Expression of telomeric repeat binding factor 2 (*TERF2*) in childhood acute lymphoblastic leukemia

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Abstract. Acute lymphoblastic leukemia (ALL) is the most frequent cancer among children. Regardless of the advances in disease treatments, approximately 10-20 % of childhood ALL (cALL) have an incidence of relapse. Therefore, identification of additional prognostic variables is essential to provide specific therapeutic intervention for each patient. *TERF2* is one of the main components of the shelterin complex (telosome) that plays a crucial role in the protective activity of telomeres. This research aims to investigate the expression level of *TERF2* and its potential as a prognostic marker in cALL patients. 88 bone marrow samples and 6 peripheral blood were used to isolated cDNA samples. Real time PCR were used to study the gene expression of *TERF2* in cALL. Results were standardized using *B2M* transcripts as an internal control. Relative quantification of the gene expression was calculated by using the delta-delta Ct method. *TERF2* was up-regulated significantly in cALL patients compared to control samples of which p-value=0.002859, (p<0.05). Over-expression of *TERF2* was observed in *TEL-AML1* subgroup of which p-value=0.0002, (p<0.05). In contrast, under-expression of *TERF2* was found in those having *BCR-ABL1* fusion transcripts of which p-value=0.0221, (p<0.05). *TERF2* also have found to have a better survival advantages for cALL patients. Over-expression of *TERF2* is associated with good prognosis in cALL whilst under-expression is associated with poor prognosis in cALL patients. Measurement of *TERF2* gene expression allows proper stratification of cALL subtypes into its respective prognostic indicator classification.

Keywords: acute lymphoblastic leukemia, childhood cancer, real-time PCR, telomeric repeat binding factor 2, *TERF2*

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common pediatric disease and occur in approximately one fourth of pediatric cancers (Kaatsch, 2010). Despite the remarkable treatment advances in the past decade and a cure rate of 80% in cALL through the effectiveness of risk-directed therapy developed and well-designed clinical trials, treatment failure remains a

formidable problem (Locatelli *et al.*, 2012). Relapsed ALL ranks as the fourth most common childhood malignancy and has an overall survival rate of only 30%. Alterations in the telomere maintenance have been associated with an adverse outcome in many cancers. Therefore, investigation of telomere length and its associated telomere complexes would lead us to understand

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its role in relapse in pediatric ALL (Avigad *et al.*, 2007; Jones *et al.*, 2012)

Mammalian telomeres are formed by tandem repeats of the TTAGGG sequence bound by a specialized six-protein complex known as shelterin, which has fundamental roles in the regulation of telomere length and telomere capping. The shelterin complex, also known as telosome consists of few interacting proteins; hTERT, TREF1, TERF2, POT1, EST1, TANK1, TANK2 and TEP (Lin *et al.*, 2013; Kaminker *et al.*, 2001; Neidle and Parkinson, 2003; De Lange, 2005). In this study, we focus on quantifying telomere repeat binding factor 2 (*TERF2*) gene expression in childhood ALL (cALL). *TERF2* enhances the t-loop formation between telomere end and interacts with ataxia telangiectasia mutated (ATM) kinase from activating DNA damage signaling at functional telomeres (Stansel *et al.*, 2001). Mutation in *TERF2* leads not only to telomeric structure destruction but also to DNA damage, apoptosis, or senescence concomitantly (Van Steensel *et al.*, 1998, Karlseder *et al.*, 2002, Wang *et al.*, 2004, Lechel *et al.*, 2005). However, over-expression of *TERF2* is associated with an increased rate of telomere shortening in primary human IMR90 fibroblasts without accelerating senescence (Karlseder *et al.*, 2002, Martínez and Blasco, 2017).

The structure of telomeres and its maintenance genes were reported to be abnormal in many cancer cell lines (Jafri *et al.*, 2016; Bernal and Tusell, 2018). *TERF2* is over-expressed in several human tumors, such as skin cancer (Biroccio *et al.*, 2006; Martínez and Blasco, 2017), hepatocarcinoma (Oh *et al.*, 2005), carcinoma of the lung (Nakanishi *et al.*, 2003) and breast carcinomas (Diehl *et al.*, 2011; Nijjar *et al.*, 2005) as well as colon cancer (Dong *et al.*, 2010) which indicates that *TERF2* may play a role in tumorigenesis. *TERF2* expression has been reported to be increased, decreased or unchanged in other solid tumors (Diehl *et al.*, 2011, Dong *et al.*, 2010, Benhamou *et al.*, 2016, Klapper *et al.*, 2003).

Characterization of *TERF2* mRNA over-expression in cALL *TEL-AML1* subgroup is concordant with the findings from several research groups (Yeoh *et al.*, 2002, van Delft *et al.*, 2005, Gandemer *et al.*, 2007). *TERF2* is one of the 40 genes that are useful to discriminate ALL from

AML (van Delft *et al.*, 2005). Hence, we would like to explore the potential value of *TERF2* as a prognostic marker which may guide patients towards suitable therapy.

MATERIALS AND METHODS

Patients and samples

After informed consent, a total of 88 diagnostics bone marrow samples and 6 peripheral blood samples were obtained from children aged between 0.1 to 16 years diagnosed with *de novo* acute lymphoblastic leukemia (ALL). These patients were recruited from University of Malaya Medical Centre (UMMC) from January 2006 to December 2010 (Table 1). All leukemic cases were classified according to FAB criteria, characterized at cytogenetic level using G-banding karyotyping and screened by real-time polymerase chain reaction (PCR) for the presence of the most significant molecular fusion transcripts namely; *BCR-ABL1*, *E2A-PBX1*, *MLL-AF4* and *TEL-AML1* as previously described in cALL (Cho *et al.*, 2012). These samples also being immunophenotyped towards pre-B ALL markers such as CD34+ and CD19+ using flow-cytometer at the Clinical Diagnostic Lab, UMMC. *TERF2* gene expression was also compared in five tumor samples (3 samples of Ewing sarcoma and a sample of hepatoblastoma) and nine samples from other hematological malignancies (7 samples from AML and a sample from CML). Three healthy bone marrow and six healthy peripheral blood samples were used as control. All bone marrow samples were also being typed for CD34+ markers. Ethical approval was obtained from the University of Malaya Medical Centre Ethics committee for both the leukemia (MEC 2006/481.01) and solid tumour samples (MEC 2006/510.7).

RNA extraction and preparation of cDNA

Mononuclear cells were isolated from 2-3 ml diagnostic bone marrow aspirate in 3 ml EDTA tube by Ficoll-Hypaque density gradient centrifugation (GE Healthcare Life Sciences, USA). Total ribonucleic acid (RNA) was extracted by using TRIZOL method (Invitrogen, USA) according to the manufacturer's recommendation

and stored at -80°C . As part of RNA quality assessment, archived samples were randomly selected, and RNA Integrity Number (RIN) were analysed on RNA 6000 Nano Labchip kit (Agilent Technologies, USA) using the Agilent 2100 Bioanalyser platform (Agilent Technologies, USA). Only RNA samples having RIN more than 8.5 were used for the study. Complementary DNA (cDNA) was synthesized from 1000 ng of RNA by reverse-transcription PCR using Omniscript RT kit (Qiagen Inc., Valencia, CA). We also included a randomly picked cDNA sample as a minus reverse transcriptase control sample to check for DNA contamination in the RNA samples.

Real-time PCR

TERF2 gene expression (NCBI accession no: NM_005652.3) was assessed and quantified using real-time quantitative PCR on a Rotorgene™ 3000 real-time thermal cycler (Corbett Research, Australia). The amplification of *TERF2* was performed with dsDNA intercalating dye, SYBR-Green using the following primers: forward primer for exon 6: 5' CAGGGAAGGAAGATAAACAGC 3' and a reverse primer for exon 6: 5' CAATGGTGGTTGGAGGATTC 3'. Amplification of *TERF2* cDNA produced a 93 bp PCR product. Beta-2-microglobulin (B2M), a cytoskeletal protein involved in cell locomotion (Zhang *et al.*, 2005) was used as the internal control gene because it is known to be moderately expressed in abundant levels in most cells, and it is found to have stable expression in hematopoietic cells (Matsuzaki *et al.*, 2015). HL60 which is derived from a patient with acute promyelocytic leukemia having no fusion transcript (Collins, 1987), are useful to study on molecular mechanisms leukemic cells of the hematopoietic lineage (Collins, 1987). The B2M forward primer sequence has 5' GAGGCTATCCAGCGTACTCCAAAGAT 3' while the reverse has 5' TGAAACCCAGACACATAGCAATTCAGG 3'. The B2M PCR amplification produced 150 bp PCR product.

As part of PCR optimization, samples were run on gel electrophoresis and specific melting temperature for each PCR product was checked using Rotor gene melt analysis software (Corbett

Research, Australia). All samples were run in triplicate.

Table 1. Clinical characteristics of cALL patients at diagnosis.

Characteristics	Value (%)
<i>Gender – Number (%)</i>	
Male	46 (63.89)
Female	26 (36.11)
<i>Age group – Number (%)</i>	
<2 yr	10(13.89)
2-9 yr	46(63.89)
≥10 yr	16(22.22)
<i>Leukocyte count ($\times 10^9$ /L) at diagnosis – Number (%)</i>	
<20	36 (50)
20-100	18 (25)
> 100	18(25)
<i>Leukocyte count at diagnosis ($\times 10^9$ /L)</i>	
Median	20.95
Range	0.6– 708
<i>Blast count in bone marrow (percentage)</i>	
Median	90
Range	30-90
<i>Hemoglobin (g/L)</i>	
Median	8.3
Range	2.2 – 116
<i>Platelets ($\times 10^9$/L)</i>	
Median	43
Range	1.58 – 573

Relative real-time PCR quantification

Relative quantification of *TERF2* gene expression was calculated using the delta delta Ct method (Livak and Schmittgen, 2001). We tested the PCR efficiencies for *TERF2* and B2M from a serially diluted RNA (from 1000ng to 0.1ng RNA). PCR efficiency from both genes was comparable having an efficiency of 90%. Furthermore, we determined the amplification efficiencies of the two target sequences by subtracting Ct values of *TERF2* and Ct values of B2M and the difference in Ct values was then plotted against the log values of the template amount. The slope of the resulting straight line was found to be 0.088 (<0.1), indicating our amplification efficiencies were comparable and gene expression analysis can be used to perform delta-delta Ct method analysis.

Receiver operating characteristic curve analysis

Receiver operating characteristic (ROC) curve

analysis was used to determine *TERF2* level that best discriminates between patients who achieved or failed day 33 remission. Only fifty-four samples were only included due to data insufficiency for the day 33 remission status. Patients who achieved remission were labeled as event ($n=0$) and those who does not achieve remission were labeled as a non-event ($n=1$). The ROC curve was generated by using MedCalc® version 11.3.8.0.

Kaplan-Meier estimation

Kaplan-Meier estimation was used to evaluate the overall survival of the patients. Kaplan-Meier analysis was performed in 72 cALL patients (all subtypes) that were stratified accordingly following ROC curve analysis.

Statistical analysis

One-way ANOVA with Bonferroni correction was used to compare *TERF2* gene expression values in cALL with various groups and *TERF2* gene expression within cALL molecular subtypes. Univariate analysis using Chi-square test was performed to determine statistical links between biological and clinical parameters.

RESULTS

TERF2 mRNA expression in childhood ALL

We studied a total of 72 *de novo* cALL diagnostic bone marrow samples for *TERF2* gene expression level. There was a broad spectrum of *TERF2* expression, ranging from 0.0072 to 4.2575 (mean=0.5736, median=0.5664). *TERF2* gene expression from diagnostic bone marrow samples was compared with normal bone marrow samples, other childhood hematological malignancies (CML and AML) and pediatric solid tumors. ANOVA one-way test shows significant differences between groups (p -value = 0.00053), $p < 0.05$ (Figure 1). Post-hoc test between groups shows significant *TERF2* expression in cALL compared with control samples p -value=0.00286, $P < 0.05$; while comparisons with groups were insignificant. Further analysis of *TERF2* expression between subtypes of cALL shows significant differences in *TEL-AML* subgroup compared to control samples with p -value=0.00023, $p < 0.05$ as shown in Figure 2. Although lower expression was

recorded in *BCR-ABL1* subtype in comparison to control, it is not-significant.

Figure 1. Bar graph showing means and standard error of relative expression of *TERF2* in 94 samples from different groups. The x-axis refers to the samples from various groups whilst the y-axis refers to relative expression of *TERF2*. ANOVA one-way test shows significant difference between groups $P = 0.00053$, $P < 0.05$ (Figure 1). Further analysis between childhood leukemia samples (cALL) subtypes shows significant differences in cALL compared to control which is non-cancerous bone marrow samples having p -value = 0.00286, $p < 0.05$. There is no significant difference between others (which comprises of CML and AML) and solid tumors with control.

Prognostic significance of *TERF2* mRNA expression in cALL

The cutoff point value was used to discriminate over-expression or under-expression of *TERF2* by using ROC curve analysis (Figure 3). Following this, the area under the curve was determined with 0.663 having significance level of p -value = 0.0273 ($p < 0.05$) (95% CI 0.521, 0.786) and the cut off value generated was 0.3439. Patients who have an expression less than 0.3439 were declared as *TERF2* under-expression and over-expression for those who have higher expression than 0.3439.

Kaplan-Meier analysis of relapse-free survival in 72 patients shows 17 out of 35 patients who under-expressed *TERF2* and 14 patients (82.35%) did not achieve clinical remission at day 33. In contrast, of 37 who over-expressed *TERF2*, 11 of them achieved clinical remission at day 33 (29.73%). The result shows a better overall

survival for cALL patients with *TERF2* over-expression while poorer overall survival for under-expressed *TERF2*. Univariate analysis using Chi-square test was carried out to evaluate the relationship between *TERF2* under and over-

expression level with clinical parameters. We found that *TERF2* gene expression was not associated with age, hyperleukocytosis, or risk groups (Table 2).

Figure 2. Shows box and whisker plot representation of relative fold of *TERF2* expression in 72 diagnostic bone marrows in cALL with different molecular subtypes (*TEL-AML1*; n=11, *BCR-ABL1*; n=7, *E2A-PBX1*; n=4, *MLL-AF4*; n=1 and negative OFT; n=49). Statistical analysis shows that *TEL-AML1* patients have significantly higher expression of *TERF2* compared to control. Significant association was not found in *BCR-ABL1* ($p>0.05$), *E2A-PBX1* ($p>0.05$), *MLL-AF4* and negative OFT subgroups ($p>0.05$). The median expression level of *TERF2* in different subgroups was: *BCR-ABL1*, 0.344; *TEL-AML1*, 2.848; *E2A-PBX1*, 0.591; *MLL-AF4*, 0.321 and those without any fusion transcripts, 0.435.

Table 2. Table shows the evaluation of *TERF2* relative gene expression.

Clinical parameters	<i>TERF2</i> over-expression (37)	<i>TERF2</i> under-expression (17)	p-value
Age <2 years	29 (29.41%)	12 (70.59%)	0.7
Leucocytosis > $5 \times 10^9/L$	15 (40.54%)	6 (35.29%)	0.9
Poor risk	3 (8.1%)	5 (29.41%)	0.09
Relapse	6 (16.22%)	4 (23.52%)	0.7

Evaluation of *TERF2* relative gene expression with different clinical parameters (Age, leucocytosis, poor risk, relapse) by using univariate analysis. None of the parameters shows association with the *TERF2* expressions.

Figure 3. The cutoff point value used to discriminate over-expression or under-expression of TERF2 by ROC curve analysis. The area under the curve was determined with 0.663 having significance level of p -value = 0.0273 ($p < 0.05$) (95% CI 0.521, 0.786) and the cut off value generated was 0.3439. Patients who have an expression less than 0.3439 were declared as TERF2 under-expression and over-expression for those who have higher expression than 0.3439.

DISCUSSION

The relationship with telomere impairment and cancer has been well debated and discussed. It is known that shorten telomere contributes toward cancer initiation and progression. DNA binding proteins TERF2 binds directly with double stranded telomere sequences and plays a crucial role for telomere end protection and helps in the t-loops conformation (Jones *et al.*, 2012). Studies shows that expression level of shelterin protein complex like TERF2 are elevated and correlated with cancer progression in many cancers as compared to the non-cancerous tissues. Our studies show found that TERF2 expression are significantly high in cancerous samples compared to the normal samples. Upregulation of TERF2 in malignant cells were well documented in previous publications (Biroccio *et al.*, 2006; Oh *et al.*, 2005; Nakanishi *et al.*, 2003; Diehl *et al.*, 2011; Nijjar *et al.*, 2005; Dong *et al.*, 2010; Benhamou *et al.*, 2016). TERF2 over-expression that were reported in the lung, gastric, breast (Savage *et al.*, 2007; Pellatt *et al.*, 2013, Diehl *et al.*, 2011), renal cancers (Chen *et al.*, 2017; Pal *et al.*, 2015), prostate (Kibel *et al.*, 2016; Chen *et al.*, 2017) and colorectal cancer (Aljarbou *et al.*, 2018) might confer proliferative advantages to tumor cells (Kumar *et al.*, 2018). Recent study also shows TERF2 occupancy at promoter were enriched in cells with short telomeres (Mukherjee *et al.*, 2018).

Unlike other studies, our research focusses on investigation of TERF2 expression in the different molecular subgroups of cALL. Analysis of TERF2 expression in cALL subgroups shows significantly higher TERF2 expression in *TEL-AML1* subgroups compared to control. *TEL-AML1* subgroup of ALL generally presents with low or medium risk features and overall has a very good prognosis. We suggest that over-expression of TERF2 in *TEL-AML1* would serve as a protective mechanism for the telomere from end to end fusion by preventing anaphase bridges from occurring and promoting chromosomal stability leading to the stable environment (Hartmann and Scherthan, 2005; Cookson and Laughton, 2009; Wang *et al.*, 2018). With the presence of intact telomere maintenance function, TERF2 triggers normal apoptosis and DNA damage mechanism via ataxia-telangiectasia mutated (ATM) and p53 signalling pathway checkpoint (Maser and DePinho, 2002). *TEL-AML1* is known as good prognostic molecular subgroup in cALL, hence this may suggests the role of TERF2 over-expression may enhance responsiveness to standard chemotherapy and patients achieve complete remission by day 33 (Subotnicu *et al.*, 2017; Seeger *et al.*, 2001).

Hypomethylation of promoter and exon 1 CpG islands of TERF2 may appear to be one of the critical epigenetic mechanisms by which TERF2 gene can be over-expressed. However, this is not the only mechanism involved in TERF2 regulation (Dong *et al.*, 2010). In *TEL-AML1* cells after it has been exposed to L-asparaginase, the genome-wide analysis revealed that TERF2 gene expression decreased significantly together with a panel of genes namely MCM3, MCM4, MCM5, CDC23 which are involved in cellular proliferation. (Lynch *et al.*, 2008). Higher expressions of TERF2 in *TEL-AML1* have been reported in (Yeoh *et al.*, 2002; Ross *et al.*, 2003; van Delft *et al.*, 2005) which suggested that the gene protects the telomere end against senescence as most leukaemic cells have short telomeres (Ross

et al., 2003). Another different study has observed the correlation between high *TERF2* expression and longer life spans of advanced stage cervical cancer patients which suggests that *TERF2* as an important factor in chromatin stability and survival (Ozden *et al.*, 2014).

TERF2 is under-express in *BCR-ABL1* patient cells. Although this is not significant in our present study due to low sample number, it may propose reason to why *BCR-ABL1* is often present with high-risk features and with poor prognosis during treatment. *TERF2* under-expression will not be able to protect the telomere causing more severe complications to the chromosomes and genomic stability. The situation in *BCR-ABL1* and *MLL-AF4* cells

becomes more complex as there is a high incidence of potential p53 pathway inactivation due to p53 mutation and MDM2 over-expression in these patients *BCR-ABL1* (Marks *et al.*, 1996). The resistance mechanism involving *TERF2* and *BCR-ABL1* and *MLL-AF4* fusions are yet to be discovered.

We managed to investigate *TERF2* gene expression in a small cALL sample size. Larger sample size will be needed to confirm potent use of *TERF2* as prognostic markers. To further understand *TERF2* gene function and mechanism as well as its correlation with telomere, telomerase and telomere maintenance in subtypes of cALL, in depth *in vitro* model experiments will be needed in the future.

Figure 4. Kaplan-Meier analysis of relapse-free survival in 72 ALL patients. Patients were stratified according to the higher and lower expression of *TERF2* in their diagnosis bone marrow samples taken at diagnosis. The x-axis refers to the period of follow-up (months) and the y-axis refers to the proportion of patients. The dashed and dark lines refer to patients who over-expressed *TERF2*, or not respectively. Analysis shows a better survival for patients who over-express *TERF2* comparatively with $p < 0.05$.

CONCLUSION

Over-expression of *TERF2* is associated with good prognosis in cALL whilst under-expression is associated with poor prognosis in cALL

patients. Measurement of *TERF2* gene expression allows proper stratification of cALL subtypes into its respective prognostic indicator classification.

Telomere haemostasis has been significantly deregulated in cALL, and each molecular subtype

have different deregulation of *TERF2* gene expression. Our result suggests that *TERF2* has a potential prognostic implication, which may tailor patients towards specific treatment decision and less invasive treatment regimen exposure. In addition, we hypothesize that *TERF2* over-expression might have protective mechanism towards *TEL-AML1* subtype. This may uncover the possibilities of telomere targeted therapies for cancer such as telomerase inhibitor. It is important to note that telomere haemostasis in cALL and adult might not be consistent due to the different disease behavior. Therefore, further investigation is needed to confirm the mechanism.

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ETHICAL APPROVAL

Approval for this project was obtained from the University of Malaya Medical Centre Ethics committee for both the leukemia (MEC 2006/481.01) and solid tumour (MEC 2006/510.7) components.

REFERENCES

- Aljarbou, F., Almousa, N., Bazzi, M., Aldaihan, S., Alanazi, M., Alharbi, O., Almadi, M., Aljebreen, A. M., Azzam, N. A., & Arafa, M. 2018. The expression of telomere-related proteins and DNA damage response and their association with telomere length in colorectal cancer in Saudi patients. *PLoS One* 13: e0197154.
- Avigad, S., Naumov, I., Ohali, A., Jeison, M., Berco, G. H., Mardoukh, J., Stark, B., Ash, S., Cohen, I. J., & Meller, I. 2007. Short telomeres: a novel potential predictor of relapse in Ewing sarcoma. *Clinical Cancer Research* 13: 5777-5783.
- Benhamou, Y., Picco, V., Raybaud, H., Sudaka, A., Chamorey, E., Brolih, S., Monteverde, M., Merlano, M., Nigro, C. L., & Ambrosetti, D. 2016. Telomeric repeat-binding factor 2: a marker for survival and anti-EGFR efficacy in oral carcinoma. *Oncotarget* 7: 44236.
- Bernal, A. & Tusell, L. 2018. Telomeres: Implications for cancer development. *International Journal of Molecular Sciences* 19: 294.
- Biroccio, A., Rizzo, A., Elli, R., Koering, C.-E., Belleville, A., Benassi, B., Leonetti, C., Stevens, M. F., D'incalci, M., & Zupi, G. 2006. TRF2 inhibition triggers apoptosis and reduces tumorigenicity of human melanoma cells. *European Journal of Cancer* 42: 1881-1888.
- Chen, W., Wang, Y., Li, F., Lin, W., Liang, Y., & Ma, Z. 2017. Expression of telomere repeat binding factor 1 and TRF2 in prostate cancer and correlation with clinical parameters. *BioMed Research International* 2017.
- Cho, Y.-U., Chi, H.-S., Park, C.-J., Jang, S., & Seo, E.-J. 2012. Rapid detection of prognostically significant fusion transcripts in acute leukemia using simplified multiplex reverse transcription polymerase chain reaction. *Journal of Korean Medical Science* 27: 1155.
- Collins, S. J. 1987. The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression.
- Cookson, J. & Laughton, C. 2009. The levels of telomere-binding proteins in human tumours and therapeutic implications. *European Journal of Cancer* 45: 536-550.
- De Lange, T. 2005. Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes & Development* 19: 2100-2110.
- Diehl, M. C., Idowu, M. O., Kimmelschue, K. N., York, T. P., Jackson-Cook, C. K., Turner, K. C., Holt, S. E. & Elmore, L. W. 2011. Elevated TRF2 in advanced breast cancers with short telomeres. *Breast Cancer Research and Treatment* 127: 623-630.
- Dong, W., Wang, L., Chen, X., Sun, P., & Wu, Y. 2010. Upregulation and CpG island hypomethylation of the TRF2 gene in human gastric cancer. *Digestive Diseases and Sciences* 55: 997-1003.
- Gandemer, V., Rio, A.-G., De Tayrac, M., Sibut, V., Mottier, S., Sunnaram, B. L., Henry, C., Monnier, A., Berthou, C., & Le Gall, E. 2007. Five distinct biological processes and 14 differentially expressed genes characterize TEL/AML1-positive leukemia. *BMC Genomics* 8: 385.
- Hartmann, N. & Scherthan, H. 2005. Characterization of the telomere complex, TERF1 and TERF2 genes in muntjac species with fusion karyotypes. *Experimental Cell Research* 306: 64-74.
- Jafri, M. A., Ansari, S. A., Alqahtani, M. H., & Shay, J. W. 2016. Roles of telomeres and telomerase in cancer, and advances in telomerase-targeted therapies. *Genome Medicine* 8: 69.
- Jones, C. H., Pepper, C., & Baird, D. M. 2012. Telomere dysfunction and its role in haematological cancer. *British Journal of Haematology* 156: 573-587.
- Kaatsch, P. 2010. Epidemiology of childhood cancer. *Cancer Treatment Reviews* 36: 277-285.
- Kaminker, P. G., Kim, S.-H., Taylor, R. D., Zebajarian, Y., Funk, W. D., Morin, G. B., Yaswen, P., & Campisi, J. 2001. TANK2, a new TRF1-associated poly (ADP-ribose) polymerase, causes rapid induction of cell death upon overexpression. *Journal of Biological Chemistry* 276: 35891-35899.
- Karlseder, J., Smogorzewska, A., & De Lange, T. 2002. Senescence induced by altered telomere state, not telomere loss. *Science* 295: 2446-2449.
- Kibel, A. S., Ahn, J., Isikbay, M., Klim, A., Wu, W. S., Hayes, R. B., Isaacs, W. B., & Daw, E. W. 2016. Genetic variants in cell cycle control pathway confer susceptibility to aggressive prostate carcinoma. *The Prostate* 76: 479-490.
- Klapper, W., Qian, W., Schulte, C., & Parwaresch, R. 2003. DNA damage transiently increases TRF2 mRNA expression and telomerase activity. *Leukemia* 17: 2007-2015.
- Kumar, R., Khan, R., Gupta, N., Seth, T., Sharma, A., Kalaivani, M., & Sharma, A. 2018. Identifying the biomarker potential of telomerase activity and shelterin complex molecule,

- telomeric repeat binding factor 2 (TERF2), in multiple myeloma. *Leukemia & Lymphoma* 59: 1677-1689.
- Lechel, A., Satyanarayana, A., Ju, Z., Plentz, R. R., Schaetzlein, S., Rudolph, C., Wilkens, L., Wiemann, S. U., Saretzki, G., & Malek, N. P. 2005. The cellular level of telomere dysfunction determines induction of senescence or apoptosis in vivo. *EMBO Reports* 6: 275-281.
- Lin, J., Countryman, P., Buncher, N., Kaur, P., E, L., Zhang, Y., Gibson, G., You, C., Watkins, S. C., & Piehler, J. 2013. TRF1 and TRF2 use different mechanisms to find telomeric DNA but share a novel mechanism to search for protein partners at telomeres. *Nucleic Acids Research* 42: 2493-2504.
- Livak, K. J. & Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta CT$ method. *Methods* 25: 402-408.
- Locatelli, F., Schrappe, M., Bernardo, M. E., & Rutella, S. 2012. How I treat relapsed childhood acute lymphoblastic leukemia. *Blood* 120(14): 2807-2816.
- Lynch, M., Sung, W., Morris, K., Coffey, N., Landry, C. R., Dopman, E. B., Dickinson, W. J., Okamoto, K., Kulkarni, S., & Hartl, D. L. 2008. A genome-wide view of the spectrum of spontaneous mutations in yeast. *Proceedings of the National Academy of Sciences* 105: 9272-9277.
- Marks, D. I., Kurz, B. W., Link, M. P., Ng, E., Shuster, J. J., Lauer, S. J., Brodsky, I., & Haines, D. S. 1996. High incidence of potential p53 inactivation in poor outcome childhood acute lymphoblastic leukemia at diagnosis.
- Martínez, P. & Blasco, M. A. 2017. Telomere-driven diseases and telomere-targeting therapies. *Journal of Cell Biology* 216: 875-887.
- Maser, R. S. & Depinho, R. A. 2002. Connecting chromosomes, crisis, and cancer. *Science* 297: 565-569.
- Matsuzaki, Y., Umemoto, T., Tanaka, Y., Okano, T. & Yamato, M. 2015. $\beta 2$ -Microglobulin is an appropriate reference gene for RT-PCR-based gene expression analysis of hematopoietic stem cells. *Regenerative Therapy* 1: 91-97.
- Mukherjee, A. K., Sharma, S., Sengupta, S., Saha, D., Kumar, P., Hussain, T., Srivastava, V., Roy, S. D., Shay, J. W., & Chowdhury, S. 2018. Telomere length-dependent transcription and epigenetic modifications in promoters remote from telomere ends. *PLoS Genetics* 14: e1007782.
- Nakanishi, K., Kawai, T., Kumaki, F., Hiroi, S., Mukai, M., Ikeda, E., Koering, C. E., & Gilson, E. 2003. Expression of mRNAs for telomeric repeat binding factor (TRF)-1 and TRF2 in atypical adenomatous hyperplasia and adenocarcinoma of the lung. *Clinical Cancer Research* 9: 1105-1111.
- Neidle, S. & Parkinson, G. N. 2003. The structure of telomeric DNA. *Current Opinion in Structural Biology*, 13: 275-283.
- Nijjar, T., Bassett, E., Garbe, J., Takenaka, Y., Stampfer, M. R., Gilley, D., & Yaswen, P. 2005. Accumulation and altered localization of telomere-associated protein TRF2 in immortally transformed and tumor-derived human breast cells. *Oncogene* 24: 3369.
- Oh, B.-K., Kim, Y.-J., Park, C., & Park, Y. N. 2005. Up-regulation of telomere-binding proteins, TRF1, TRF2, and TIN2 is related to telomere shortening during human multistep hepatocarcinogenesis. *The American Journal of Pathology* 166: 73-80.
- Ozden, S., Tiber, P. M., Ozgen, Z., Ozyurt, H., Serakinci, N., & Orun, O. 2014. Expression of TRF2 and its prognostic relevance in advanced stage cervical cancer patients. *Biological Research* 47: 1-7.
- Pal, D., Sharma, U., Singh, S. K., Kakkar, N., & Prasad, R. 2015. Over-expression of telomere binding factors (TRF1 & TRF2) in renal cell carcinoma and their inhibition by using SiRNA induce apoptosis, reduce cell proliferation and migration invitro. *PLoS One* 10: e0115651.
- Pellatt, A. J., Wolff, R. K., Torres-Mejia, G., John, E. M., Herrick, J. S., Lundgreen, A., Baumgartner, K. B., Giuliano, A. R., Hines, L. M., & Fejerman, L. 2013. Telomere length, telomere-related genes, and breast cancer risk: the breast cancer health disparities study. *Genes, Chromosomes and Cancer* 52: 595-609.
- Ross, M. E., Zhou, X., Song, G., Shurtleff, S. A., Girtman, K., Williams, W. K., Liu, H.-C., Mahfouz, R., Raimondi, S. C., & Lenny, N. 2003. Classification of pediatric acute lymphoblastic leukemia by gene expression profiling. *Blood* 102: 2951-2959.
- Savage, S., Chanock, S., Lissowska, J., Brinton, L., Richesson, D., Peplonska, B., Bardin-Mikolajczak, A., Zatonski, W., Szeszenia-Dąbrowska, N. & Garcia-Closas, M. 2007. Genetic variation in five genes important in telomere biology and risk for breast cancer. *British Journal of Cancer* 97: 832-836.
- Seeger, K., Kreuzer, K.-A., Lass, U., Taube, T., Buchwald, D., Eckert, C., Körner, G., Schmidt, C.-A., & Henze, G. 2001. Molecular quantification of response to therapy and remission status in TEL-AML1-positive childhood ALL by real-time reverse transcription polymerase chain reaction. *Cancer Research* 61: 2517-2522.
- Stansel, R. M., De Lange, T., & Griffith, J. D. 2001. T-loop assembly in vitro involves binding of TRF2 near the 3' telomeric overhang. *The EMBO Journal* 20: 5532-5540.
- Subotnicu, M., Ivanov, A., Ivanov, I., Mocanu, A., Dumitras, S., Antonela, C., Schmidt, M., Rusu, C., & Miron, I. 2017. P378 Molecular diagnostic markers for childhood acute leukaemia—a single centre experience. BMJ Publishing Group Ltd.
- Van Delft, F. W., Bellotti, T., Luo, Z., Jones, L. K., Patel, N., Yiannikouris, O., Hill, A. S., Hubank, M., Kempinski, H., & Fletcher, D. 2005. Prospective gene expression analysis accurately subtypes acute leukaemia in children and establishes a commonality between hyperdiploidy and t(12; 21) in acute lymphoblastic leukaemia. *British Journal of Haematology* 130: 26-35.
- Van Steensel, B., Smogorzewska, A. & De Lange, T. 1998. TRF2 protects human telomeres from end-to-end fusions. *Cell* 92: 401-413.
- Wang, L., Tu, Z., Liu, C., Liu, H., Kaldis, P., Chen, Z., & Li, W. 2018. Dual roles of TRF1 in tethering telomeres to the nuclear envelope and protecting them from fusion during meiosis. *Cell Death & Differentiation* 25: 1174.
- Wang, R. C., Smogorzewska, A., & De Lange, T. 2004. Homologous recombination generates T-loop-sized deletions at human telomeres. *Cell* 119: 355-368.
- Yeoh, E.-J., Ross, M. E., Shurtleff, S. A., Williams, W. K., Patel, D., Mahfouz, R., Behm, F. G., Raimondi, S. C., Relling, M. V., & Patel, A. 2002. Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. *Cancer Cell* 1: 133-143.
- Zhang, X., Ding, L., & Sandford, A. J. 2005. Selection of reference genes for gene expression studies in human neutrophils by real-time PCR. *BMC Molecular Biology* 6: 1-7.