: a biomarker of cell proliferation, not malignant transformation. *Proc Natl Acad Sci USA* 1997; **94**: 13677–13682.
19 Holt SE, Aisner DL, Shay JW, Wright WE. Lack of cell cycle regulation of telomerase activity in human cells. *Proc Natl Acad Sci USA* 1997; **94**: 10687–10692.
20 Kannan S, Tahara H, Yokozaki H, Mathew B, Nalinakumari KR, Nair MK, Tahara E. Telomerase activity in premalignant and malignant lesions of human oral mucosa. *Cancer Epidemiol Biomarkers Prev* 1997; **6**: 413–420.
21 Kyo S, Takakura M, Kohama T, Inoue M. Telomerase activity in human endometrium. *Cancer Res* 1997; **57**: 610–614.
22 Engelhardt M, Kumar R, Albanell J, Pettengell R, Han W, Moore MA. Telomerase regulation, cell cycle, and telomere stability in primitive hematopoietic cells. *Blood* 1997; **90**: 182–193.
23 Ohyashiki JH, Sashida G, Tauchi T, Ohyashiki K. Telomeres and telomerase in hematologic neoplasia. *Oncogene* 2002; **21**: 680–687.
24 Ulaner GA, Hu JF, Vu TH, Giudice LC, Hoffman AR. Telomerase activity in human development is regulated by human telomerase reverse transcriptase (hTERT) transcription and by alternate splicing of hTERT transcripts. *Cancer Res* 1998; **58**: 4168–4172.
25 Hathcock KS, Hemann MT, Opperman KK, Strong MA, Greider CW, Hodes RJ. Haploinsufficiency of mTR results in defects in telomere elongation. *Proc Natl Acad Sci USA* 2002; **99**: 3591–3596.
26 Mitchell JR, Wood E, Collins K. A telomerase component is defective in the human disease dyskeratosis congenita. *Nature* 1999; **402**: 551–555.
27 Collins K, Mitchell JR. Telomerase in the human organism. *Oncogene* 2002; **21**: 564–579.
28 Mason PJ. Stem cells, telomerase and dyskeratosis congenita. *Bio Essays* 2003; **25**: 126–133.
29 Hathcock KS, Hemann MT, Opperman KK, Strong MA, Greider CW, Hodes RJ. Haploinsufficiency of mTR results in defects in telomere elongation. *Proc Natl Acad Sci USA* 2002; **99**: 3591–3596.
30 Liu Y, Snow BE, Hande MP, Yeung D, Erdmann NJ, Wakeham A *et al*. The telomerase reverse transcriptase is limiting and necessary for telomerase function *in vivo*. *Curr Biol* 2000; **10**: 1459–1462.
31 Kharbanda S, Kumar V, Dhar S, Pandey P, Chen C, Majumder P *et al*. Regulation of the hTERT telomerase catalytic subunit by the c-Abl tyrosine kinase. *Curr Biol* 2000; **10**: 568–575.
32 Bakalova R, Ohba H, Zhelev Z, Kubo T, Fujii M, Ishikawa M *et al*. Antisense inhibition of Bcr-Abl/c-Abl synthesis promotes telomerase activity and upregulates tankyrase in human leukaemia cells. *FEBS Lett* 2004; **564**: 73–84.

33 Kilian A, Bowtell DD, Abud HE, Hime GR, Venter DJ, Keese PK *et al*. Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types. *Hum Mol Genet* 1997; **6**: 2011–2019.
34 Colgin LM, Wilkinson C, Englezou A, Kilian A, Robinson MO, Reddel RR. The hTERTalpha splice variant is a dominant negative inhibitor of telomerase activity. *Neoplasia* 2000; **2**: 426–432.
35 Ulaner GA, Hu JF, Vu TH, Oruganti H, Giudice LC, Hoffman AR. Regulation of telomerase by alternate splicing of human telomerase reverse transcriptase (hTERT) in normal and neoplastic ovary, endometrium and myometrium. *Int J Cancer* 2000; **85**: 330–335.
36 Lingner J, Hughes TR, Shevchenko A, Mann M, Lundblad V, Cech TR. Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science* 1997; **276**: 561–567.