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A gene expression signature classifying telomerase and ALT immortalisation reveals an hTERT regulatory network and suggests a mesenchymal stem cell origin for ALT

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Abstract

Telomere length is maintained by 2 known mechanisms, activation of telomerase or alternative lengthening of telomeres (ALT). The molecular mechanisms regulating the ALT phenotype are poorly understood and it is unknown how the decision of which pathway to activate is made at the cellular level. We have shown previously that active repression of telomerase gene expression by chromatin remodelling of the promoters is one mechanism of regulation, however other genes and signalling networks are likely to be required to regulate telomerase and maintain the ALT phenotype. Using gene expression profiling we have uncovered a signature of 1305 genes to distinguish telomerase positive and ALT cell lines. By combining this with gene expression profiles of liposarcoma tissue samples we refined this signature to 297 genes. Network analysis of known interactions between genes within this signature revealed a regulatory signalling network consistent with a model of hTERT repression in ALT cell lines and liposarcomas. This network expands on our existing knowledge of hTERT regulation and provides a platform to understand differential regulation of hTERT in different tumour types and normal tissues. We also show evidence to suggest a novel mesenchymal stem cell origin for ALT immortalisation in cell lines and mesenchymal tissues.

Keywords

Telomerase; ALT; mesenchymal; microarray; signalling; c-Myc

INTRODUCTION

A central hallmark of cancer cells is their capacity for unlimited proliferation, made possible partly by telomere length maintenance. Most human tumours maintain telomeres by

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activating telomerase, however in a smaller subset of tumours maintenance occurs in the absence of telomerase through a recombination-based mechanism termed Alternative Lengthening of Telomeres (ALT). ALT is characterised phenotypically by long, heterogeneous telomeres and the presence of ALT associated promyelocytic leukaemia bodies (APBs), which contain telomeric DNA, telomere binding proteins TRF1 and TRF2 and proteins implicated in DNA recombination and replication including MRN complex proteins MRE11, RAD51, and NBS (Henson, Neumann et al., 2002; Yeager, Neumann et al., 1999). Association of these proteins with the ALT phenotype made them attractive as potential markers or regulators of ALT (Jiang, Zhong et al., 2007); indeed, the MRN complex is required for APB formation and telomere maintenance in ALT cells (Zhong, Jiang et al., 2007).

Whilst the overall prevalence of ALT in tumours is relatively low it is observed at high frequency in those of mesenchymal origin. However, even within this group the prevalence of ALT varies. 77% of malignant fibrous histiocytomas (Henson, Hannay et al., 2005) and 47%-66% of osteosarcomas (Henson, Hannay et al., 2005; Ulaner, Huang et al., 2003) were shown to be ALT positive, while 25% of glioblastoma multiforme (Hakin-Smith, Jellinek et al., 2003) and liposarcomas (Costa, Daidone et al., 2006; Johnson, Varkonyi et al., 2005) and no Ewings sarcomas (Ulaner, Hoffman et al., 2004) showed evidence of ALT. Since the prognostic significance of ALT varies, signifying a good prognosis for patients with glioblastoma multiforme but a poor prognosis for those with liposarcoma (Cairney, Hoare et al., 2008; Costa, Daidone et al., 2006), understanding the molecular details regulating ALT immortalisation and the cell of origin may impact future diagnosis or treatment of these malignancies.

A possible explanation for the prevalence of ALT in mesenchymal malignancies is that the telomerase genes are more tightly repressed in mesenchymal tissues over those of epithelial origin (Henson, Neumann et al., 2002). We previously reported that both hTR and hTERT are actively repressed at the chromatin level in ALT cell lines (Atkinson, Hoare et al., 2005), suggesting forced repression of telomerase as one potential molecular mechanism promoting immortalisation by ALT. However, other genes and signalling networks also regulate telomerase and may contribute to the decision to select ALT versus telomerase. In this study we investigate the gene expression profiles of telomerase positive and ALT cell lines and liposarcoma tissue samples in order to better understand the molecular mechanisms regulating the decision to activate telomerase or ALT. Additionally we show a link between mesenchymal tumours and human mesenchymal stem cells (hMSC) as the target cell for transformation in ALT immortalisation.

Materials and Methods

Cell lines and RNA extraction—The ALT cell lines used were: SKLU (lung adenocarcinoma), SUSM1 (liver fibroblasts), KMST6 (lung fibroblasts), WI38-SV40 (SV40 immortalised lung fibroblasts), SAOS (Osteosarcoma) and U2OS (Osteosarcoma). Telomerase positive cells used were: C33a (cervical carcinoma), HT1080 (fibrosarcoma), A2780 (ovarian carcinoma) and 5637 (bladder carcinoma). Normal cell strains used were WI38 (normal lung fibroblasts) and IMR90 (normal lung fibroblasts). Bone marrow hMSC were isolated as previously described (Serakinci, Hoare et al., 2006) and cultured in DMEM-LG plus Glutamax supplemented with 17% Hyclone FBS (Thermo Fisher Scientific, Waltham, MA) at 5% CO_2.

RNA was extracted using the Nucleospin II RNA extraction kit (Macherey-Nagel, Duren, Germany) following manufacturers' instructions.

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Liposarcoma cases and RNA extraction—A subset of 17 liposarcoma samples (9 ALT and 8 telomerase positive) from a larger study population previously described (Cairney, Hoare et al., 2008) were used for gene expression analysis.

Frozen tissue was disrupted using a Hybaid Ribolyser at a setting of 5.5 for 5 × 10 second pulses with 30 second pauses in between and RNA was extracted using the RNeasy Lipid kit (Qiagen Inc, Hilden, Germany) as per manufacturers' instructions.

Gene expression microarray hybridisation, normalisation and quality control —4 ALT cell lines (WI38-SV40, KMST6, SKLU, SUSM1) and 4 telomerase positive cell lines (A2780, C33a, HT1080, 5637), 2 normal fibroblasts (WI38, IMR90), 4 hMSCs and 17 liposarcomas samples were examined using gene expression microarrays. Two separate RNA samples for each were amplified and labelled using the Agilent Low RNA Input Linear Amplification Kit PLUS, One-Colour and hybridised to Agilent whole human genome 4 × 44K gene expression arrays as per manufacturers' instructions. Raw data was extracted from scanned images using Agilent feature extraction software (Agilent Technologies, Santa Clara, CA).

All array data was then imported into GeneSpring GX (version 7.3.1, Agilent Technologies, Santa Clara, CA) and normalised to the 50^{th}, for cell line arrays, or 70^{th} percentile for hMSC and liposarcoma arrays respectively to ensure equal medians across all samples. Further quality control involved filtering based on flag values where only those genes that had non-absent flag values for at least half of the samples were included in downstream analysis. A box plot of the distribution of the resulting genome (figure 1a) was plotted using Minitab version 14 to assess comparability of groups. Data files submitted to GEO for Public access under accession number GSE14533.

Statistical analysis, signature generation and clustering—All statistical analysis and signature generation was carried out within GeneSpring GX. The 1305 gene signature was generated from cell line expression profiles using a WELCH ANOVA with a false discovery rate of 0.05 and Benjamini and Hochberg multiple testing correction. A similar analysis in liposarcoma samples generated no results therefore to generate the refined 297 gene signature a Fishers Exact Test using a p-value of ≤0.05 was performed using the liposarcoma expression profiles to test for significant association of gene expression with telomere maintenance mechanism. From this analysis 422 genes were also present within the 1305 signature. Those genes with fold change values in the same direction for both cell line and liposarcoma data (ie an increased or decreased expression in ALT compared to telomerase positive) were included in the refined signature.

All hierarchical clustering was performed using the Spearman correlation, average linkage and merging branches with a similarity correlation of 0.001 or less.

Q-PCR Validation of microarray data—Expression of some genes within the 1305 signature was validated by quantitative PCR using the DyNAmo Hot star SYBR Green kit (Finnzymes, Espoo, Finland) and Opticon 2 DNA Engine from MJ Research. Primer sequences for QPCR validation were as follows: MYEOVF: 5′-TGGGAGGACACGCAAGTT, MYEOVR: 5′-CCAGCAGCCAAAGCAAAG, WNT5BF: 5′-AGGAGGGAGGTTGTGGTT, WNT5BR: 5′-GAACCGTGGAGGATGAAG, DSC54F: 5′-ACCCTTCTACGAAATGGA, DSC54R: 5′-ACTGTGGCTTATTCCCAT, NSUN5F: 5′-TGAGACCACACTCAGCAG, NSUN5R: 5′-GAGAGGACAGGCATCTTC.

Expression of each gene was normalised to GAPDH as a loading control: GAPDHF: 5′ACCACAGTCCATGCCATCAC, GAPDHR: 5′TCCACCACCCTGTTGCTGTA.

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Analysis of Public Data—Normalised gene expression data for a compendium of 61 samples from various normal cell types was downloaded from NCBI GEO (series GSE3239). Comparison with cell line and hMSC data was carried out in Microsoft Excel.

Network analysis in Metacore—Expression data from the 297 gene signature was converted to fold change in ALT over telomerase positive and uploaded into Metacore analytical suite (Genego Inc, St Joseph, MI). Network analysis was performed using the analyse network algorithm within the software.

Western blotting validation of Network Analysis—Fifteen micrograms of protein equivalents were separated on 10% Bis-Tris gels using NuPAGE MES running buffer (Invitrogen, Renfrewshire, UK, then blotted onto nitrocellulose membrane (Millipore, Watford, UK) and blocked in PBS-Tween 20 containing 5% nonfat dried milk. Membranes were probed with the following antibodies respectively: HDAC5 rabbit polyclonal (CONC) (Active Motif, Rixenstart, Belgium) PKC alpha rabbit polyclonal (CONC) and KAT2A/GCN5 rabbit polyclonal (CONC) (both Abcam, Cambridge, UK) and secondary Anti-Rabbit IgG HRP-linked Antibody (New England Biolabs UK, Hitchin, UK). Following visualisation membranes were stripped of bound primary and secondary antibodies by submerging in 1% SDS and 0.2M glycine (pH2.5) and shaken for 1 hour at room temperature, rinsed and re-blocked before probing with loading control antibody ERK1 rabbit polyclonal (1:3000) (Santa Cruz Biotechnology, Heidelberg, Germany).

c-MYC DNA binding ELISA—Nuclear extracts of approximately 8×10^8 cells of each of the 6 ALT cell lines (WI38-SV40, KMST6, SKLU, SUSM1, SAOS, U2OS) and 4 telomerase positive cell lines (A2780, C33a, HT1080, 5637) were extracted in triplicate using an Active motif nuclear extraction kit as per manufacturer's instructions. c-MYC DNA binding ELISAs (Active Motif, Rixenstart, Belgium) were performed on three separate occasions with four technical replicates of each cell line as per manufacturor's instructions. A standard curve of 5, 2.5, 1.25 and 0.625 ng/well recombinant c-MYC protein (Active Motif, Rixenstart, Belgium) was run on each assay to allow relative quantification. Results of all ALT and telomerase positive cells were grouped and a T-test performed in Minitab (version 14).

RESULTS

Gene expression analysis distinguishes telomerase and ALT cell lines

We initially investigated the presence of a telomere maintenance mechanism (TMM) specific gene expression signature by generating expression profiles for 4 ALT and 4 telomerase positive cell lines using Agilent whole human genome 1-colour microarrays. A detailed description of the quality control measures and the normalisation options applied can be found in materials and methods, however boxplots show that the normalised data is equally spread and comparable between the 2 groups (figure 1a) with virtually equal medians of 0.78 and 0.79 for ALT and telomerase positive cell line groups respectively.

To explore the possibility that expression of individual genes within these large profiles is responsible for defining either telomerase positive or ALT cells we performed a Welch ANOVA test to look for significant differences in gene expression between the 2 groups (figure 1b, left panel). A list of 1307 probes corresponding to 1305 differentially expressed genes with a p-value ≤0.05 was generated. Focussing on the expression values for individual genes within the larger 1305 gene signature it was clear that where expression was high in the ALT cell lines it was low in the telomerase positive cell lines and *vice versa* (figure 2a). Furthermore hierarchical clustering of the cell lines based on this signature accurately

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separated out ALT and telomerase positive cell lines into 2 separate groups (figure 2b), suggesting that the genes responsible for defining telomerase or ALT or those involved in regulating the decision of which TMM to activate may lie within this signature.

Clustering using the 1305 gene signature is suggestive of a mesenchymal stem cell origin for ALT

The fact that ALT is predominantly found in tumours of mesenchymal origin prompted us to investigate whether ALT is a function of the cell of transformation and if mesenchymal stem cells could be the potential cell of origin for ALT tumours. We performed hierarchical clustering analysis using the 1305 gene signature to investigate any relationship between telomerase positive, ALT and normal fibroblast cell lines and hMSC (figure 2c). The signature accurately separates out telomerase positive and ALT cell lines, normal fibroblasts and hMSC. However, while the telomerase positive cell lines cluster together on a separate branch the ALT cell lines, normal fibroblasts and hMSC all cluster together. Normal fibroblasts are more directly related to hMSC than ALT, however fibroblasts and hMSC are equally related to the ALT cell lines, suggestive of a mesenchymal stem cell origin for ALT. This may be as predicted, however to our knowledge this is the first time any association between mesenchymal stem cells and ALT has been shown.

Analysis of the signature revealed several genes associated with stem cell maintenance and self-renewal processes were differentially expressed between telomerase positive and ALT cell lines. Four of these genes with strong differences in expression were chosen for validation by quantitative PCR (figure 3a). Three of these were significantly up-regulated in ALT cell lines and barely expressed in telomerase positive. DSC54 is a novel mesenchymal stem cell protein for which little information exists; WNT5b is a well-known regulator of stem cell function implicated in oncogenesis and development; MYEOV is overexpressed in myeloma and has a role in promoting invasion and proliferation. The final gene NSUN5 is a proliferation associated nucleolar antigen, deletion of which may contribute to the premature aging effects of the developmental disorder Williams syndrome. NSUN5 is significantly up-regulated in telomerase positive compared to ALT cell lines.

To further explore the link between ALT and mesenchymal stem cells we examined the expression of these genes within a variety of normal tissues of differing embryonic origin and human mesenchymal stem cells (hMSC). Publicly available gene expression profiles from normal fibroblasts, smooth muscle, stromal, ectoepithelial, epithelial and endothelial tissues were downloaded from the NCBI GEO database and expression of DSC54, WNT5b and MYEOV in these tissues was compared to that of hMSC, telomerase positive and ALT cell lines (figure 3b). No comparable data was available for NSUN5, therefore this gene could not be included in the analysis.

Consistent with the Q-PCR validation expression of all 3 genes is higher in ALT than telomerase positive cell lines. When comparing all of the expression patterns DSC54 is only high in ALT cell lines and hMSC, consistent with a mesenchymal stem cell origin for ALT. WNT5b on the other hand shows varying expression across the different tissue types and cell lines with highest expression in hMSC, ALT and mesenchymally derived tissues and lowest expression in telomerase positive and epithelial tissues. MYEOV distinguishes ALT from telomerase positive cell lines, however a similar low level of expression is seen across the various other tissue types.

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Refinement of the 1305 gene signature using liposarcoma gene expression improves separation of ALT and telomerase positive liposarcomas and suggests a mesenchymal stem cell origin for ALT in this mesenchymal malignancy

Liposarcomas are tumours of mesenchymal origin. In order to refine the cell line derived signature with data from primary tumours we investigated the power of the 1305 gene expression signature to distinguish telomerase positive and ALT in liposarcomas. Gene expression profiles were generated for a group of 17 previously characterised liposarcoma samples, of which 9 were ALT and 8 were telomerase positive. Unsupervised clustering shows some split in the samples depending on their TMM, which was not improved when the 1305 gene signature was applied. Furthermore hMSC do not cluster with any liposarcoma samples, but cluster together on a separate branch (figure 4 compare a and b).

Although the 1305 signature does not improve clustering, the liposarcoma samples, for the most part, cluster together according to their telomere maintenance mechanism. The obvious separation between ALT and telomerase positive tumours in the clustering diagram led us to believe that differences in gene expression exist between the 2 groups, although no significant differences were found. In order to explore this further we used a Fisher's exact test to test for any association between gene expression level and telomere maintenance mechanism. From this analysis 8227 probes corresponding to 6719 genes were found to be significantly associated with TMM in ALT and telomerase positive liposarcoma samples (figure 1b right-hand panel).

To further refine this large signature we looked for any overlap with the 1305 gene signature determined from the cell lines previously. Of these 1305 genes 422 genes are also present in the liposarcoma signature and therefore have a significant association with telomere maintenance mechanism in liposarcoma and cell lines. Further refinement of the signature was carried out by looking at the direction of gene expression in telomerase positive and ALT tumours in comparison to the cell line data. 297 of the 422 genes had gene expression that was comparable to the cell line data, 152 genes up in ALT, down in telomerase positive and 145 genes down in ALT, up in telomerase positive (figure 1b centre panel).

Hierarchical clustering of ALT and telomerase positive liposarcoma samples using this refined 297 gene signature showed a clear separation between the 2 groups with all but 2 ALT samples clustering on one branch while all telomerase positive samples clustered together on a separate branch (figure 4c). Furthermore, consistent with the hypothesised mesenchymal stem cell origin for ALT seen within the cell line data, hMSC cluster with the ALT liposarcomas using this refined signature rather than as a separate group when the 1305 signature was applied (figure 4 compare b and c).

To further verify the refined signature we applied it back to the cell line data for hierarchical clustering. As predicted it accurately separates out telomerase positive from ALT cell lines (data not shown), further validation that this refined signature holds true and no power is lost by reducing gene number.

The refined 297 gene signature is involved in telomerase gene regulation and highlights lower c-MYC activity in ALT

Given the ability of the refined 297 gene signature to separate liposarcomas by TMM we hypothesised that the genes within the signature may comprise of functional regulatory networks involved in aspects of TMM. In order to explore this we performed network modelling using Metacore from GeneGo, allowing us to build a candidate network indicating possible interactions between genes from the 297 signature mined from published data. A regulatory network involving hTERT and telomeric DNA was revealed by this analysis (figure 4d). Expression data from the 297 gene signature was converted to fold

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change in ALT over telomerase positive, uploaded into Metacore analytical suite and overlaid on the direct interactions network. As can be seen from Figure 4d, by combining interactions between known signalling pathways and experimentally defined levels of expression for regulatory genes this approach allows for predictions relating to hTERT regulation and repression in ALT cells. hTERT expression is reduced in ALT cells and tumours in relation to telomerase positive samples. Consistent with this, expression of E2F1 a known repressor of hTERT, is up-regulated in ALT samples, whereas chromatin modifying enzymes with roles in gene activation such as GCN5 are down-regulated in ALT, in agreement with the decreased association of acetylated histones and low hTERT expression in ALT cell lines as we have previously shown. Western Blotting of HDAC5 PKCa and GCN5 seen in Figure 5a shows that the expression differences highlighted in this network are also seen at the protein level.

The analysis also indicates that c-Myc regulation may contribute to the signature. Although c-Myc was not itself differentially expressed, 21 signature genes including hTERT are predicted transcriptional targets of c-Myc. Interestingly, most signature genes expected to be activated by c-Myc are repressed, while those expected to be inhibited are mainly up-regulated in ALT relative to telomerase positive samples, suggesting that c-Myc activity may be suppressed in ALT. Functinonal examination of c-Myc activity levels using DNA binding activity ELISAs confirms that this is indeed the case, as a significantly lower level of c-Myc activity is seen in the ALT cell lines ($p=0.015$), see Figure 5b. Aside from hTERT, another down-regulated c-Myc target that may play a role in telomere maintenance is hnRPA3. It was recently shown that hnRPA3 binds the single stranded telomere repeat in vitro, protects against nuclease activity and inhibits extension by telomerase (Huang, Tsai et al., 2008).

Importantly however, network analysis expands our understanding of TERT regulation beyond previously recognised mechanisms to new pathways upstream of those already known to be involved. Overall this network highlights a potential mechanism of regulating the ALT phenotype through repression of hTERT and provides a platform for further expansions of regulatory mechanisms present in tissue or tumour specific situations to enable us to understand the differential regulation of biological processes and how they vary in different tumour types and normal tissues.

Discussion

The molecular mechanisms regulating the decision to activate telomerase or ALT during tumourigenesis are currently poorly understood. We have shown previously that lack of expression of the telomerase genes hTR and hTERT is associated with chromatin remodelling at the promoters, suggesting that forced repression of these genes may cause the cells to utilise the ALT mechanism for immortalisation (Atkinson, Hoare et al., 2005). In this study we have used gene expression profiling of telomerase and ALT cell lines and liposarcomas to investigate other signalling pathways and networks that may be operating to control the ALT phenotype and the decision to activate telomerase or ALT for immortalisation. To our knowledge this is the first study of global gene regulation of telomere maintenance mechanisms.

We uncovered a gene expression signature with the power to distinguish telomerase positive and ALT through hierarchical clustering methods in tumour cell lines. Further refinement of this signature using gene expression profiles from liposarcoma tissue samples revealed a 297 gene signature that has significant association with TMM. While the role of these signatures in regulation of the ALT phenotype remains to be fully investigated, by combining clinical samples with cell line profiles we have uncovered some of the underlying biology. Network analysis of interactions within the refined signature highlighted a signalling network

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involved in repression of hTERT in ALT liposarcoma samples and cell lines. Western blot validation of 3 of the molecules in the network confirmed that this pattern can also be observed at the protein level. Consistent with our previous work (Atkinson, Hoare et al., 2005; Cairney, Hoare et al., 2008) this again points to forced repression of hTERT in ALT and may in part explain the decision to activate telomerase or ALT at the molecular level. Although hTERT expression alone is insufficient to discriminate ALT and telomerase positive in clinical samples, hTERT repression is clearly important for regulation of the ALT phenotype. This network of interactions also highlighted the potential for lower c-Myc activity in cells using the ALT mechanism. Upon direct investigation using c-Myc activity ELISAs a lower level of c-Myc activity in ALT was confirmed. This is consistent with the fact that c-Myc is a known hTERT transcriptional activator (Hao, Nancai et al., 2008) and may show a further mechanism by which the decision to activate either ALT or telomerase is influenced.

This candidate network further highlights the importance of a global analysis of gene expression. Where significant expression of one gene may be of importance in certain circumstances, it is more likely, as evidenced by this example, that small changes in a combination of genes in a signalling pathway are responsible for defining a phenotype. By investigating signalling networks on a global scale we are at a better advantage to discover the biology underlying the ALT phenotype and its regulation in mesenchymal malignancies.

In addition to the hTERT regulatory network a number of stem cell related genes were also highlighted within the large gene signature. The possibility that the decision to activate either telomerase or ALT is made at the cellular level is an interesting hypothesis to consider. Cancer biology in many ways parallels that of stem cell biology as pathways regulating the self-renewal phenotype and replicative lifespan of stem cells are commonly deregulated in cancer. With the growing interest in stem cells as the cell of origin for certain tumours, investigating the potential origin for ALT immortality may improve our understanding of the regulation of telomere maintenance. The preponderance of ALT in mesenchymal malignancies prompted us to investigate any relationship between human mesenchymal stem cells (hMSC) and ALT or telomerase positive cell lines and liposarcoma tissues. hMSC are an adult stem cell population with limited replicative lifespan and no detectable telomerase activity (Zimmermann, Voss et al., 2003), which is due at least in part to active repression of the telomerase genes at the chromatin level, similar to the situation in ALT cell lines (Cairney & Keith, 2008; Serakinci, Hoare et al., 2006). hMSC do not however display characteristic molecular markers of ALT (Bernardo, Zaffaroni et al., 2007; Zhao, Li et al., 2008). It is therefore possible that hMSCs upon transformation could become either ALT or telomerase positive tumours. There is however a need for accurate models of the molecular details of the decision between these two mechanisms, such as ours, before manipulation of the resulting telomere maintenance mechanism can be achieved.

Hierarchical clustering showed that the expression profile of the signature genes in hMSC was more closely related to ALT than to telomerase positive cell lines with the larger signature and also to ALT liposarcomas when the refined 297 gene signature was utilised, suggesting a mesenchymal stem cell origin for ALT.

Mesenchymal stem cells are known to be potential targets for transformation *in vitro*. Lack of any telomere maintenance mechanism in these cells may be a tumour suppressor mechanism as transduction with hTERT has been shown to extend their lifespan and induce neoplastic characteristics following long term culture *in vitro* and tumour formation *in vivo* (Serakinci, Guldberg et al., 2004). In addition several studies have shown the ability of MSC to transform spontaneously following long term culture *in vitro* in murine systems (Miura, Miura et al., 2006; Zhou, Bosch-Marce et al., 2006), although the situation in human

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systems remains unclear with conflicting reports suggesting that the capacity for spontaneous transformation may be dependent on the tissue of origin (Bernardo, Zaffaroni et al., 2007; Miura, Miura et al., 2006; Rubio, Garcia-Castro et al., 2005; Wang, Huso et al., 2005). More recently several studies have linked hMSC with mesenchymal malignancies including Ewing's sarcoma (Riggi, Suva et al., 2008; Tirode, Laud-Duval et al., 2007) and malignant fibrous histiocytoma (Matushansky, Hernando et al., 2007). Stem-like tumour initiating cells have also been isolated from various mesenchymal tumours (Gibbs, Kukekov et al., 2005; Wu, Wei et al., 2007). Taken together this data suggests that the stem cell origin for cancer extends to mesenchymal malignancies.

Although the preponderance of ALT in mesenchymal tissues has been documented previously on numerous occasions, to our knowledge this is the first time the link to a mesenchymal stem cell origin for ALT has been made. Furthermore this is not simply reflective of the mesenchymal origin of ALT cell lines as both the ALT and telomerase positive liposarcoma samples are mesenchymally derived and only the ALT liposarcomas cluster with hMSC. The significance of this association is unknown at present, but certainly warrants further investigation.

In conclusion we have uncovered a gene expression signature capable of distinguishing telomerase positive from ALT in cell lines and liposarcoma tissue samples. This signature contains a regulatory signalling network involving hTERT repression in ALT and is indicative of a novel hMSC origin for ALT. The results presented allow us to postulate 2 potential models for the target cell of origin for ALT and telomerase immortalisation. Either (1) two separate cells of origin exist for telomerase positive and ALT expressing malignancies or (2) telomerase positive and ALT tumours arise from the same cell of origin. In this latter case the cell of origin would be the hMSC, whereby telomerase positive tumours have obtained molecular profiles over time that diversify them from the hMSC origin, while ALT tumours maintain the stem cell profile, perhaps in part through mechanisms such as repression of the telomerase genes. The data presented here favour this latter scenario, however further investigation is required in the other tumour types known to utilize the ALT mechanism such as glioma (Chen, Hakin-Smith et al., 2006), Adrenocortical carcinoma, Breast carcinoma, Malignant melanoma, Lung carcinoma, Ovarian carcinoma (Bryan, Englezou et al., 1997) and Renal Carcinoma (Mehle, Piatyszek et al., 1996). A better understanding of the regulation of TMM in the cell of origin will increase our knowledge of the biology underlying these tumour types and may highlight novel areas for therapeutic intervention.

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References

Atkinson SP, Hoare SF, Glasspool RM, Keith WN. Lack of telomerase gene expression in alternative lengthening of telomere cells is associated with chromatin remodeling of the hTR and hTERT gene promoters. Cancer Res. 2005; 65:7585–7590. [PubMed: 16140922]

Bernardo ME, Zaffaroni N, Novara F, Cometa AM, Avanzini MA, Moretta A, et al. Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms. Cancer Res. 2007; 67:9142–9149. [PubMed: 17909019]

Bryan TM, Englezou A, Dalla-Pozza L, Dunham MA, Reddel RR. Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. Nat Med. 1997; 3:1271–1274. [PubMed: 9359704]

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Cairney CJ, Hoare SF, Daidone MG, Zaffaroni N, Keith WN. High level of telomerase RNA gene expression is associated with chromatin modification, the ALT phenotype and poor prognosis in liposarcoma. Br J Cancer. 2008; 98:1467–1474. [PubMed: 18414473]

Cairney CJ, Keith WN. Telomerase redefined: integrated regulation of hTR and hTERT for telomere maintenance and telomerase activity. Biochimie. 2008; 90:13–23. [PubMed: 17854971]

Chen YJ, Hakin-Smith V, Teo M, Xinarianos GE, Jellinek DA, Carroll T, et al. Association of mutant TP53 with alternative lengthening of telomeres and favorable prognosis in glioma. Cancer Res. 2006; 66:6473–6476. [PubMed: 16818615]

Costa A, Daidone MG, Daprai L, Villa R, Cantu S, Pilotti S, et al. Telomere maintenance mechanisms in liposarcomas: association with histologic subtypes and disease progression. Cancer Res. 2006; 66:8918–8924. [PubMed: 16951210]

Gibbs CP, Kukekov VG, Reith JD, Tchigrinova O, Suslov ON, Scott EW, et al. Stem-like cells in bone sarcomas: implications for tumorigenesis. Neoplasia. 2005; 7:967–976. [PubMed: 16331882]

Hakin-Smith V, Jellinek DA, Levy D, Carroll T, Teo M, Timperley WR, et al. Alternative lengthening of telomeres and survival in patients with glioblastoma multiforme. Lancet. 2003; 361:836–838. [PubMed: 12642053]

Hao H, Nancai Y, Lei F, Xiong W, Wen S, Guofu H, et al. siRNA directed against c-Myc inhibits proliferation and downregulates human telomerase reverse transcriptase in human colon cancer Colo 320 cells. J Exp Clin Cancer Res. 2008; 27:27. [PubMed: 18694522]

Henson JD, Hannay JA, McCarthy SW, Royds JA, Yeager TR, Robinson RA, et al. A robust assay for alternative lengthening of telomeres in tumors shows the significance of alternative lengthening of telomeres in sarcomas and astrocytomas. Clin Cancer Res. 2005; 11:217–225. [PubMed: 15671549]

Henson JD, Neumann AA, Yeager TR, Reddel RR. Alternative lengthening of telomeres in mammalian cells. Oncogene. 2002; 21:598–610. [PubMed: 11850785]

Huang PR, Tsai ST, Hsieh KH, Wang TC. Heterogeneous nuclear ribonucleoprotein A3 binds single-stranded telomeric DNA and inhibits telomerase extension in vitro. Biochim Biophys Acta. 2008; 1783:193–202. [PubMed: 17919748]

Jiang WQ, Zhong ZH, Henson JD, Reddel RR. Identification of candidate alternative lengthening of telomeres genes by methionine restriction and RNA interference. Oncogene. 2007; 26:4635–4647. [PubMed: 17297460]

Johnson JE, Varkonyi RJ, Schwalm J, Cragle R, Klein-Szanto A, Patchefsky A, et al. Multiple mechanisms of telomere maintenance exist in liposarcomas. Clin Cancer Res. 2005; 11:5347–5355. [PubMed: 16061847]

Matushansky I, Hernando E, Socci ND, Mills JE, Matos TA, Edgar MA, et al. Derivation of sarcomas from mesenchymal stem cells via inactivation of the Wnt pathway. J Clin Invest. 2007; 117:3248–3257. [PubMed: 17948129]

Mehle C, Piatyszek MA, Ljungberg B, Shay JW, Roos G. Telomerase activity in human renal cell carcinoma. Oncogene. 1996; 13:161–166. [PubMed: 8700542]

Miura M, Miura Y, Padilla-Nash HM, Molinolo AA, Fu B, Patel V, et al. Accumulated chromosomal instability in murine bone marrow mesenchymal stem cells leads to malignant transformation. Stem Cells. 2006; 24:1095–1103. [PubMed: 16282438]

Riggi N, Suva ML, Suva D, Cironi L, Provero P, Tercier S, et al. EWS-FLI-1 expression triggers a Ewing's sarcoma initiation program in primary human mesenchymal stem cells. Cancer Res. 2008; 68:2176–2185. [PubMed: 18381423]

Rubio D, Garcia-Castro J, Martin MC, de la Fuente R, Cigudosa JC, Lloyd AC, et al. Spontaneous human adult stem cell transformation. Cancer Res. 2005; 65:3035–3039. [PubMed: 15833829]

Serakinci N, Guldberg P, Burns JS, Abdallah B, Schrodder H, Jensen T, et al. Adult human mesenchymal stem cell as a target for neoplastic transformation. Oncogene. 2004; 23:5095–5098. [PubMed: 15107831]

Serakinci N, Hoare SF, Kassem M, Atkinson SP, Keith WN. Telomerase promoter reprogramming and interaction with general transcription factors in the human mesenchymal stem cell. Regen Med. 2006; 1:125–131. [PubMed: 17465827]

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Tirode F, Laud-Duval K, Prieur A, Delorme B, Charbord P, Delattre O. Mesenchymal stem cell features of Ewing tumors. Cancer Cell. 2007; 11:421–429. [PubMed: 17482132]

Ulaner GA, Hoffman AR, Otero J, Huang HY, Zhao Z, Mazumdar M, et al. Divergent patterns of telomere maintenance mechanisms among human sarcomas: sharply contrasting prevalence of the alternative lengthening of telomeres mechanism in Ewing's sarcomas and osteosarcomas. Genes Chromosomes Cancer. 2004; 41:155–162. [PubMed: 15287028]

Ulaner GA, Huang HY, Otero J, Zhao Z, Ben-Porat L, Satagopan JM, et al. Absence of a telomere maintenance mechanism as a favorable prognostic factor in patients with osteosarcoma. Cancer Res. 2003; 63:1759–1763. [PubMed: 12702558]

Wang Y, Huso DL, Harrington J, Kellner J, Jeong DK, Turney J, et al. Outgrowth of a transformed cell population derived from normal human BM mesenchymal stem cell culture. Cytotherapy. 2005; 7:509–519. [PubMed: 16306013]

Wu C, Wei Q, Utomo V, Nadesan P, Whetstone H, Kandel R, et al. Side population cells isolated from mesenchymal neoplasms have tumor initiating potential. Cancer Res. 2007; 67:8216–8222. [PubMed: 17804735]

Yeager TR, Neumann AA, Englezou A, Huschtscha LI, Noble JR, Reddel RR. Telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body. Cancer Res. 1999; 59:4175–4179. [PubMed: 10485449]

Zhao YM, Li JY, Lan JP, Lai XY, Luo Y, Sun J, et al. Cell cycle dependent telomere regulation by telomerase in human bone marrow mesenchymal stem cells. Biochem Biophys Res Commun. 2008; 369:1114–1119. [PubMed: 18339310]

Zhong ZH, Jiang WQ, Cesare AJ, Neumann AA, Wadhwa R, Reddel RR. Disruption of telomere maintenance by depletion of the MRE11/RAD50/NBS1 complex in cells that use alternative lengthening of telomeres. J Biol Chem. 2007; 282:29314–29322. [PubMed: 17693401]

Zhou YF, Bosch-Marce M, Okuyama H, Krishnamachary B, Kimura H, Zhang L, et al. Spontaneous transformation of cultured mouse bone marrow-derived stromal cells. Cancer Res. 2006; 66:10849–10854. [PubMed: 17108121]

Zimmermann S, Voss M, Kaiser S, Kapp U, Waller CF, Martens UM. Lack of telomerase activity in human mesenchymal stem cells. Leukemia. 2003; 17:1146–1149. [PubMed: 12764382]

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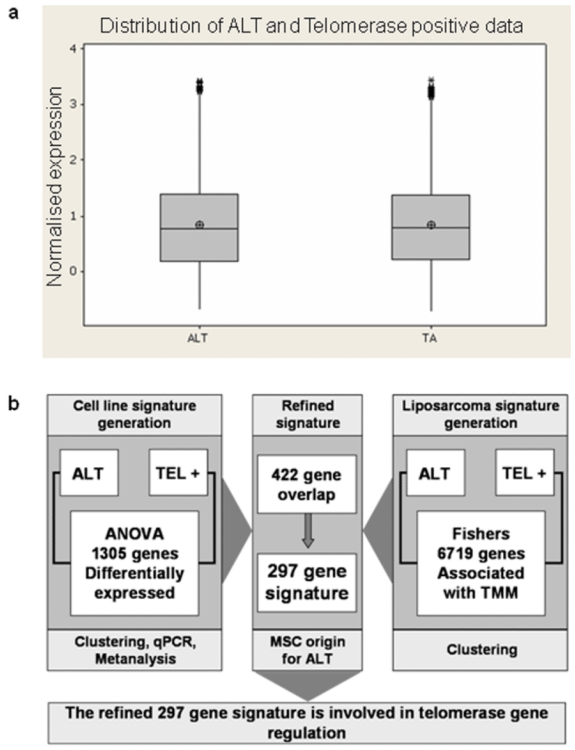

Figure 1.

Gene expression analysis of ALT and telomerase positive cell lines.

(a) Boxplots show distribution of normalised data in ALT and telomerase positive cell line groups. Grey boxes define 25 and 75% quartiles, while error bars represent the 1st and 99th percentiles of the distribution. Dots represent outliers, black line represents the median, while the cross represents the mean of the distribution.

(b) Overview of signature generation from cell line and liposarcoma tissue samples. A refined 297 gene signature was generated from a combination of the 1305 gene cell line signature and the 6719 gene liposarcoma signature. This signature shows a potential mesenchymal stem cell origin for ALT and is involved in telomerase gene regulation. Gene Expression Data submitted to NCBI GEO http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE14533.

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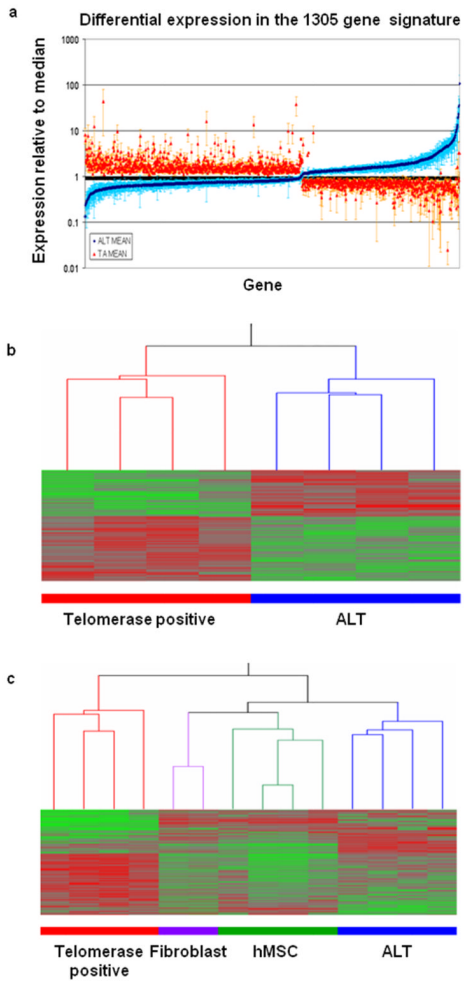

Figure 2.

Gene expression profiling distinguishes telomerase positive and ALT cell lines and is suggestive of a mesenchymal stem cell origin for ALT.

(a) Scatter plot representing normalised microarray expression values for the 1305 gene signature in ALT (blue) and telomerase positive (red) cell lines relative to overall median expression value. Each dot represents the mean gene expression values for a gene, while error bars represent the standard error.

(b) Hierarchical clustering of the cell line data performed using the Spearman correlation, average linkage and merging branches with a similarity correlation of 0.001 or less using the 1305 signature accurately separates telomerase positive (red) from ALT (blue) cell lines.

(c) Hierarchical clustering of telomerase positive (red) and ALT (blue) cell lines, normal fibroblasts (purple) and hMSC (green) performed using the Spearman correlation, average linkage and merging branches with a similarity correlation of 0.001 or less with the 1305 gene signature.

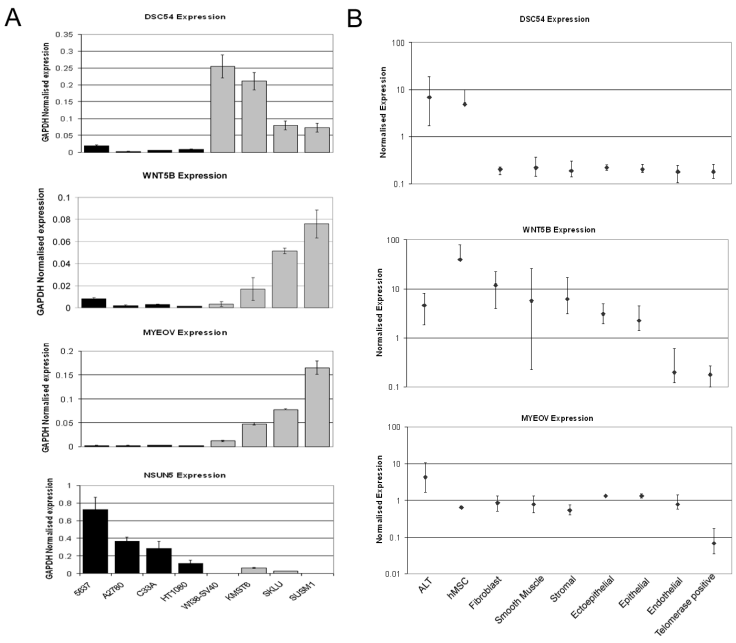

Figure 3.

Validation of the 1305 gene expression signature highlights a stem cell link.

(a) Expression levels of DSC54, WNT5B, MYEOV and NSUN5 were validated by quantitative PCR in telomerase positive cell lines 5637, A2780, C33a and HT1080 (black bars) and ALT cell lines WI38-SV40, KMST6, SKLU, SUSM1 (grey bars) cell lines. Each bar represents the mean and standard error of triplicate reactions from a representative experiment normalised to GAPDH.

(b) Expression values for DSC54, WNT5B and MYEOV in various normal tissues extracted from publicly available microarray expression data, compared to those for ALT and telomerase positive cell lines and hMSC. Dots represent the median while error bars represent the maximum and minimum normalised expression values.

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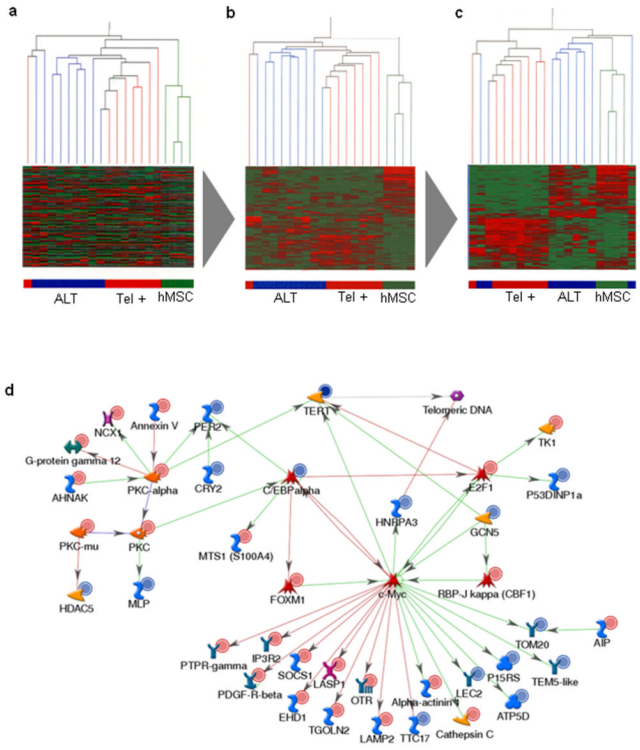

Figure 4.

Hierarchical clustering of ALT and telomerase positive liposarcoma samples and hMSC distinguishes telomerase positive from ALT and highlights an hMSC origin for ALT. Liposarcoma samples were previously determined as ALT (blue) or telomerase positive (red) by classical methods. Hierarchical clustering of these samples and hMSC (green) was performed using the Spearman correlation, average linkage and merging branches with a similarity correlation of 0.001 or less using (a) all genes (b) 1305 gene signature or (c) the refined 297 gene signature.

(d) Network analysis of the 297 gene signature shows hTERT regulation.

Signalling network of known direct interactions between genes from the 297 gene signature drawn using the analyse network building algorithm within Metacore. Green arrows represent positive, red negative and grey unspecified interactions. Red and blue circles next to network objects represent expression data. Red: up in ALT and down in telomerase positive liposarcoma samples and cell lines; Blue: down in ALT and up in telomerase positive liposarcoma samples and cell lines. The network highlights that a number of molecules activated by c-Myc have reduced expression in ALT cells and those inhibited by c-Myc have increased expression in ALT cells. This is suggestive of lower c-Myc activity in cell using ALT.

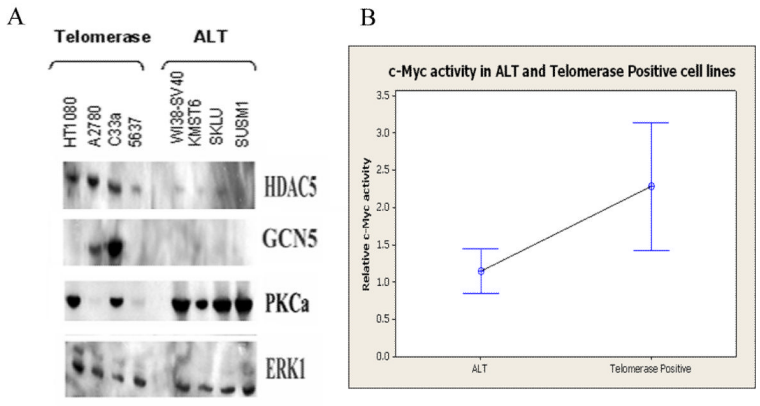

Figure 5.

The TERT regulatory network is shown at the protein level and predicted c-MYC activity is confirmed as significantly lower in ALT.

(a) Western blotting shows protein level differences in 3 molecules of the 297 gene network. 15 μg of cell extracts were run on NuPAGE 4-12% Bis-Tris gels, transferred to Millipore nitrocellulose membrane and probed with appropriate antibodies. Blots were then stripped and reprobed with ERK1 loading control. Panels shown are representative panels of 2 separate blots.

(b) c-Myc activity ELISA shows significantly lower activity in ALT cells. Interval plot shows the average of 6 ALT cell lines (WI38-SV40, KMST6, SKLU, SUSM1, SAOS and U2OS) and 4 telomerase cell lines (A2780, C33a, HT1080 and 5637) on 3 separate occasions with 4 replicates of each cell line. Crosshairs show mean expression for each group and error bars show 95% confidence intervals of the mean. T-test of the results were T-Value = −2.51 P-Value = 0.015 DF = 51.

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