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NEAR EAST UNIVERSITY

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Telomere dynamics in patients suffering from recurrent miscarriage

A THESIS SUBMITTED TO THE GRADUATE INSTITUTE OF

HEALTH SCIENCES NEAR EAST UNIVERSITY

BY:

Rameez Hassan Pirzada

In Partial Fulfillment of the Requirements for the Degree of

Master of Science in Medical Biology and Genetics

NICOSIA 2016

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Advisor:

Prof. Dr Nedime Serakıncı

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Acknowledgement

All praise is due to God, who supplied me with the courage, the guidance, and the love to complete this research. Firstly, I would like to express my sincere gratitude to my advisor Prof. Dr Nedime Serakinci for the continuous support, useful comments, remarks and engagement during my Masters Research study. Her supervision helped me throughout the research and writing of this thesis. I could not have imagined having a better advisor and mentor. Secondly, I would like to thank Dr. Oya Orun and Dr. Can Erzik for their guidance and support. They provided me with an opportunity to use their lab at the Marmara University Faculty of Medicine, Istanbul.

I would also like to thank the faculty members of Medical Biology and Genetics department Near East University for their valuable help and encouragement. I also thank all my family members, especially my mother for unceasing encouragement and support.

Last but not the least my sense and gratitude to one and all who, directly or indirectly, have lent their support and helping hand in this venture.

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Abstract

Telomeres are the tandem repeats (TTAGGG) present at the ends of the chromosomes that ensure the chromosome stability and also prevent chromosome ends from degradation. Telomeres in human cells shorten with each cellular division and are linked to cellular senescence. Surprisingly, there are few studies showing an association of telomere length and associated shelterin protein complex with various reproductive disorders such as miscarriage. The study included patients (n=20) and control group (n=10). The telomere length was measured using Universal short telomere elongation length assay (U-STELA) and Telomere restriction fragment length (TRF) analysis. The gene expressions of important shelterin protein complex (TRF1, TRF2, POT1, and TPP1) were measured using Real time quantitative reverse transcriptase PCR (qRT-PCR).

The study shows down regulation of *TRF2* and *TPP1* and an overall decline in average telomere length clearly indicates the significant ($p \leq 0.05$) correlation between abnormal telomere dynamics and recurrent pregnancy loss.

In conclusion, shorter telomere length and inappropriate binding of the TRF2 and TPP1 at chromosomal ends appears to play a critical role in recurrent pregnancy loss. The down-regulation of 2 major shelterin proteins (TRF2, TPP1), contributes to incomplete capping known to have led to chromosomal instability through Breakage-fusion-bridge (BFB) cycle. An improper binding of shelterin complex leaves ends of the chromosomes uncapped which could trigger unwanted DNA damage response pathways (ATM and ATR) and may induce cellular senescence by considering telomeres ends as a double strand break.

The definite cause between abnormal telomere length dynamics in inducing idiopathic recurrent pregnancy loss needs further research and evaluation by enrolling a larger number of studies to further endorse the findings of this study.

Keywords: **Idiopathic Recurrent Pregnancy loss, Universal STELA, TRF, Shelterin proteins**

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LIST OF ABBRREVATIONS

S. #	ABBREVATIONS	EXPLANATION
1	iRPL	Idiopathic recurrent pregnancy loss
2	FISH	Fluorescence *in situ* hybridization
3	FSH	Follicle-stimulating hormone
4	GnRH	Gonadotropin releasing hormone
5	HPO	Hypothalamus-pituitary-ovarian
6	LH	Luteinizing hormone
7	RM	Recurrent misscarriage
8	MTHFR	Methylenetetrahydrofolate reductase
9	qPCR	Quantitative polymerase chain reaction
10	ROS	Reactive oxidative species
11	STELA	Single Telomere Length Analysis
12	U-STELA	Universal Single Telomere Length Analysis
13	TRF	Terminal Restriction Fragment
14	TIF	Telomere Induced Foci
15	MI	Meiotic division
16	UTR	Untranslated region
17	TRF1	Telomere restriction fragment length 1
18	TRF2	Telomere restriction fragment length 2
19	POT1	protection of telomeres 1
20	TPP1	Tripeptidyl peptidase I
21	RAP1	Repressor Activator Protein
22	DSB	Double stranded break
23	ATM	Ataxia telangiectasia mutate

24	IUGR	Intrauterine growth restriction

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1. Introduction

Recurrent miscarriage (RM) is a multifactorial disorder with complex etiology and many related factors. Most often it is difficult to locate the exact cause of idiopathic recurrent pregnancy loss iRPL, although there are several factors such as endocrinological, immunological, anatomical and genetic factors governing RM (Venkatesh et al., 2011). Telomeres are the regions present at the ends of the chromosomes, which are enriched in the sub telomeric region, CpG islands and are supposed to contain highest gene density (Saccone, De Sario, Della Valle, & Bernardi, 1992). They play a significant role in maintaining the chromosomal integrity by providing the capping function that ensures chromosomal protection from degradation and end to end fusion. With each cell division chromosomal ends become shorter due to end replication problem which results in really short telomeres and this causes cellular senescence (Baird, 2005). In germ cell lines they play significant role in meiotic recombination and ensure proper pairing of homologous chromosomes (L. Liu et al., 2004). Extreme telomere shortening triggers aneuploidies and non-reciprocal translocations which leads to chromosomal abnormalities and DNA damage (Murnane, 2006) (Krizhanovsky et al., 2008). Telomeres are highly prone to oxidative stress by creating a buffer zone that protects the chromosomal ends and ensures genomic and cellular integrity (Coluzzi et al., 2014) .

Abnormal telomere length and expression of shelterin protein complex has been identified to be responsible for oocyte or sperm cleavage therefore causing an abnormality in chromosome structure; and also negatively influence the fertilization process as demonstrated in knock-out mice (L. Liu, Blasco, Trimarchi, & Keefe, 2002a). The cytoplasmic fragmentation in oocytes which is responsible for apoptosis can be predicted by measuring the telomere length in human embryos (David L Keefe et al., 2005). Pronuclear development during fertilization the telomeres present in male sperms respond first to oocyte signals (Zalenskaya, Bradbury, & Zalensky, 2000a).

In 2004, a study by Liu et al has revealed that short telomeres are found to be related to plenty of defects such as impaired chromosome synapsis, aneuploidies, reduced quality of oocyte, increased apoptotic activity, premature aging and defects in oogenesis (L. Liu et al., 2004).

Short telomeres induce DNA defragmentation in mice and they may also cause abnormal embryonic development in humans (Rodríguez et al., 2005). Telomere and associated shelterin

protein complex (telomere binding protein) play a key role in telomere protection and maintenance by facilitating replication and regulating telomerase access. They also facilitate the chromosomal localization, whereas abnormal chromosomal localization may leads to meiotic errors and results in aneuploidies due to sperm abnormality (Scherthan et al., 1996) (Barlow & Hultén, 1996) (Kato, Nakayama, Agata, & Yoshida, 2012) **.**

Recurrent miscarriage is a multifactorial condition and the diagnosis is difficult as it is often identified in women with normal pregnancies and there are few therapies available for a woman with RM (A.-W. Tang & Quenby, 2010). Spontaneous pregnancy loss is commonly occurred in 15 % of the clinically observed pregnancies (Ford & Schust, 2009).

A recurrent miscarriage is an important area of research due to its stressful implications on both couple and physician. In the introduction of this thesis, the complex etiology of spontaneous abortions will be included, with an emphasis on telomere biology and the role of short telomeres and associated proteins in recurrent miscarriages.

1.1 Aims

1. The comparative study of telomere length dynamics between control group and patients suffering from recurrent miscarriage provides insight into the abnormal telomere dynamics that may be related to aneuploidies, miscarriages and other such disorders caused due to chromosomal instability.
2. The aim is to analyze the expression pattern of telomere binding proteins due to their direct impact on the stability of chromosomes and telomere length dynamics. The expression analysis provides better insight into complex regulatory networks which will lead to the identification of novel biological processes or implicated in the disease development such as recurrent miscarriage (Aithal & Rajeswari, 2015).
3. The study will help to use telomere as a biomarker for better prognosis of Idiopathic recurrent miscarriages and will help to develop new treatment modalities.

1.2.0 Recurrent miscarriage and maternal factors

Recurrent miscarriage is a condition characterized by two or more failed pregnancies and is a multifactorial disorder with complex etiology and many related factors. The multifactorial nature of the disease makes it difficult to locate the exact cause of IRPL, although there several factors such as endocrinological, immunological , anatomical , and genetic factors governing this condition (Venkatesh et al., 2011).

Recurrent miscarriages can be caused due to various maternal factors out of which primary factors associated with RM can be categorized as chromosomal, anatomical, endocrinological and immunological (Ford & Schust, 2009)**.** These primary factors alone or in combination resulted in recurrent miscarriages as observed in various previous studies however various other factors also play role in causing RM. The etiology of RM miscarriage is complex making it difficult to clinically evaluate the causes governing RM (Courtney Wood Hanna, 2013). The disorder of reproductive system poses a significant amount of challenge to clinicians and researcher's. Some of the most prevalent causes of miscarriages is as followed.

1.2.1 Chromosomal

There are various causes of recurrent miscarriage such as uterine anomalies, endocrinological, chromosomal translocations (Sugiura-Ogasawara, Ozaki, & Suzumori, 2014) . Balanced chromosomal rearrangements has been observed in approximately 3.5 % of couples with recurrent miscarriage (RM) (Clifford, Rai, Watson, & Regan, 1994)**.** A balanced translocation in which section of chromosome changes its position from one chromosome to another within a chromosomal map without causing any gain or loss of important genetic material; are common cause of recurrent miscarriages.(Kavalier, 2005). Data obtained from various studies showed that approximately 1/3 of miscarriages occurred due to unbalanced chromosomal rearrangements. As it is known that telomeres protect the chromosomes ends and provides chromosomal stability so short telomere also induces chromosomal aberrations and may results into miscarriage. Short telomere leads to chromosomal instability and studies have indicated that shorter length of telomeres appears to play an important role in recurrent pregnancy loss (Thilagavathi et al., 2013a)**.**

1.2.2 Anatomical

Anatomical abnormalities also account for recurrent miscarriage with an incidence ranging between 1.8 to 16 % (Stephenson, 1996). The most common anatomical abnormalities are septate uterus (35%), Bicornuate Uterus (25%), Arcuate Uterus (20%). The exact mechanism of this various abnormalities and recurrent miscarriage is an area of further research whereas poor implantation and physical hindrance in the progression of pregnancy has been proposed among other anatomical abnormalities resulting in miscarriage (Y. Y. Chan et al.). Uterine abnormalities account for RM, but many women with same anomalies have successful pregnancies and further study is needed to determine the effectiveness of surgical interventions to improve pregnancy rates (Ford & Schust, 2009) (A.-W. Tang & Quenby, 2010).

1.2.3 Endocrinological

Maternal endocrinological factors play a significant role in successful growth and development of the fetus, whereas abnormality in the endocrine system may cause recurrent miscarriage. Statistical data suggests that approximately 8 % to 12% of miscarriages are due to abnormality in the endocrine system (Pluchino et al. 2014). Abnormality in maternal endocrine system will lead to abnormal level of progesterone secretions, elevated androgen level, diabetes mellitus, hyperprolactinaemia, hyperinsulinaemia and polycystic ovarian syndrome, may affect the pregnancy (Bajaj, Rajput, & Jacob, 2013).

1.2.4 Immunological

The exact physiological mechanism which allows a mother to surpass her semi-allogeneic fetus is not known clearly and presently an area open to further research (Sharma, 2014). Mother normal immunological response plays a fundamental role to carry out successful pregnancy whereas abnormality in mother's immunological response during pregnancy causes serious harm to fetus and may result in a miscarriage. The relationship between recurrent miscarriage and immunological origin is currently an area of further research so that such an events can be

forecast and in turn prevent them from occurring (Beaman et al., 2012). The coexistent of genetically different fetus is only possible when mechanism to suppress maternal immune system is active and other complex series of events are functioning normally (Mor & Cardenas, 2010).

During implantation which is an early phase of pregnancy the uterine killer cells seems to play an important role. During the luteal phase the numbers of uterine killer cells (uNK) increase whereas apoptosis has been observed in uNK cells before entering into the next follicular phase (King, Balendran, Wooding, Carter, & Loke, 1991)**.** During fertilization increment in the uNK cells has been detected throughout the first 20 weeks of pregnancy, massive increment of these cells has been identified at the sites of placental invasion (Bulmer & Lash, 2005)**.** In the peripheral blood an increment in natural killer cells has been observed in women suffering from recurrent miscarriage **(**King et al. 2010). So increment in the proportion of natural killer cells in the peripheral blood can cause RM. Uterine mucosa and natural killer cells (NK) plays significant part in cytokine response at the maternal- fetal interface (T –helper 1 and 2) (Chetty & Duncan, 2015).

1.3 Genetics and epigenetic causes of recurrent miscarriages

Recurrent miscarriage is a multifactorial disorder with strong genetic basis, as it has been observed that sisters of patients suffering from recurrent miscarriages are approximately six times more likely to suffer from RM than normal control women (Christiansen, Mathiesen, Lauritsen, & Grunnet, 1990). Both environmental and genetic factors in combination or alone may result into RM by negatively impacting different phases of oogenesis or fetal development (Rull, Nagirnaja, & Laan, 2012). In the following section the evidences that verify the contribution of genetic and epigenetic factor contribute to risk for RM.

1.3.1 Aneuploidy in Miscarriages

Aneuploidy is the presence of abnormal sets of chromosomes; it is among the second major category of chromosome mutations (Griffiths, Miller, Suzuki, Lewontin, & Gelbart, 2000a). The aneuploidy chromosomes differs from the wild type by single or a minute number of chromosomes (Griffiths et al., 2000a). Non disjunction of homologous pair of chromosomes can cause loss or gain of chromosome or abnormality in segregation of sister chromatids during

first meiotic division (MI). Moreover error can arise before the formation of zygote (post zygotic) in few early stages of cell division after fertilization (Bean, Hunt, Millie, & Hassold, 2001). The contribution of various factors such as meiotic, maternal and paternal error for every chromosome differs among miscarriages (Hassold & Hunt, 2001a). It has been calculated that approximately around 15 to 20% of all pregnancies in humans terminated due to RM or spontaneous abortions (Ghazaey et al.).

1.3.2 Chromosome Missegregation and Maternal risk

Chromosome missegregation involves an impaired or defective separation of chromosome in cellular reproduction and division. Increased maternal age has always been associated with improper segregation of chromosomes due to deterioration of the meiotic machinery (Hassold & Chiu, 1985)(Jones & Lane, 2013). Meiotic non-disjunction arises due to two essential primary risk factors first one is advanced maternal age and the other is aberrant recombination of chromosomes (Nagaoka, Hassold, & Hunt, 2012). To validate these primary hypotheses linked with chromosomal missegregation further research and detailed investigation in human oocyte is needed. The separation of sister chromatids is regulated by cohesion protein complex. The reduction in cohesion protein complex with an increasing age negatively influences the tethering of sister chromatids together (Michaelis, Ciosk, & Nasmyth, 1997). Animal models displayed reduction in cohesion proteins in the oocyte of aged females which resulted in higher rates of aneuploidy (L. Liu & Keefe, 2008) (Subramanian & Bickel, 2008). Disagreement exists among the scientific community as to whether cohesion proteins are produced during adulthood or established during fetal development. A study conducted by Garcia Cruz et al. showed the production of cohesion protein during early adulthood (Garcia-Cruz et al., 2010). The destruction in the cohesion protein complex is irreversible as it was shown in a mice study that suggested where the impaired cohesion proteins were replaced but did not show any improvement in changing the phenotype (Tachibana-Konwalski et al., 2010). In mice model the heterozygous mutation in cohesion gene displayed elevated levels of aneuploidy that increases with maternal age (Murdoch et al., 2013). Advanced maternal age also suggests weakened cohesion between sister chromatids (Jessberger, 2012). Aberrant recombination leads to achisamate formation and abnormal crossover during MI results in aneuploidy due to an error in chromosome segregation

(Lamb, Sherman, & Hassold, 2005). The incidence of achismate is higher in oocytes during MI (Oliver et al., 2008).

1.3.3 The genetic control of meiosis

Variation in the genes involved in meiosis may predispose some women to increasing rates of aneuploidies due to chromosomal non disjunction. To date few genes such as synaptonemal complex protein 3 (SYCP3) has been investigated and linked with recurrent miscarriages (Bolor et al., 2009). SYP3 genes plays essential role in organization by tethering the homologous chromosome together during meiosis 1 prophase (Yuan et al., 2000). The novel change in PLCD4 gene and a rare variation in the OSBPL5 gene that has been observed to play an important role in oocyte maturation and in the early embryonic development may be the cause of recurrent miscarriage due to recurrent triploidy (Filges et al., 2015). Further research is needed to identify more genes responsible for controlling meiosis.

1.3.4 Genes involves in immune functions

One cause of recurrent miscarriages is an abnormality in maternal immune system which results in abnormal production of cytokines and immune cells (Laird et al.). Studies revealed difference in expression of few immunological cells in women with RM; alterations have been observed in CD56+ cell populations and Th1, Th2 cytokines peripheral blood monocytes. HLA alleles are linked with RM (Meuleman et al., 2015).

HLA-G plays essential role in inhibiting T and B lymphocytes. During pregnancy maternal immune system modulates its immune response by immunosuppression which is believed to be mediated by HLA-G molecule due to an expression in cytotrophoblast and placenta (Mosaferi et al., 2013). The polymorphism in HLA-G genes may cause complications in pregnancy due to alteration in its pattern of expression.

1.3.5 Genes involved in endocrine system

Female fertility is controlled by a hypothalamic–pituitary–gonadal axis (HPO axis) and its imbalance seems to disrupt the women's hormonal system. Genetic polymorphisms in HPO axis controlling genes can cause recurrent miscarriages. A study revealed variation in the aromatase genes (SNP rs10046) during the study of estrogen synthesis pathway (Cupisti et al., 2009)(Litridis et al., 2011). Missense mutation in the chorionic gonadotropin (CGB5/8) has been associated with RM (Nagirnaja et al., 2012). Another study revealed that variation in HPO axis controlling genes (ESR2, PRLR, GCCR and ACVR1) are associated with recurrent miscarriages (Courtney W Hanna, Bretherick, Liu, Stephenson, & Robinson, 2010) whereas polymorphism in progesterone and estrogen receptor genes revealed no association with recurrent miscarriage (Su, Lin, & Chen, 2011). The data obtained from various studies suggest that variation in the genes related to HPO axis may influence risk for recurrent miscarriages whereas further research is needed to understand the mechanism behind female hormones and recurrent miscarriage. While these data suggest that genetic variation in receptors and regulatory genes may influence the risk for RM, a more comprehensive study is needed.

1.3.6 Thrombophilia associated genes and recurrent miscarriage

Thrombophilia can be a lethal condition that predisposes patients to arterial and venous thromboembolic events (Kutteh & Triplett, 2006). It is among the major reasons behind the pregnancy loss and accounts for around 40 % of cases worldwide (Bogdanova & Markoff, 2010). There has been a detailed study to understand the dynamics and the genetics of inherited Thrombophilia with primary focus on genes associated with two important pathways that influence 1) Blood clot formation 2) coagulation folate cycle and fibrinolysis (Krabbendam, Franx, Bots, Fijnheer, & Bruinse, 2005). The cohort study of more than 32,000 women revealed that the two gene variants; prothrombin (F2) G20210A and factor V Leiden (F5) G1691A have been linked with high risk for late miscarriage which is greater than or equal to 10 weeks of gestation and also these variants were associated with recurrent miscarriage (RM) (Lissalde-Lavigne et al., 2005)(Bradley, Palomaki, Bienstock, Varga, & Scott, 2012).

1.4 Telomeres

Telomeres are the repeat sequence (TTAGGG) present at the ends of the chromosomes that function to protect the chromosomes and ensure the chromosomal integrity. They are also identified as a marker of biological aging. The length of the telomeres declines with every cellular division due to end replication problem (T. de Lange, 2009).

In addition, exposure to oxidative stress also accounts for telomere decline apart from shortening induced by normal cellular division (H. Li et al., 2015) so the phenomena of telomere shortening in non-replicating cells such oocyte is caused due to oxidative stress.

The cells expressing telomerase are capable of elongating telomeres through the process of reverse transcription however majority of the somatic cells do not code for this enzyme (Cong, Wright, & Shay, 2002).

Apart from protecting chromosome ends telomere also contributes significantly in positioning the chromosomes during meiosis by letting the chromosome pairs to tether together and align properly within the cell for recombination (Cooper & Sandler, 1998). In many organisms telomeres are vital in bringing the chromosomes together during meiosis (Calderón, Rey, Cabrera, & Prieto, 2014)

In mouse irregular telomere shortening seems to cause abnormal recombination and synapses formation in females (L. Liu et al., 2004) similarly age related telomere shortening also show similar phenotype. Various studies led to formulation of the hypothesis which states that the occurrence of age related aneuploidies are due to the reduction in telomere length (David L Keefe, Marquard, & Liu, 2006). When mouse exposed to an agent that reduces the harmful effects caused by oxidative stress an increment in telomere length in the ovaries and enhanced egg quality is observed (J. Liu et al., 2012). The IVF procedure can be a predictor of pregnancy outcome by analyzing telomere length in sister oocytes (D L Keefe, Liu, & Marquard, 2007), these and various other studies suggest the importance of telomere length dynamics in human reproduction.

The importance of shelterin protein complex in the protection of telomeres comes from the observation that this complex affects the structure of telomeric DNA. Shelterin determines the

structure of telomere terminus, it is implicated in the generation of t-loop, and it controls the synthesis of telomeric DNA by telomerase (Titia de Lange, 2005a).

1.4.1 Structure of Telomeric DNA

Telomeres are present at the end of the chromosomes and contain tandem TTAGGG repetitive sequence bound with specialized complex shelterin protein complex (Patel, Vasan, Gupta, Patel, & Trivedi, 2015) .The ends of eukaryotic chromosomes were initially seen to contain tandem repeats of short, G-rich sequences in Tetrahymena (Blackburn & Gall, 1978), and these tandem repeat sequences ensures to provide chromosome end protection (Szostak & Blackburn, 1982)**.** All vertebrates have telomeres that are made out of hexameric 5' TTAGGG 3' double stranded tandem repeats (Meyne, Ratliff, & Moyzis, 1989). The length of the telomere repeats array varies among different species, with rodents having the longest telomeres of up to 150 kb (Meyne et al., 1989)**;**(Makarov, Lejnine, Bedoyan, & Langmore, 1993) and humans having telomeres that range in size from 2-30 kb (Cooke & Smith, 1986)**;**(T de Lange et al., 1990). Telomeres continue to grow in few organisms such as Daphnia and insects that grow continuously due to telomerase; the activity was observed using telomerase activity assay (Schumpert, Nelson, Kim, Dudycha, & Patel, 2015).

Telomere length in humans is an inherited characteristic (Njajou et al., 2007). Mammalian telomeric DNA ends in a 3' G-rich single-stranded overhang (Makarov, Hirose, & Langmore, 1997) Telomerase plays an important role in proper telomere structure and function. Telomerase is not needed for overhang generation, proposing that there must be an alternating mechanism which controls overhang generation and processing (Hemann & Greider, 1999) (Huffman, Levene, Tesmer, Shay, & Wright, 2000). Determining the length and nature of mammalian telomeres has been challenging because of the specialized and technical restrictions inherent to the diverse techniques that have been developed. Therefore, making it difficult to assess the telomere structure and processes related to overhang production. Electron microscopy of purified telomeres was utilized to recognize long overhangs of approximately 75-300 nucleotides on one telomere end (W. E. Wright, Tesmer, Huffman, Levene, & Shay, 1997), however this assay

requires high quantities of starting material (making it extremely difficult for routine use); the failure to detect overhangs shorter than 75 nucleotides using electron microscopy could not preclude the presence of shorter overhangs (W. E. Wright et al., 1997). Two independent assays based on hybridization to the G-rich overhang were able recognize overhangs as short as 12 nucleotides (non-denaturing hybridization assay), (McElligott, 1997) and 20 nucleotides (hybridization insurance assay), (Tahara, Kusunoki, Yamanaka, Matsumura, & Ide, 2005), however these assays can just measure the relative signal intensity of overhangs regarding total DNA, and may not be extremely accurate. A few molecular assays have been produced and used to obtain overhang length measurements that range from 45-300 nucleotides, yet each one of the assays has its own inherent difficulties, mutually they have had poor resolution to recognize short overhangs, and the data has been conflicting (Chai, Du, Shay, & Wright, 2006)**.** The recent overhang assay leaves single stranded telomere overhang, and it is comprised of DNA with a novel protein and duplex specific nuclease (DSN). The overhang is then investigated by gel electrophoresis and tested with a C-rich region in order to focus on the required overhang lengths (Zhao, Hoshiyama, Shay, & Wright, 2008)**.** The DSN technique is useful and precise in analyzing the overhang and it can interpret of overhangs that are as short as 12 nucleotides sequence. The G rich overhang plays a fundamental role in telomere end protection and also helps to evade DNA damage pathways by forming t-loop (Greider, 1999)**.**

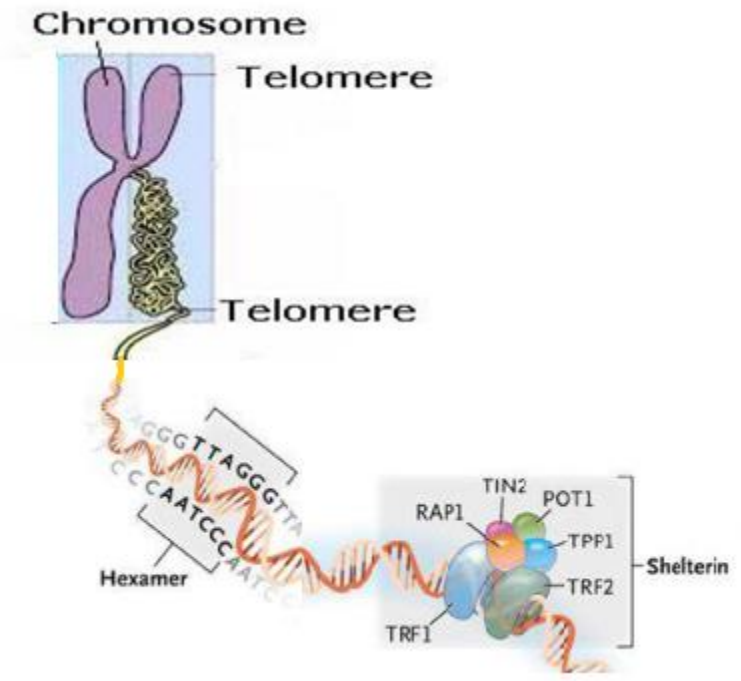


Figure 1 Telomeres are made up of hexameric repetitive sequence present at the end of the chromosomes that along with the shelterin protein complex (TRF1, TRF2,RAP1,POT1,TIN2,TPP1) protects the chromosome and ensures overall genomic stability.

1.4.2 T-Loops

T-loops were first identified in human telomeres by utilizing electron microscopy to visualize telomeric DNA (J. D. Griffith et al., 1999). The t-loop appeared as a rope or lariat-like structure at the end of telomeres in electron micrographs, and is presumed to be structured by strand invasion of the 3' overhang into the preceding double stranded telomeric DNA, and stabilized by group of associated telomeric proteins (Greider, 1999). Apart from human, T-loops were also identified in various other living organisms, including *Trypanosome brucei* (Munoz-Jordan et al., 2001), *Oxytricha nova* (Murti & Prescott, 1999), *Candida parapsilosis* (Tomaska, Makhov, Griffith, & Nosek, 2002), *Caenorhabditis elegans* (Raices et al., 2008) *Pisum sativa* (Cesare, Quinney, Willcox, Subramanian, & Griffith, 2003) and in mouse and chicken (Nikitina & Woodcock, 2004). The size of T-loops depends on the length of telomeric DNA and varies depending on the length of telomeric DNA. (J. D. Griffith et al., 1999) . The t-loop structure is

considering to be an essential component of the end protection properties of telomeres, providing machinery for physically protecting the double strand break at the telomere ends by sequestering the 3' overhang. At the base of T-Loop, the G strand displaces the double stranded duplex to form a displacement loop (D-Loop) (De Lange, 2004). The formation of D-loop has been observed during the initiation of homologous recombination (Verdun, Crabbe, Haggblom, & Karlseder, 2005). The 5' strand seems to be involved in this invasion process by forming a structure similar to Holliday junction (HJ) from G-quadruplexes (Webb et al., 2013).

The proper t-loop formation requires an overhang of minimum 6 nucleotides (R M Stansel, de Lange, & Griffith, 2001) and TRF2, a telomere binding protein provides the stability of the structure by binding to the t-loop junction (R M Stansel et al., 2001). Structural studies have demonstrated that TRF2 might induce t-loop arrangement by enabling strand invasion of the overhang, and by helicase enzyme activity (Nora, Buncher, & Opresko, 2010). G-quadruplexes might likewise be involved in stabilizing the t-loop structure, as recent data suggests that G-quadruplexes structure presents preferentially at the 3' termini of telomeres (J. Tang et al., 2008), and studies showed that a G-quadruplex like structure is also found in telomeres and had the potential to form t-loop-like structure in vitro (Xu & Blackburn, 2007). It has been observed that G-quadruplex structure is widely distributed in the important functional regions such as gene exon, promoter, telomere and Untranslated region (5, 3 UTR) and also plays vital role in telomere extension, DNA replication, transcription, meiosis and other important living processes (Gao, Yuan, & Xu, 2014). On the other hand, the knowledge about the mechanism and timing of t-loop formation in vivo is restricted due to the absence of an easy, sensitive assay for detecting t-loop formation. Currently, the main method used to assess the structure of t-loops is electron microscopy (J. D. Griffith et al., 1999), which requires extensive sample preparation and considerable amount of starting material. A direct, cell biology based super-resolution fluorescence imaging method, stochastic optical reconstruction microscopy (STORM) has been employed to visualize the function and structure of telomeres and it has also been observed that TRF2 uniquely serves to protect telomere ends and is required for the proper formation of T-loop (Doksani, Wu, de Lange, & Zhuang, 2013).

1.4.3 Structure of the Telomeric Protein Complex

The DNA contain several repeats of TTAGGG telomeric sequence and associated bonded proteins known as shelterin protein. It comprises of the complex of six proteins, namely TRF1, TRF2, TIN2, POT1, TPP1 and RAP1(Patel et al., 2015). A complex of six proteins is highly specific to mammalian telomeres, with orthologs in several other eukaryotes (Titia de Lange, 2005b). TRF1 and TRF2 bind to dsDNA whereas Pot1 binds to single stranded G-rich DNA sequence loop**.** TIN2 protein binds to TRF1, TRF2 which provides stability and affinity to telomeric DNA. TPP1 binds to TIN2 and Pot1 and facilitates the binding of six proteins together, and anchoring all the six proteins leads to an association between single and double stranded proteins of the telomere t-loop. Rap1 binds to TRF2 and may have other unidentified protein interactions. Shelterin protein complex plays an essential role in the formation of t-loop and other aspects of telomere biology (Palm and de Lange, 2008; Smogorzewska and de Lange, 2004).

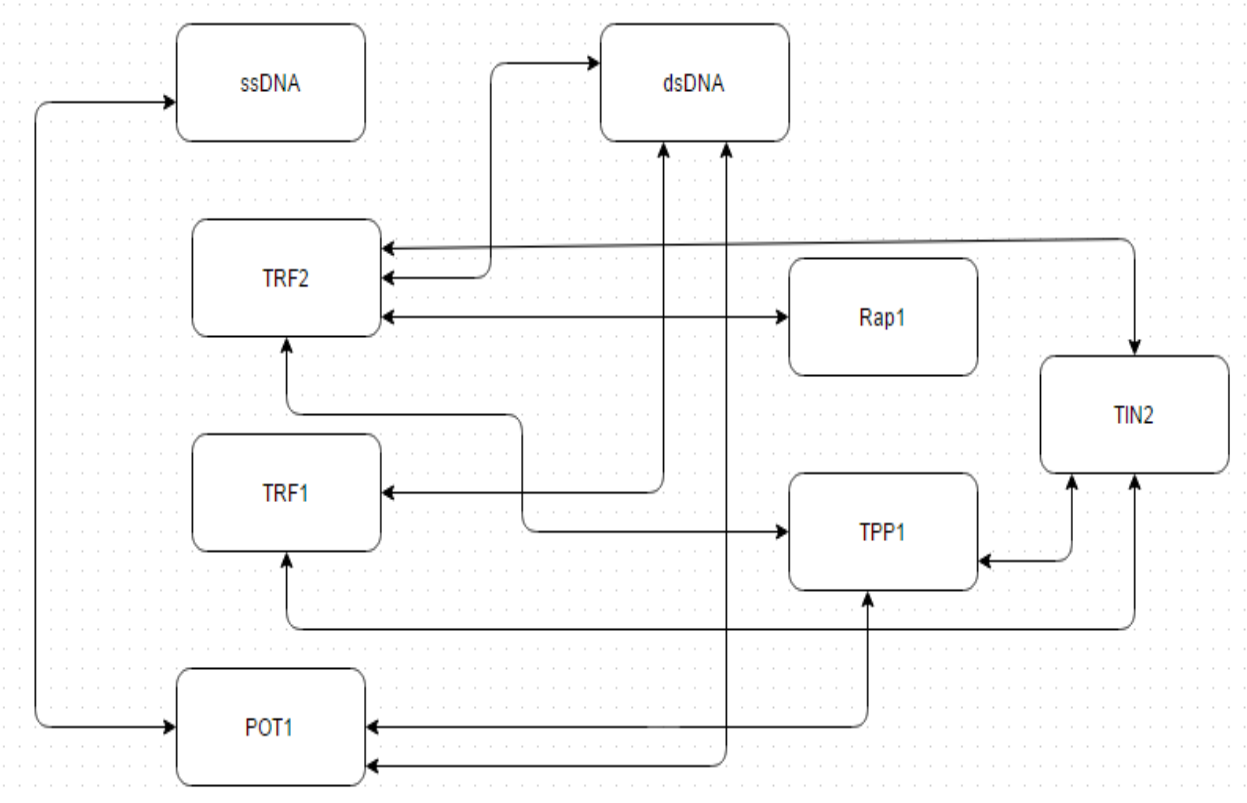


Figure 2 Interaction of Shelterin protein complex with Single and double stranded DNA.

1.5 Shelterin protein complex

1.5.1 Telomeric Repeat Binding Factor 1 (TRF1)

Telomeric Repeat Binding Factor 1 is consisted of 439 amino acids residues (UNIPROT-P54274 (TERF1_HUMAN)). As we know that telomeres protects the chromosomes from DNA damage machinary, the binding of shelterin protein complex ensures the stability of telomeres and chromosomes. Both TRF1 and TRF2 are important members in this regard as the stable interaction of other shelterin protein mainly depends on the proper binding of both of these proteins (Galati et al., 2015). TRF1 binds to double strands of TTAGGG repeats at the telomeres (Garton & Laughton, 2013).

TRF1 was first identified in HeLa cells nuclear extracts (Zhong, Shiue, Kaplan, & de Lange, 1992). The chemical composition of TRF1 consists of an amino terminal acidic domain, and a carboxyl terminal myb domain which binds to duplex telomeric sequence (Bianchi, 1997). TRF1 plays a significant role by preserving genomic stability, maintaining the proliferative capacity, and preventing the activation of DNA-damage cell cycle checkpoints (Zhou, Perrem, & Lu, 2003)

The TRF homology (TRFH) domain which is also known as dimerization domain can be found in mammalian TRF1 and TRF2 (Bibo Li, Espinal, & Cross, 2005). Biochemical nature of TRF1 and TRF2 is similar but their roles in telomere function are distinct. Electron microscopy revealed the interaction of telomeric T- loop with TRF2 at the loop junction between the duplex repeats and the 3′ single-stranded overhang (R M Stansel et al., 2001).

The dimerization or TRFH domain has been found to be significant in stimulating TRF1 to provide telomere structural stability (Okamoto & Shinkai, 2009) and also facilitates in the recruitment of TIN2 (Y. Chen et al., 2008). A homodimer is formed by TRF1 through TRFH dimerization domain (J. Griffith, Bianchi, & de Lange, 1998) and binds to 5'-YTAGGGTTR-3' sequences (Bianchi et al., 1999). The TRF1 homodimer has an higher binding affinity due to the ability of the dimer to bind to two sequences together and induce bending in telomeric DNA (Bianchi, 1997) and the bending property as induce by TRF1 provides overall stabilization. TRF1 found to be bound to the telomere at all phases of the cell cycle (Broccoli et al., 1997) (Scherthan et al., 2000) and TIN2 and ubiquitin ligase appears to regulate different levels of

TRF1 (Zeng et al., 2010). TRF1 is a negative feedback regulator for telomere length (A Smogorzewska et al., 2000) and induce efficient DNA replication (Ohishi, Muramatsu, Yoshida, & Seimiya, 2014)

1.5.2 Telomeric Repeat Binding Factor 2 (TRF2)

Telomeric Repeat Binding Factor 2 is consist of of 542 amino acids residues (UNIPROT-Q15554 (TERF2_ HUMAN)). TRF2 is a sequence specific DNA binding protein that recognizes double stranded telomeric repeat sequence and due to its DNA-binding Myb domain it is biochemically similar to TRF1 but protein-protein interactions of TRF1 and TRF2 are different(F. Liu et al., 2012). TRF2 also interacts with two other shelterin proteins TIN2 and Rap1 (Ye & de Lange, 2004). TRF1 induce DNA bending whereas TRF2 do not induce any DNA bending or cause any distortions in DNA (Court, Chapman, Fairall, & Rhodes, 2005). TRF2 functions by providing structural organization by binding to double/single stranded DNA junction (R M Stansel et al., 2001)**,** and facilitates the invasion of 3' G-rich overhang (Amiard et al., 2007). TRF2 induces the protection of the Holliday junctions thus provides more telomere stability and also plays a significant role in chromatin remodeling at the site of telomeres as a study by Nora at al revealed an aberrant nucleosomal organization at the telomeres due to an overexpression of TRF2 in mouse keratinocytes (Nora et al., 2010). NoRC is the chromatin remodeling complex that induces genomic stability due to the formation of heterochromatin and it has been observed that TRF2 along with TIP5 plays a significant role in the recruitment of NoRC to telomeres (Postepska-Igielska et al., 2013)**.** The proper functionality of TRF2 gene is necessary as it has been observed that TRF2 knockout mice displayed an embryonic fatal phenotype similar to TRF1 (Karlseder et al., 2003). TRF2 is not only restricted to telomeres as it has been observed that TRF2 also accumulates at the site of double strand breaks at the non-telomeric region of the DNA (Bradshaw, Stavropoulos, & Meyn, 2005), Apart from double strand breaks the accumulation of TFR2 at non telomeric site may have other factors in play for its accumulation (Williams et al., 2007). TRF2 is significant in inhibiting NHEJ (Agata Smogorzewska, Karlseder, Holtgreve-Grez, Jauch, & de Lange, 2002)**.** Ataxia telangiectasia mutated (ATM) is a protein kinase which is activated by DNA double strand breaks, TRF2

seems to play an important role in the regulation of ATM (Huda, Tanaka, Mendonca, & Gilley, 2009). DNA damage in the non telomeric region might be repaired by TRF2 by facilitating the early stages of homologous recombination (Mao, Seluanov, Jiang, & Gorbunova, 2007). The combination of Rap1 and TRF2 suppresses the homology directed repair mechanism in KU 70/80 heterodimer (Janoušková et al., 2015). Thus TRF2 provides the stability and protection to chromosomes by maintaining the telomere structure.

1.5.3 RAP1

Human Rap1 has been identified to be a mammalian ortholog of the yeast protein and is considered to be an important part of a shelterin protein complex that plays a major role in DNA regulation (Krauskopf & Blackburn, 1998) (Feldmann & Galletto, 2014). RAP1 does not bind directly to DNA and instead it interacts with the telomeric DNA through TRF2 (Janou kova et al., 2015). The possible association of other unknown proteins with Myb DNA domain could be important in the regulation of telomeric DNA or its BRCT domain (B. Li, 2003) (Hanaoka et al., 2001). The complex that is formed by TRF2-Rap1 suppresses non-homologous end joining (NHEJ) by interacting with DNA-dependent protein kinase catalytic subunit (DNAPK-C) to prevent end joining (Arat & Griffith, 2012). Rap1 also plays a critical role in telomere heterogeneity (B. Li, 2003). Human telomeric protein complex including TRF1, TRF2 and TIN2 does not have any recognized budding yeast orthologs whereas human RAP1 has an ortholog of yeast telomeric protein scRap1p (Bibo Li, Oestreich, & de Lange, 2000). Further research is required to fully understand the function of Rap1.

1.5.4 TIN2

TIN2 interacts with TRF1 and is an important telomeric DNA binding protein which negatively affects telomere elongation by a telomerase dependent mechanism (Chiang, Kim, Tessarollo, Campisi, & Hodes, 2004). TIN2 simultaneously binds to both TRF1 and TRF2 and forms a bridge that may provide stability to the telomere structure (Houghtaling, Cuttonaro, Chang, & Smith, 2004), direct interaction of TNI2 with TPP1 and TRF2 helps in maintaining the central

hub of the shelterin complex. TIN2 also may also contributes in the repression of ATM pathway by TRF2 (Takai, Kibe, Donigian, Frescas, & de Lange, 2011). The detailed biochemical and structural analysis revealed that TIN2 protects TRF1 from Fbx4 mediated degradation (Zeng et al., 2010). Both TRF1 and TRF2 is associated with TIN2 and is considered to be a negative regulator of telomere length and an over expression of TIN2 shelterin protein may leads to shortening of telomeres (Frescas & de Lange, 2014b). TPP1 is another important shelterin protein subunit that interacts with TIN2 directly (Frescas & de Lange, 2014a). Structurally TPP1 is composed of two domains; carboxy-terminal domain and central domain. The central domain binds to POT1 and plays a significant role in maintaining the 6 subunit shelterin complex at telomeres by providing stability to TRF-TIN2-TRF2 complex (O'Connor, Safari, Xin, Liu, & Songyang, 2006). The recruitment of telomerase is controlled by TPP1-POT1 interaction therefore plays a role in maintaining the telomere length (Xin et al., 2007).

1.5.5 POT1 (Protection of Telomeres 1)

Protection of telomeres 1 or POT1 is an important shelterin protein complex that binds to a mammalian single stranded ends of the telomeric DNA and is homologous to TEBPα which is a ciliate telomere binding protein (P Baumann & Cech, 2001). POT1 plays a significant role in chromosome end protection in telomere maintenance. It also has an oligosaccharide/oligonucleotide-binding (OB) folds (Peter Baumann & Price, 2010a). POT1 is found to be highly specific for telomeric 5'-TAGGGTTAG-3' (Loayza, Parsons, Donigian, Hoke, & de Lange, 2004). POT1 binds to a single stranded 3' G-rich overhang throughout the cell cycle; the G rich strand is known as Displacement loop (D-loop) structure (Wei & Price, 2004). The presence of POT1 during cell cycle is critical and the absence of this key protein may results in cell cycle arrest and DNA damage response (Churikov & Price, 2008).

Cell cycle machinery has different surveillance pathway to protect the integrity of genetic material but to prevent telomeres from these surveillance pathways shelterin complex plays an important role (Abraham, 2001). ATR pathway is one of the surveillance pathways and to prevent the activation of this pathway the single stranded telomere allows the binding activity of

TPP1-POT1a; POT1a prevents the binding of RPA proving the repression of ATR pathway due to exclusion of RPA binding protein (Takai et al., 2011)**.**

The POT1 and TPP1 interaction takes a part in the regulation of the telomere length by recruiting telomerase to the telomere, stabilizing the telomere primer binding and increasing the telomerase binding efficiency and translocation (Latrick & Cech, 2010).

Mutation induced in the OB folds which codes for the telomeric single stranded DNA (ssDNA) induces telomere elongation and therefore plays a critical role in telomere length regulation (Kendellen, Barrientos, & Counter, 2009a)**.** The ability of POT1 in regulating the telomere length is highly dependent on its association with TPP1 through an interaction with TRF2 (Kendellen et al., 2009a).

Knockdown of human POT1 protein is identified to induce telomere elongation as oppose to telomere degradation and rarely cause telomere fusion (Peter Baumann & Price, 2010b)**.**

Replication protein A (RPA) is among the other important DNA damage sensor proteins and it has been observed that POT1 plays a significant role in out-competing this protein (Flynn, Chang, & Zou, 2012)

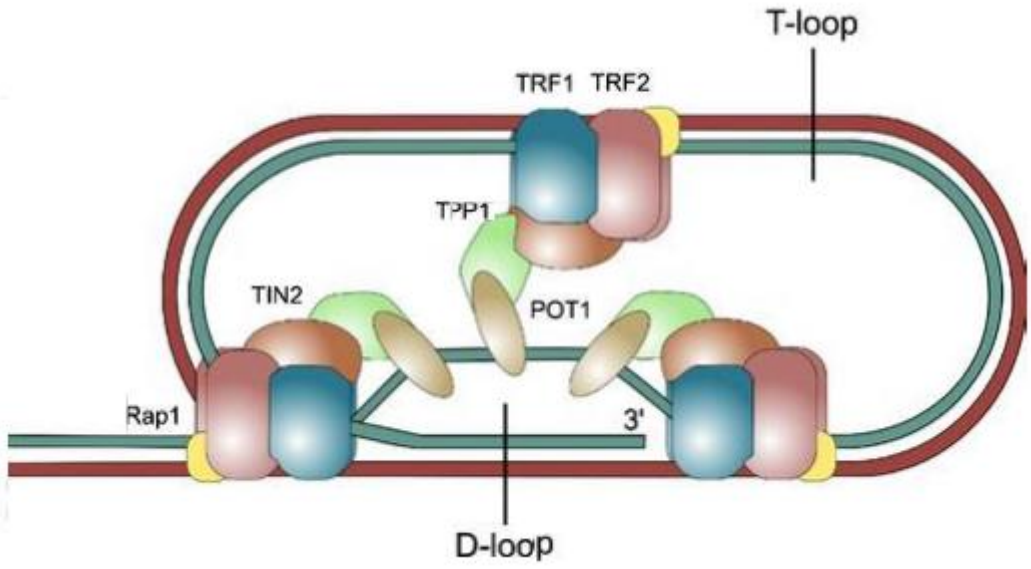


Figure 3. The Diagram depicts double and single stranded overhang (T and D loop) conformation along with the binding sites of shelterin protein complex**.**

1.6 Telomeres End Protection Mechanism

The process of making a new DNA copy is known as replication, a process in which double strand DNA is denatured with the help of topoisomerases and helicases. This allows polymerase and other DNA replication machinery to access replication sites for catalyzing the synthesis of new DNA strand from the parent strand (Griffiths, Miller, Suzuki, Lewontin, & Gelbart, 2000b). The direction of DNA polymerase is in 5'→ 3' direction which is known as leading strand (Ohki, Tsurimoto, & Ishikawa, 2001). The strand that runs antiparallel is lagging strand, the synthesis of this strand is discontinuous as it needs short RNA fragments (Okazaki fragments) (Kitani, Yoda, Ogawa, & Okazaki, 1985). The 5' end of the lagging strand is lost at-least the size of RNA primer which is known as end replication problem**.** Some studies revealed that the length of lost end at the 5' terminus is longer than the size of RNA primer which negatively impacts the polymerase activity causing the loss of sequence at the extreme ends of the telomeric region of chromosome (Ohki et al., 2001). The presence of TRF1/ TRF2 also seems to interfere the replication fork (Ohki and Ishikawa, 2004) or the slipping of the replication fork is due to the presence of G-quadruplexes which seems to be responsible in telomere loss during genome replication (Webb, Wu, & Zakian, 2013)**.** By using stable transfection method it has been

observed that the long term overexpression of TRF1 or TRF2 resulted in a gradual shortening of telomeres in human cells with positive telomerase activity (A. Smogorzewska et al., 2000).

The early eukaryotic model revealed the presence of heterochromatin at the ends of the chromosomes to protect the chromosome from degradation due to end replication problem, however telomerase does not only augment the protection against DNA attrition, but also adds the telomeric repeat sequence in order to establish the telomere length homeostasis (S. W.-L. Chan & Blackburn, 2002). The presence of shelterin protein complex protects the chromosomes ends from DNA damage machinery by making a difference between double stranded breaks (DSB) and telomeres (T. de Lange, 2009).

The overexpression of TRF2, a negative regulator of telomere length, lacking basic Myb domain induces the separation of shelterin protein complex (Celli and de Lange, 2005) and the development of telomere dysfunction induced foci (TIF) characterized by localization of proteins such as Rad17, p-ATM, gamma-H2AX, 53BP1 (Silverman, 2004). The deletion of TRF2 leads to an accumulation of TIF at telomeres ends similar to what it has been observed during the loss of normal TIN2 and POT1 activity (Celli & de Lange, 2005) (Hockemeyer et al., 2005). Telomere dysfunction results in the generation of DNA damaging signals which in response activates ATM and ATR pathway (Denchi & de Lange, 2007). Both of these pathways become activated in response to breaks in DNA double strand and for normal functioning telomeres must evade both of these pathways. The ability of TRF2 to repress ATM signaling is due to its property of promoting the formation of t-loop (Poulet et al., 2009). The formation of t-loop seems to provide a physical barrier between the telomeres ends and the surrounding protein responsible for recognizing the DSB. The MRN protein complex gets activated due to any harm to the genetic information, so that the information gets fixed prior to passing them to the offspring.

The ATR pathway is controlled at the extreme telomeric ends of the chromosomes by POT1 (Denchi & de Lange, 2007). The knockdown of POT1 initiates the formation of TIF which results in the shortening of 3 overhangs, alternation in the processing of 5' C rich strand and telomere fusion (Yang, Zheng, & Harris, 2005). The ATR pathways get activated by a single stranded protein known as Replication protein A (RPA), whereas in functional telomere the POT1 binds to single stranded loop structure (D and T loop) and stops the binding of RPA

resulting in the repression of ATR signaling (Flynn et al., 2012). RPA protein seems to accumulate in the absence of properly functional POT1 (Barrientos et al., 2008). The interaction of POT1 with other shelterin protein complex through TPP1 plays key role in the repression of ATR signaling (Hockemeyer et al., 2007a)**.**

Telomeres since it has been identified in the first cytogenetic studies revealed a distinctive nature as compared to the rest of the chromosomes. The distinctive nature of telomeres is due to their suppressing capacity of two major DNA damage signaling pathways (ATM, ATR) as well as the capacity to evade the DNA repair pathways NHEJ and HR. *In vitro* study revealed that p53 can bind to the telomeric DNA and enhance the formation of t-loop indicating the role of p53 pathway in proper functioning of telomeres (Rachel M Stansel, Subramanian, & Griffith, 2002). Additional data is important in elucidating the mechanism of telomere end protection in great details.

Chapter 2: Telomere Dynamics in Female Reproduction

1. Telomere Function in Germ Cells

Telomere length in the germ cells are important to determine as they are transferred to next generation (L. Liu, Blasco, et al., 2002a). The development of ovaries are significantly influenced by maternal nutrition and under nutrition of women negatively affects the normal folicular development, ovarian size and onset of puberty (Engelbregt, et al. 2002) (Bernal,et al. 2010). The telomerase activity is present during the development of oocyte but it gradually diminishes upon maturation of oocyte (Brenner, 1999). Mouse studies showed that the absence of functional telomerase activity displays infertility due to plenty of factors such as impaired meiotic synapsis, cell arrest at early phase of meiosis (L. Liu et al., 2004) (D L Keefe et al., 2007). Furthermore in another study female rats have been exposed to under nutrition during the fetal development and the study showed the properties of reproductive senescence at earlier age as compared to control group (Chernoff et al., 2009). The loss of telomerase activity or short telomere length in human germ line also showed the sign of infertility (Hassold & Hunt, 2001b). Women with delays in pregnancy may face problem in conceiving due to the presence of Reactive oxygen species (ROS) which induces telomere shortening in oocyte or in preimplantation embryos leading to cellular senescence or apoptosis (L. Liu, et al 2002). Treatment with antioxydants N-acetyl-L-cysteine (NAC) displayed delay in oocyte aging (Y. Wang et al., 2005). Stem cells are also prone to aging due to oxidative stress both in human and mice which induces telomere shortening ultimately affecting the proliferative and self-renewal capacity of the stem cells (Yahata et al., 2011). In addition, studies have demonstrated that there is a significant relationship between birth weight and the concentrations of endothelial progenitor cells in the cord blood, as Low birth weight (LBW) infants have the lowest concentrations as compared to higher birth weight infants (Aroviita, et al. 2004). These studies suggest that normal telomere dynamics plays a significant role in the development of normal germ line cells.

2. Telomere dynamics during oogenesis

The bouquet like formation is controlled by telomeres which plays a significant role during oogenesis (Tomita & Cooper, 2007). During the leptotene stage of meiosis 1 the telomeres are localized at the nuclear periphery and they gradually become tightly packed and are localized at one pole in late leptotene stage to form a bouquet like arrangement. At the later stages such as zygotene and pachytene, the clustering of telomeres at one pole become loosely attached and disperse lately at the pachytene. At the diplotene stage the telomeres are dispersed throughout the nucleus (Roig et al., 2004). It has been observed that proper functioning of telomeres is significantly important for recombination and meiotic synapsis (L. Liu et al., 2004).

3. Telomere and telomerase dynamics in placenta

Placenta is an organ that is attached to the maternal lining and is responsible for transporting oxygen supply to fetus. In the first week of gestation there is a rapid growth of cells in a relatively hypoxic environment and the telomerase activity is high in placental trophoblast (Meekins, Pijnenborg, Hanssens, McFadyen, & van Asshe, 1994) (Kudo, Izutsu, & Sato). The level of normal telomerase activity is correlated with HIF-1α expression, and it was shown to stimulate hTERT expression by binding to two HIF-1α consensus-binding sites in the hTERT promoter region (Hirotaka Nishi et al., 2004). After the initiation of the maternal arterial circulation, threefold increment in oxygen levels is being observed (Burton, Jauniaux, & Charnock-Jones, 2010), which induces a decrease in telomerase activity through HIF-1α from 93.5% positive telomerase activity in first trimester (three months) chorionic villi versus 62.5% from second and third trimester chorionic villi (Kudo et al. 2000).

In pregnancies complicated with IUGR or preeclampsia decreased telomerase activity has been observed in placental trophoblasts between 26 and 39 weeks (Biron-Shental et al., 2010), moreover decrease or complete absence of telomerase enzyme activity has been linked with intrauterine death of the fetus or recurrent pregnancy loss (R.-J. Chen, et.al 2002). On the

contrary, an increase or elevation in telomerase activity has been observed in hydatidiform molar pregnancies which are characterized by the formation of cluster of cells around the fertilized egg which otherwise would have developed into placenta (H Nishi et al., 1998).

In a study to demonstrate the relationship between low birth weight and telomerase activity kim et al carried out a twin study where they have found that in a smaller twin low placental telomerse activity has been observed as compared to the normal birth weight twin (Kim et al., 2006). Telomere shortening seems to induce a decrease in antiapoptotic protein Bcl-2 and upregulation of senescent markers p16 and p21 in IUGR placenta (Davy, Nagata, Bullard, Fogelson, & Allsopp, 2009).

4. Telomere length and fetal development

The analysis of telomere length dynamics during fetal development is difficult, due to limitation in sample material and also due to the limited number of studies performed relating to this topic. In a study conducted by Youngren *et al* in which telomere length was measured in different tissues isolated from fetuses between 15 and 19 weeks gestation period and the results revealed variable telomere length between fetuses which was not linked with the gestational age of the fetus (Youngren et al., 1998). It has also been observed that telomere length is synchronous during fetal development whereas during extra uterine development the synchronous nature of telomere length dynamics is lost (Youngren et al., 1998).

Fluctuations in the telomere length has been observed during 23 and 36 weeks gestation but increases in the overall telomere length has been observed (Holmes et al., 2009).

Telomerase activity has been observed in many fetal tissues including lungs, liver, intestine, kidney up to 16th week of gestation and later activity diminishes completely or significantly reduces to an extent that it becomes completely undetectable (Ulaner & Giudice, 1997).

5. Telomere homeostasis in the newborn

Telomere length remains stable during fetal life and after birth started to decline as observed in a study conducted by Holmes et al in which they have found a gradual decrement in telomere length following birth onwards whereas the telomere length in utero of age matched fetuses remained constant (Holmes et al., 2009). Maintaining constant telomere length is significant in providing a suitable environment for normal fetal development as it has been observed that oxidative stress during fetal development can lead to anomalies such as rupture in amniotic membrane and premature birth (Menon et al., 2012). Normal pregnancies require a constant maintenance of chromosomes during in utero life and variations in telomere length may lead to reproductive complications.

6. Female reproductive aging and pregnancy complications

Female fertility decreases as oocyte reserves deplete gradually with growing age and other combined factors decrease the rate of conception which results in the increment of pregnancy (te Velde & Pearson). Age related reduction in ovarian follicles leads to aneuploidy which may result in pregnancy loss and other age related fertility problems (Faddy, 2000) . Menopause occurs between the age of 40 to 60 years (te Velde & Pearson), (Nicolaides, Spencer, Avgidou, Faiola, & Falcon, 2005). The variability in reproductive senescence is due to the variations in environmental and genetic factors that may affects the individual rate of cellular aging (Rodríguez-Rodero et al., 2011). Epidemiological data suggests that longevity is associated with increased life span as observed in human and animal models (Nagai, Lin, & Sabour, 1995) (Hutchinson & Rose, 1991). Delay in the gonadal maturity leads to delay in puberty thus extending the life span (Tabatabaie et al., 2011). Experiencing menopause at older age is positively correlated with an increase in life span or longevity (Cooper & Sandler, 1998) (Jacobsen, Knutsen, & Fraser, 1999). The capacity of female centenarians to reproduce in their forties indicates the reproductive longevity which is greater than women who are living until the age of 73 (Perls, Alpert, & Fretts, 1997).

There are various possible factors that may define the relationship between menopause and longevity 1) Long-term exposure to estrogen is associated with delayed menopause and may have a positive effect on life expectancy or on generalized longevity (Perls et al., 1997) 2) Age of ovary could be directly proportional to the life expectancy (Mason, Cargill, Anderson, & Carey, 2009) (Cargill, Carey, Müller, & Anderson, 2003). Telomere length is highly variable among individuals and may accounts for variability in reproductive life of women (Kalmbach et al., 2013)**.** At conception every human is born with variable lengths of telomeres, telomerase activity during early development and rate of cell loss varies between individuals. The mitotic capability of primordial germ cells may be limited due to short telomeres during fetal development therefore the size of follicular reserves may get restricted (David L Keefe et al., 2006). Studies related to telomere length and reproductive aging revealed variable contradictory results in which telomere length dynamics has both positively and negatively related with different measures of reproductive aging (Aydos, Elhan, & Tükün, 2005) (Dorland, van Kooij, & te Velde, 1998). Some studies reported delay in puberty which is linked to longevity whereas other studies did not showed any relationship between reproductive delay and longevity.

Unbalanced subtelomeric changes such as translocation in the subtelomeric region 11p;17p have been identified to cause recurrent miscarriages (Joyce, Dennis, Howard, Davis, & Thomas, 2002).

Experimental shortening that is induced in mice models showed the phenotype of reproductive aging similar to as the phenotype observed in women's reproductive aging (L. Liu et al., 2004)

Telomere shortening in mice oocyte appears to be similar to the telomere theory of reproductive aging in women (David L Keefe et al., 2006). The process of telomere shortening in oocyte is more consistent as compared to somatic cells and it continues to get shorter with an advancing age (F. Wang et al., 2013). Studies have revealed that women with advanced maternal age hold oocyte with short telomeres that are often unable to support the process of fertilization and embryogenesis. This is often considered to be an evolutionary advantage for women in old age as a natural contraceptive to protect them from the risks related with pregnancy and old age (F. Wang et al., 2013). Most often the women with advanced maternal age are more prone to various reproductive anomalies, the factors behind this often includes depletion in oocyte numbers and

its quality (Munné et al., 2005)**;** (Navot et al., 1991). Oocytes of women with older age carry error from meiosis causing aneuploidies and mostly accounts for pregnancy loss during first trimester (Allison, Sherwood, & Schust, 2011)**.** Telomeres play an important role in recombination, synapses and segregation during meiosis (L. Liu et al., 2004)**.**

Several complications has been observed during pregnancies such as high blood pressure, gestational diabetes, pre-eclampsia, infection, Intrauterine growth restriction (IUGR) (Deshpande, Harris-Hayes, & Schootman, 2008) (Unterscheider et al., 2014). Short telomere length can also be a factor that can be associated with abnormal tissue function and may leads to pregnancy related complications. Oxidative stress induces telomere shortening and cause cellular senescence in placenta resulting in the growth restriction of the fetus and cause intrauterine growth restriction (Toutain et al., 2013). Telomere rupture at preterm stages significantly affects the telomere length in the new born (Menon et al., 2012).

Endometriosis is a disorder of reproductive system in which endometrial cells grow outside the cavity that normally lies inside female uterus (Metzger & Haney, 1989). The condition affects 10% to 15% of women in reproductive age and up to 40% of infertile women (Metzger & Haney, 1989). Endometriosis sometimes completely blocks the fallopian tube and may cause infertility but the exact phenomena are still unclear (Goyal, Maheshwari, Kaur, & Kaur, 2015), but the telomere biology may provide the underlying mechanism governing the process (Bulletti, Coccia, Battistoni, & Borini, 2010)**.** Surprisingly endometrium is among tissues that express high levels of telomerase (Yokoyama et al., 1998), the reason behind the production of high levels of telomerase is significant to support the process of menstrual cycle. The relationship between variations in telomerase and the progression of endometriosis has also been observed (Mafra et al., 2014)**.** Variations in telomere length homeostasis pathways involved in telomere-length homeostasis may impact the survival of ectopic endometrial and hematopoietic stem cells while also affecting fertility.

Chapter 3: Various methods to measure telomere length

3. Methods for measuring telomere length

There are several methods available to measure telomere length dynamics that utilize a variety of various molecular techniques including FISH, flow cytometry, PCR and Southern blotting (Canela et al., 2007). The different methods of telomere length measurement are described below.

3.1 Telomere Restriction Fragment (TRF) Analysis/ Southern blotting

TRF analysis is a most commonly adopted method used to measure telomere length dynamics that utilizes southern blotting technique (Kimura et al., 2010) for telomere containing genomic DNA. The process includes following steps (fig.4) (Bendix, Horn, Jensen, Rubelj, & Kolvraa, 2010b).

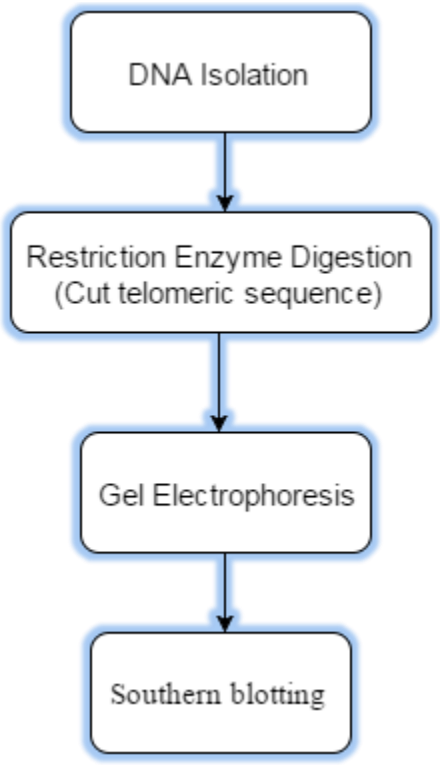


Figure 4 Schematic diagram indicating the workflow of telomere restriction fragment length analysis.

The result generated after southern blotting produces signals that represent the variable telomere length dynamics and help in estimating the telomere length. The major drawbacks in TRF analysis are data interpretation and the nature of restriction digestion as it includes the sub-telomeric region of unknown length (Steinert, Shay, & Wright, 2004).

3.2 Hybridization protection assay (HPA)

An alternative method used instead of TRF to measure telomere length is HPA which is considered to be faster and easier to perform, and can also be performed with relatively lower concentration of DNA

1. The assay based technique involves incubating a small amount of genomic DNA with telomere repeat or *AluI* probe labeled with an acridinium ester.
2. Chemiluminescence reading allows the calculation of the ratio of telomere, AluI sequence and then TA ratio is normalized to telomere length as measured by TRF (Nakamura et al., 1999).
3. It is considered to be an indirect method of measuring telomere length but provide an edge over TRF as it requires less starting material and less completion time. In clinical settings this technique proves to be useful where usually the sample size is small (bioposy or swab) (Nakamura et al., 1999).

3.3 Quantitative Flourescent In-Situ Hybridization (Q-FISH)

For the direct labeling of the telomere sequence FISH technique is employed at the level of individual cell. Florescent probes are designed to bind to telomeric DNA using peptide nucleic acid probes (PNA) (Genet, Cartwright, & Kato, 2013)

Cells are collected at the metaphase stage and incubated with telomeric probe stained with counterstaining protocol (DAPI and PI) and the visualization is carried using fluorescent microscopy and the resulting fluorescent signals were used to measure telomere length (P Slijepcevic, 2001). The classic methodology employs southern analysis for telomere measurement which can only measure average telomere length whereas Q-FISH allows the measurement of telomere length in each individual chromosome with the resolution of 200bp (Predrag Slijepcevic, n.d.)

The probes designed for Q-FISH are very sensitive in telomere assessment due to their small size that allows an excellent penetration through cells. Many modifications have been developed for Q-FISH strategy. TELI-FISH which employs both immunostaining and FISH techniques (Meeker et al., 2002) and Flow-FISH technique employs both FISH and flow cytometry technique that allows the measurement of telomere length in actively dividing cells instead of cells arrested only at metaphase stage (Lauzon, Sanchez Dardon, Cameron, & Badley, 2000).

3.4 Primed in situ (PRINS)

PRINS method can be applied to measure telomere repetitive sequence present at the ends of each chromosome (Montpetit et al. 2014). In this method a synthetic primer binds complementarily to the telomeric sequence during metaphase spread and it is incubated along with fluorescently labeled nucleotides, and DNA polymerase. The florescent labeled nucleotides will produce signals which can then be used for the quantitative analysis of the telomeric sequence (Lavoie, Bronsard, Lebel, & Drouin, 2003). The efficiency of the method can only be detected by its ability to produce signals by fluorescently labeled nucleotides. Low signal strength indicates either short telomeres or error in proper primer annealing (Lin & Yan, 2005). The new technique based on PRINS technique enables simultaneous detection of multiple tandem repeat elements (Serakinci et al., 2002).

3.5 Quantitative Polymerase Chain Reaction (Q-PCR)

Q-PCR technique is used to measure the telomere length but there are numerous difficulties inherent to this technique. The foremost challenge is designing the forward and reverse primers so that the formation of primer dimers could be avoided (Cawthon, 2002). To avoid the formation of primer dimers, the primers are designed to be the variant of telomeric sequence by inducing a mismatch in the sequence which is sufficient to prevent the primer dimer formation (Gil & Coetzer, 2004). The Q-PCR based method is used to measure relative telomere length by measuring T/S ratio which is the ratio of telomere (T) signal to that of single copy gene (S) (Cawthon, 2002). This method is easy to perform and can be used for genetics and epidemiological studies.

3.6 Universal STELA (U-STELA)

Telomeres are present at the ends of the chromosomes and ensure genomic stability, whereas short telomeres do not protect the chromosomal ends from degradation. Cellular senescence is a phenomenon known to occur due to short telomeres that are unable to protect the chromosomes. Measuring the load of short telomeres is an area of active research therefore various techniques have been employed to measure short telomeres. One such recent technique used is Universal STELA which is a variant technique of STELA. Unlike STELA this technique (U-STELA) can measure load of short telomeres regardless of chromosomes in a cell population. The universality of this technique makes it an ideal tool to measure short telomeres and cellular senescence (Bendix et al., 2010b).

The following diagram (Figure 5) shows stepwise explanation of Universal STELA which has been deduced from (*bendix et al*, 2010).

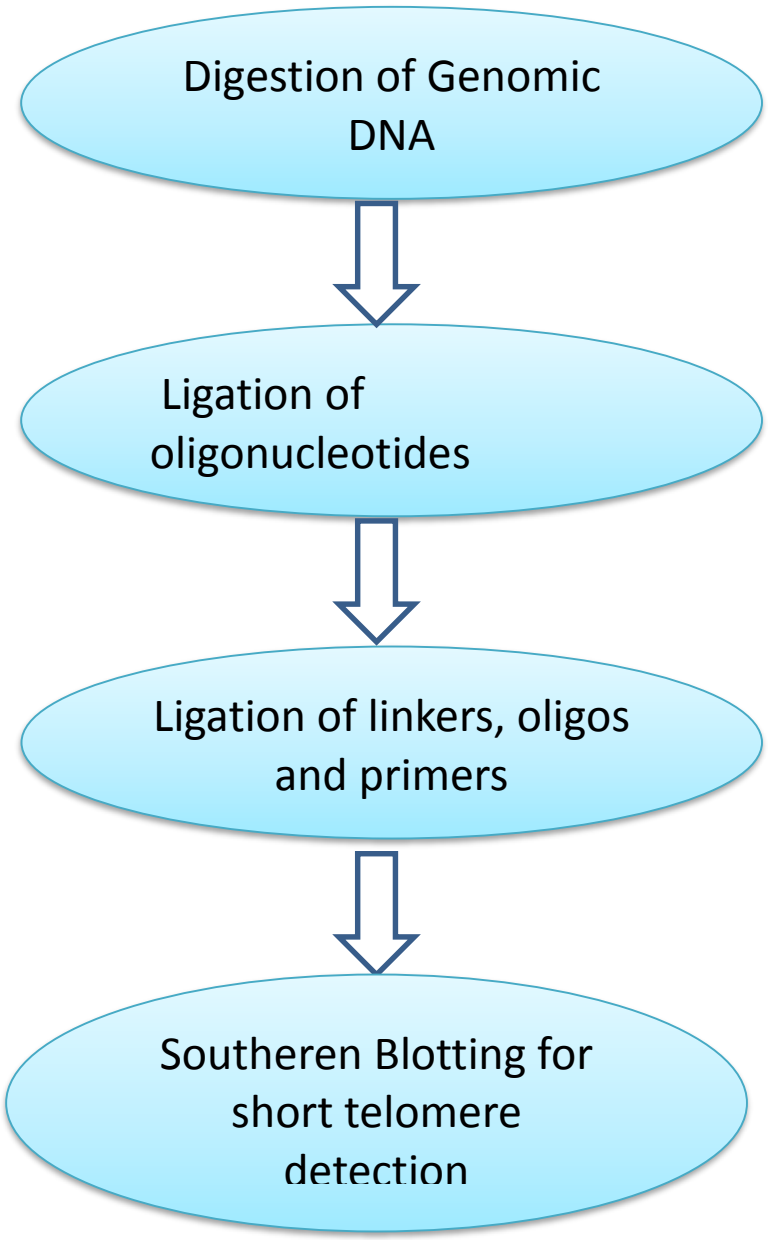


Figure 5 Schematic diagram indicating the protocol to measure short telomeres using the Universal STELA technique (Bendix et al, 2010)

Techniques to measure telomere length	Advantages	Disadvantages	References
Telomere Restriction Fragment (TRF)	-Easy to perform for routine analysis. - It only measures the mean telomere length.	-Measure average telomere length including small but unkown length of the subtelomeric region. -Biased against detection of short telomeres	Harley et al., 1990
Hybridization protection assay (HPA)	-Easy and relatively quick to perform. -As compared to TRF it requires less starting material (DNA).	-Difficult to interpret the ratio values due to variation in Alu repeat seuqence. -It is an indirect method of measuring telomeres	(Nakamura et al., 1999) (Allison et al., 2011)
Primed *in situ* (PRINS)	-Alternate technique of Q-FISH employed for for quantitative *in situ* telomere length analysis	-Cells must be arrested at metaphase stage -Poor telomere specific primer annelaing produces weak signals	(Lavoie et al., 2003;
Quantitative Polymerase	-Easy to perform.	-Indirect method of	Cawthon, 2002; Gil and

Chain Reaction (Q-PCR)	-Suitable for epidemiological studies and clinical studies. -In comparison to TRF analysis Q-PCR donot include subtelomeric region in actual measurement.	telomere measurement but more precise in comparison to HPA indirect measurement method.	Coetzer, 2004). (Allison et al., 2011)
Quantitative Flourescent In Situ Hybridization (Q-FISH)	-Telomere can be measured in fixed tissues. -Used for the analysis of cells during metaphse and interphase stages.	-This technique can be only employed to non-cycling or arrested cells.	(Slijepcevic, 2001) (Heaphy & Meeker, 2011)
Single Telomere Length Analysis (STELA)	-It requires less starting material or DNA. - It measures short telomeres.	-It only measures short telomeres of specific chromosomes. For example, XpYp chromosome etc. -It is labor intensive technique.	(Vera & Blasco, 2012)
Universal STELA	-It measures the load of short telomeres of all chromosomes	-Labour intensive technique.	(Vera & Blasco, 2012)

Table 1 Comparative analysis of the advantages and disadvantages of various techniques adapted to measure telomere length dynamics.

4.0 The Rationale behind Universal STELA

Sudden telomere erosion that is mainly caused due to oxidative stress generates critically short telomeres ultimately inducing replicative senescence through cell cycle arrest **(**di Fagagna et al., 2003). Measuring the mean telomere length with other techniques such as TRF analysis or Q FISH generates bias when short telomeres are measured. The experimental evidence suggests the link between high quantities of short telomeres with senescence has been a challenge due to an inability of a previously available methods, even a detailed analysis of TRF length analysis is not able to identify critically short telomeres (Kimura et al., 2008). When Cells arrest at metaphase stage for Q FISH they often produce signal free ends that indicate short telomeres but the inherent problem with this technique is that it requires actively dividing cells as direct comparative study is not possible between dividing and senescent cells **(Graakjaer et al., 2003)**. Flow fish has the capability to measure the percentage of cells with short mean telomere length but does not have the capacity to measure short telomere in a single cell **(Rufer et al., 1998**). The technique I adopted for my research project is Universal STELA which accurately measures the single short telomere in a cell and gives us a reliable information regarding to short telomeres in a cell population (Vera & Blasco, 2012)**.**

Chapter 5: Materials and Methods

5.0 Study Subjects

The samples were collected from the control (n=10) and patients (n=20) suffering from idiopathic recurrent miscarriage that were admitted to Near East University. The present study was approved by the University ethics committee

5.1 Material Used

5.1.1 General chemicals and Consumables

1. Tris-Acetate
2. EDTA
3. Hydrochloric acid
4. Sodium hydroxide
5. Sodium chloride
6. Tris base
7. Tri-sodium citrate
8. 100% pure ethanol
9. Phenol: chloroform: isoamyl alcohol (25:24:1) – Sigma
10. Whatman® cellulose chromatography papers- Aldrich
11. Nylon membrane filter (Amersham hybond N+)- GE Healtcare
12. LightCycler 480 SYBR Green I Master - Roche

5.1.2 Complete kits

1. TeloTAGGG Telomere Length Assay – Roche
2. High Pure RNA Tissue Kit- Roche

5.1.3 Enzymes

1. MseI- NEB
2. NdeI- NEB
3. T4 DNA Ligase- NEB

5.1.4 Molecular size standards

1. DIG labelled DNA molecular weight marker (23.1 – 1.1 kb) – Roche

5.1.5 Primers and Probes

1. TRF1 (Forward, Reverse) - Metabion
2. POT1 (Forward, Reverse) - Metabion
3. TRF2 (Forward, Reverse) - Metabion
4. TPP1 Forward, Reverse) - Metabion
5. Anti-digoxigenin-AP fab fragments – Roche
6. Digoxigenin (DIG) labelled telomere probe (TTAGGG) 7 – Roche
7. Teltail primer Sequence 5'-3'Digoxigenin- Metabion
8. Adapter primer- Metabion
9. Telorette- Metabion
10. 42-mer –Panhandle- Metabion
11. 11 + 2-mer – Panhandle- Metabion

5.1.6 Solutions

1. NBT/BCIP Stock Solution- Roche
2. FailSafe PCR 2X PreMix- Epicentre
3. FailSafe Enzyme Mix- Epicentre

Methods

5.2 DNA Extraction

DNA isolation was carried out by adopting the manual DNA isolation method. The cells were treated with 5ml of lysis buffer, 50µl of proteinase K and incubated at 37C for 2 hours. The lysate was centrifuged at 13000 rpm for 5 minutes at 4C. The supernatant was discarded and pellet was washed with 5ml prechilled RBC lysis buffer, add 330 µl of 6 M NaCl and shake vigorously for 15 seconds (Do not vortex). Centrifuge for 5 min at 13000 rpm at 4C and remove the supernatant, add 2 volume of ice cold absolute alcohol. Invert the tube to precipitate the DNA, until the DNA becomes visible, but if the DNA is not visible place the tube at -80 C for 20 min. Centrifuge the DNA for 5 min at 13000 rpm, discard the supernatant and wash the DNA with 70 % alcohol. Dissolve the DNA in 50 or 100 µl of TE buffer.

5.3 RNA Isolation

RNA extraction was carried out utilizing Roche high pure RNA tissue kit, which provided fast processing and effective purification of RNA from cells. The procedure was performed according to the manufacturer's instructions which few modifications. The cells were trypsinized and washed with 5ml of cold PBS. The cells lysate was pipetted 10- 20 times through a 2ml gauge needle. Centrifuge the lysate for 2 min at 13000 rpm, collect the supernatant and add 0.5 volumes of absolute ethanol to further lysate the supernatant. Collect the supernatant and pipet the entire lysate in the upper reservoir of the filter tube and centrifuge at 13000 rpm for 30 seconds and discard the leftover. Pipet 90 µl of DNase incubation buffer and combine the filter tube with collection tube. Pipet 90 µl of DNase incubation buffer into 1.5 ml of reaction tube and stored at -20 C and mix. Pipet the entire solution in the upper filter tube and incubate for 15 minutes at 20 C. wash the entire solution with the wash buffer 1, wash buffer II and spin at 8000 x g for 15 seconds. Add elution buffer to the filter tube and centrifuge. Use 10 ul of eluted RNA for RT-PCR or stored at -80 C.

5.4 cDNA synthesis

cDNA was carried out utilizing Helixcript first strand cDNA synthesis kit (nano helix). The procedure was performed according to the manufacturer's instructions which few modifications.

5.5 Primer design

Primers were designed for RT-qPCR using primer-BLAST at: www.ncbi.nlm.nih.gov. Primer parameters were set as follows: -

- Primer Tm (melting temperature): min (57°C) – max (63°C), optimum (60°C)

- Primer length: min (18bp) – max (22) bp, optimum (20)

- Primer GC% content: min (45%) – max (55%)

- Amplicon product size: min (50bp) – max (200bp)

Primer Name	Primer sequence (5---3)	Product length (bp)	Acession number
TRF1	**Forward primer:** CACCCCCAAATCCTGTTCCA **Reverse primer:** ACAGGGTTGAGGTCAGCCTA	101	NM_003218.3
POT1	**Forward primer:** TGTCCCCCATCATCGACT **Reverse primer:** GGGCTTCCTGAAGGCAGAA	200	NM_015450.2
TRF2	**Forward primer:** GTCCAAGGACCCCACAACTC **Reverse primer:** TTTTTGGCCATCGTGAGGAG	180	XM_005256123.1
TPP1	**Forward primer:** AGGTACTACAGGACGCCGAG **Reverse primer:** GGAAGCCGAACTCCTTCTCC	168	NM_001082486.1

Table 2 Primer sequences used in Real-Time PCR

5.6 Real-Time quantitative RT-PCR (qRT-PCR)

Light cycler 480 SYBER green 1 master (Roche) was used to perform real-time PCR (qPCR). The reactions were carried out in 96-well plates (Roche). A final volume of 10µl pre-mix was prepared containing 5µl of 2x SYBR® green master mix, 1µl of 5µM of forward and reverse primers (See Table 2 for primers used), 1µl of cDNA, and distilled water to make the final volume up to 10µl. Target and endogenous pre-mixes were prepared separately and 9µl of reaction pre-mix was aliquoted into each of 96-well plate; 1µl of cDNA was immediately added and the plate was sealed utilizing an optical adhesive film (Roche). Samples were minimized to light exposure. After that, 96-well plate was centrifuged at 13000rcf for 60 seconds at room temperature.

The default PCR conditions are as listed below

Stage	Cycle	Temperature	Time
Pre-incubation	1 cycle	95°C	600 sec
Amplification	55 cycles	95°C	10 sec
		95°C	10 sec
		58°C	10 sec
		72°C	20 sec
Melting	1 cycles	95°C	1 sec
		65°C	15 sec
		95°C	20 sec
Cooling	1 cycles	40°C	10 sec

Temperature profiles

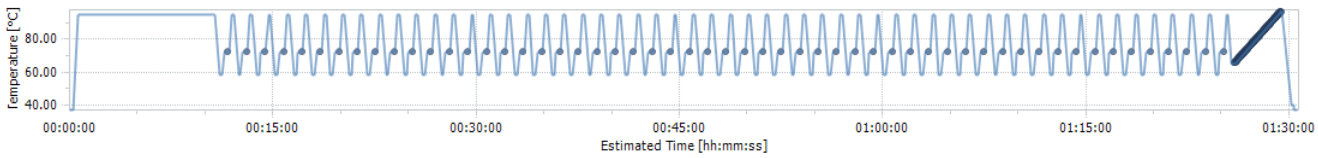


Table 3 Reverse transcriptase real time PCR temperature profiles during different cycles.

Finally, the dissociation curve was constructed after the PCR run to verify and check the results. Dissociation curves were important to detect primer dimmers and non-specific amplification that may affect the quality of the data. The relative expression level of genes of interest was calculated using ΔΔ method. In this way, the mRNA expression level of each shelterin associated genes were calculated using qRT-PCR and compare it with the gestational age matched control group with normal pregnancy, housekeeping β-actin gene is used as a reference gene in the study.

To quantify the expression level of Shelterin protein complex (TRF1, TRF2, TPP1, POT1) expression levels, the Ct values obtained for each mRNA were calculated using ΔΔ method which expresses the difference between the cycle threshold between the mRNA expression of patients and control group. Each sample was run in thrice to ensure the reproducibility and accuracy of the results. qPCR data was analyzed by calculating the fold difference individually for each housekeeping *beta actin* and similarly compare it with the cells isolated through aminocentosis from female control group (normal pregnancy). The expression of target gene in all other samples is expressed as an increase or decrease relative to the calibrator and control group. The Cycle threshold (Ct) is defined as the number of PCR cycles at which the fluorescence signal rises above the threshold value and is inversely proportional to the amount of template present in the reaction. Ct values of Shelterin complex and normal control group samples were compared and the fold difference calculated by the equation:

Gene being Tested Experimental (TE), Housekeeping Gene Experimental (HE)

1. ΔCTE= TE-HE

Gene being Tested Control (TC), Housekeeping Gene Control (HC)

2. ΔCTC= TC-HC
3. Double Delta Ct Value (ΔΔCt) = (ΔCTE-ΔCTC)

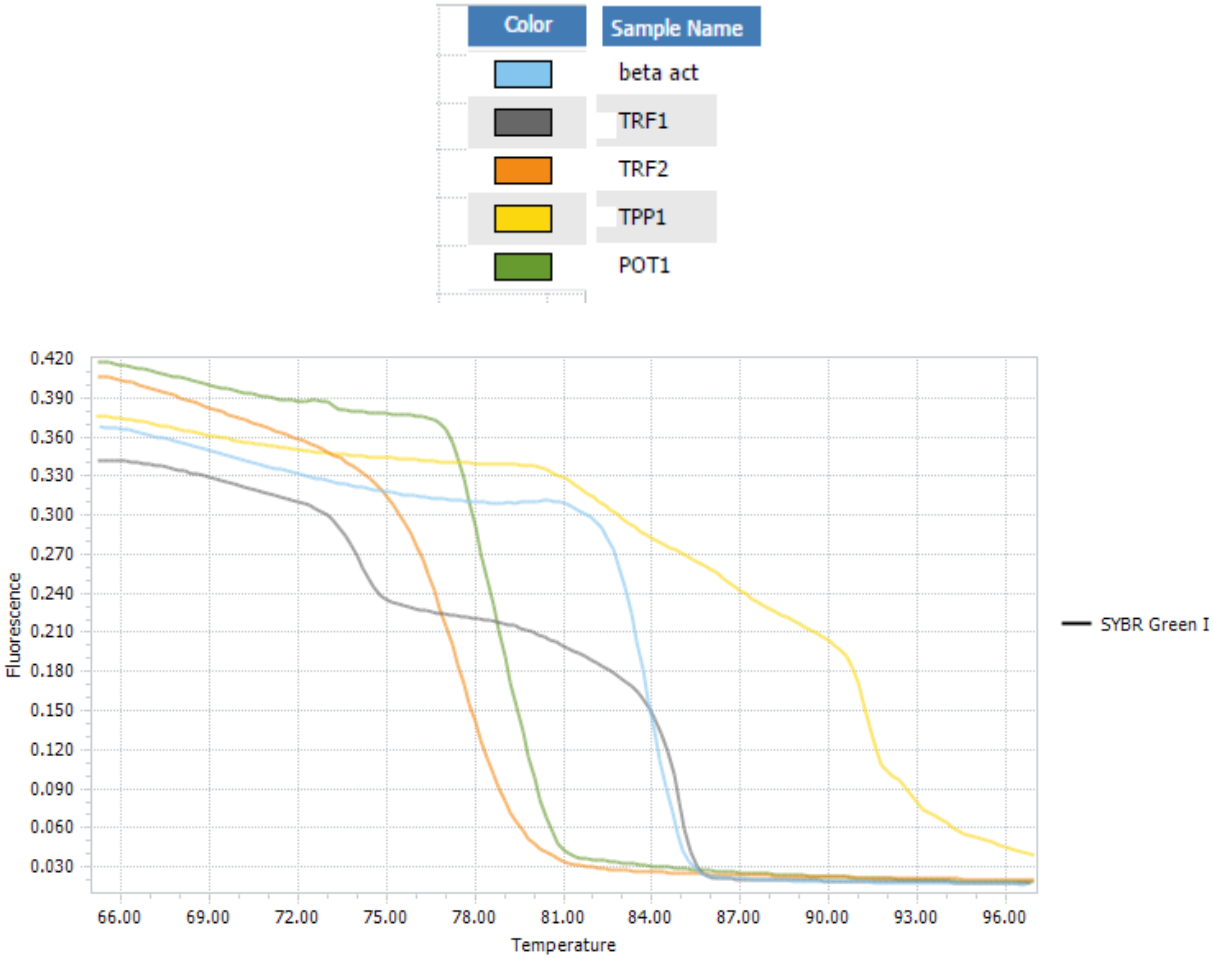


Figure 6 A Dissociation Curve reveals the change in fluorescence of shelterin complex (TRF1, TRF2, TPP1, and POT1) with increasing temperature. As the temperature is increased, the amplicon strands separates causing the fluorescent intercalating dye to dissociate from the DNA and stop fluorescing.

5.7 Telomere restriction fragment (TRF) analysis

The restriction endonucleases *Hinf* I and *Rsa* I were used because they do not contain recognition sequences that will cut within the telomeric repeat sequences. The enzyme used are unique in a way that it only allows the digestion of non-telomeric DNA, leaving only telomeric DNA intact. This DNA left after digestion is reffered to as a Telomere Restriction Fragment (TRF). The Telo TAGGG (Roche) kit has been used with few modifications

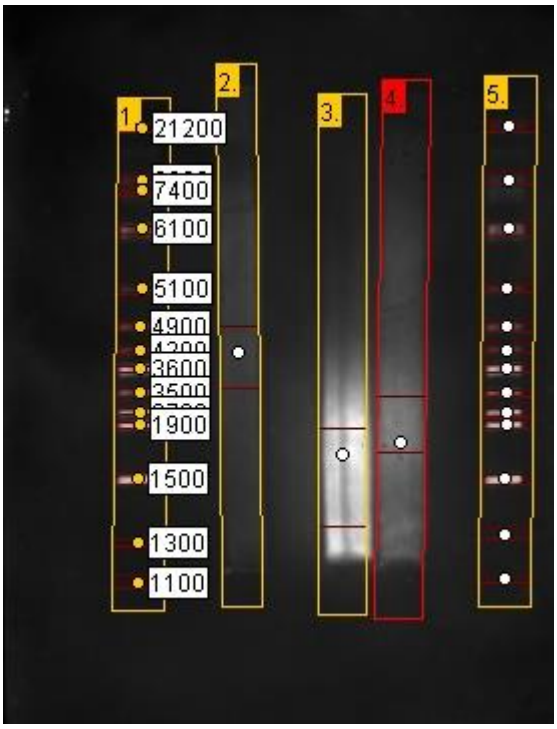
For each sample in a reaction vial add 1.5 µg DNA and control with 2 µl of nuclease free water to produce a final volume of 17 µl, than 2 µl of digestion buffer 10X alongwith 1 µl of *Hinf* I/*Rsa*ı enzyme mixture is added. The mixture was incubated for 5 hours at 37C , to stop the reaction add 5 µl of gel electrophoresis loading buffer and quick spin the reaction vial. If digests were not immediately used, they were stored at -20°C for up to one month.

Digested DNA was separated out on a 0.8% agarose gel made by dissolving 2g of agarose in 250ml of 1X TAE buffer. 3µl of bromophenol blue loading dye was added to each digested DNA sample and 20µl of this mixture was added to each lane. Two marker lanes containing a DIG labelled DNA molecular weight marker (23.1 kb – 1.1 kb, Roche) were also run on each gel. Each marker lane contained 10µl of a mixture containing the marker, distilled water and bromophenol blue loading dye in a 5:5:1 ratio. The gel was run at 50V for approximately 3.5 hours.

After gel electrophoresis the agarose gel was pretreated with Hcl for 5 minutes at +15 to +25 C for depurination. The gel was rinsed in distilled water and placed in the denaturation solution for 1 hour at +15 to +25 C and rinsed with distilled water before placing it into the neutralization solution with the same conditions as was used in the denaturation step.

The DNA from the pretreated gel was transferred to a positively charged nylon membrane (Amersham) by use of the vacum blotting following a standard protocol available with the Vacu-Blot (Biometra) with minor changes. The vacum pressure forced the buffer (20x SSC) through the gel and membrane rapidly as compared to conventional way of capillary action, enableling transferred time to be reduced as little as 30 min (*Encyclopedia of Life Sciences*, 2001).

The blotted membrane was incubated with digoxigenin (DIG) labelled telomere probe, which hybridizes to the telomeric region of the digested DNA. A digoxigenin (DIG) labelled specific antibody coupled covalently to alkaline phosphates was added which converts the substrate CDP-Star into a signal, indicating the location of the telomere on the blot. The blot was exposed to the imaging device for 1 hour and photographed. The DNA containing telomere repeats was visible as a smear, the images were edited as needed for visualization. The intensity of TRF smears at different molecular sizes was calculated using TotalLab software (Photoretix) and GelAnalyzer software.



Densitometry analysis of mean TRF length

Figure 7: The Southern Blot is shown with the telomere specific smear in white. Lane 1 and 5 contains the size standard ranging from 21.2kb to 1.1kb. Lanes 2-4 contain Patients genomic DNA samples. The principles of how the analysis (5.7.1) is performed using the GelAnalyzer software.

5.7.1 Calculating the Mean TRF Length

Mean TRF length is calculated according to the following formula.

TRF= Σ (ODi) / Σ (ODi /Li)

Where ODi is Chemiluminnescent signal and length Li is the length of TRF at position i *(Figure 8)*

The background density was calculated using the average density of several boxes that did not contain any signal (telomere specific), and subtracted from each box that did have a signal. For

each box containing DNA, the mean density (ODi) and corresponding length (Li) using the molecular weight at the mid-point of the box was calculated and then substituted into the above mentioned formula for TRF measurement.

6.0 Universal STELA protocol

U-STELA is a technique used for the measurement of short telomeres which has been adopted in this thesis to measure really short telomeres in an attempt to find a correlation between short telomeres and recurrent miscarriage. The same protocol was followed as devised by (Bendix et al., 2010b) with few modifications. The detailed explanation is as followed.

1. Digest 1 µg of DNA with 1 µl of MseI and 0.5 µl of NdeI in 50 µl of volume containing 5 µl cutsmart buffer. Incubate it at 37 C for 1 hour followed by 20 minutes of inactivation at 65 C.
2. 0.5 µl of digested DNA is mixed with 3 µl of 12 mer and 42 mer panhandles and 0.5 µl of dH2O to make 7 µl of volume.
3. Use centrifuge to decrease the temperature from 65 to 16 in 49 minutes.
4. Add 0.5 µl (20 units) of T4 DNA ligase quickly with 1.5 µl (1*) T4 DNA ligase reaction buffer and 6 µl dH2o to make it 15 µl of volume and incubate the sample overnight at 16 C
5. Add 0.5 µl (20 units) of T4 DNA ligase with 2.5 µl telorette working solution, 1 µl (1*) T4 DNA ligase reaction buffer and 6 µl of water to make it 25 µl volume and incubate at 35C overnight followed by 20 min of inactivation at 65C.
6. Prepare a diluent of the reaction at 1:20 using 5 µl from the stock
7. Prepare the PCR reaction with 1.2 µl of adapter and teltail (0.1 µM), 2 µl ligated DNA (40 pg), 6 µl of failsafe master mix, 0.5 µl of failsafe enzyme and 1.1 µl of dH2O.

	Number of Cycle	Temperature	Time
1	1 cycle	68^{c}	5 minutes
2	1 cycle	95^{c}	2 minutes
3	26 cycle	95^{c}	15 seconds
4	26 cycle	58^{c}	30 seconds
5	26 cycle	72 ^{c}	12 minutes
6	1 cycle	72^{c}	15 minutes
7	1 cycle	4^{c}	∞

Table 4 The PCR conditions for Universal STELA (U-STELA)

After PCR southern blotting, chemiluminescence and hybridization performed as we did in TRF analysis (5.7)

7.0 Results

7.1 Telomere Restriction Fragment Length Analysis

Telomere restriction fragment length analysis was used to analyze mean telomere length from cells isolated from the abortion material in women suffering from idiopathic recurrent pregnancy loss and control with normal pregnancy. The statistical parameter adopted to compare mean telomere length between the two groups by using Mann-Whitney U-Test which is a version of the independent samples t-Test and the result is significant $p \leq 0.05$. The data obtained from this study clearly indicates the decline in mean telomere length in patients with iRPL as compared to the control as shown in Figure 9. The calculated mean had a longer calculated mean in control group (15.5 kb) than the patients group (2.5 kb); the Mann-Whitney U-Test was employed. The U-value is 14.5. The critical value of U at $p \leq 0.05$ is 62. Therefore, the result is significant at $p \leq 0.05$.

The clear pattern of difference in mean telomere length between both groups supports the hypothesis that short mean telomeres may have induced the cellular senescence in developing fetus resulting into a miscarriage.

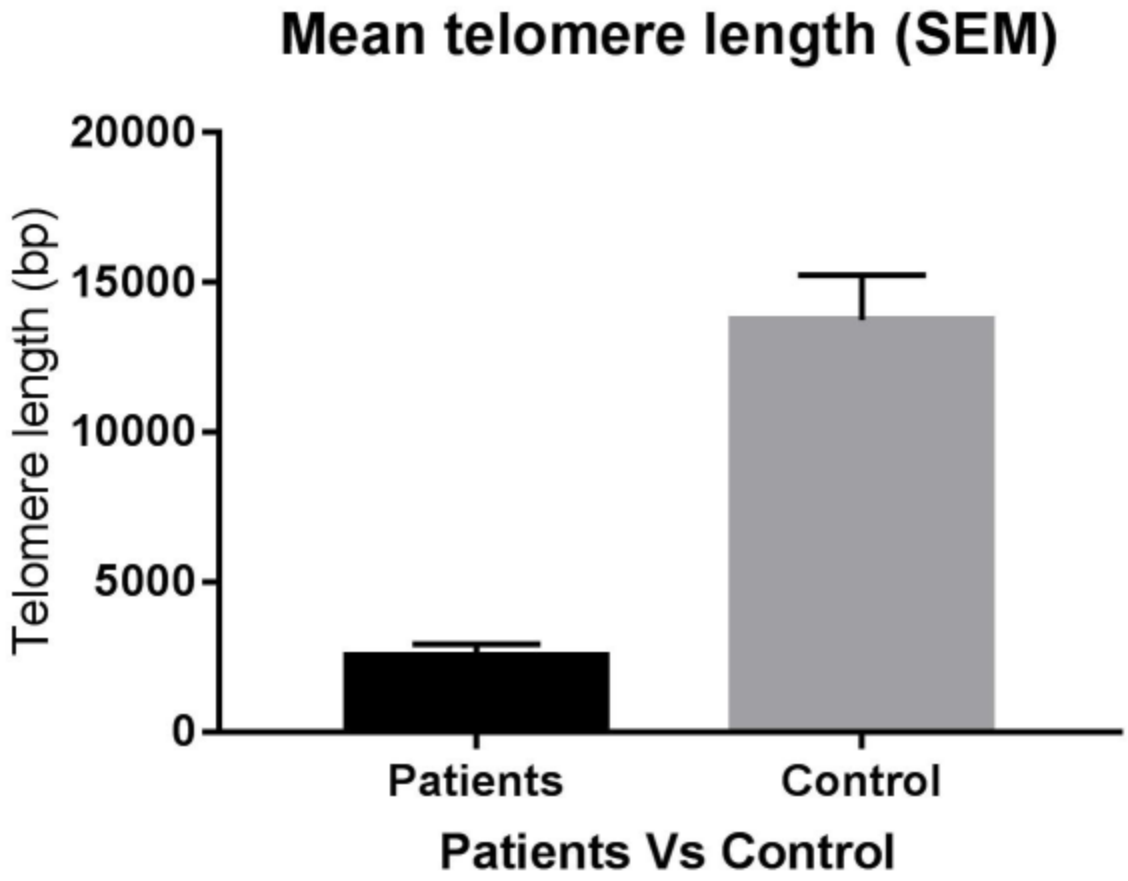


Figure 8 Mean telomere lengths of Patients (iRPL) vs. Normal control populations. Mean telomere lengths were calculated by adopting TRF length analysis for each sample, and means were compared using a Mann Whitney u test. The result is significant at $p \leq 0.05$.

Southern blot data also reveals short telomeres in patients with iRPL as compared to normal control group. The threshold set for short telomeres were set according to previous studies ≤ 2000 bp. And it is clearly visible from the blot image that the telomere smears lie around 2000bp (A), whereas long telomeres have been observed in control with normal pregnancy.

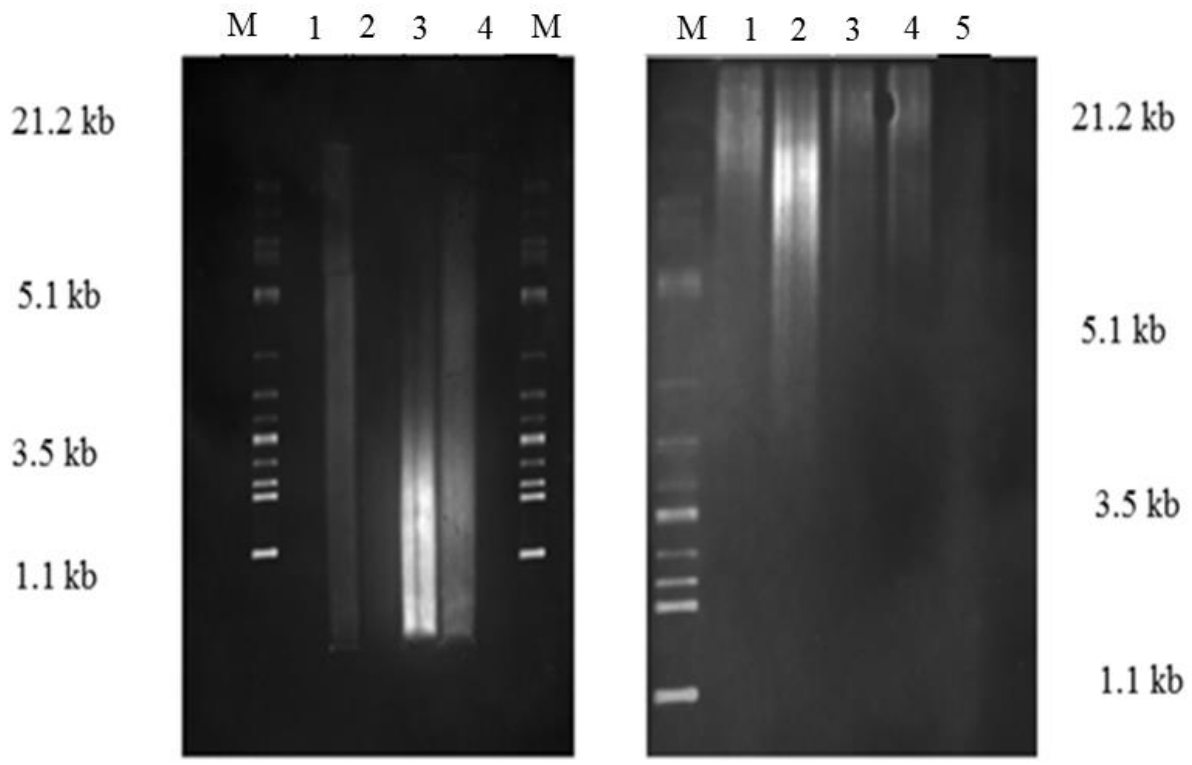


Figure 9: Southern blot including samples from (A) iRPL Patients and (B) Normal control group

Lane M contains the size standard ranging from 21.2kb to 1.1kb. **Group (A)**: Lanes 1-4 contain different DNA samples with variable telomere length, **Lane 1**: 2 kb, **Lane 2**: 2.5 kb, **Lane 3**: 1.4 kb, **and Lane 4**: 1.2 kb. **Group (B)** Lanes 1-5 contain different DNA samples from normal control with variable telomere length: **Lane 1**: 18 kb, **Lane 2**: 15 kb, **Lane 3**: 20 kb, **Lane 4**: 17.21 kb, **Lane 5**: 10 kb. The variation in telomere length within and between both groups indicates telomeres of variable lengths; the decline in telomere length is clearly visible in patients suffered from iRPL whereas long telomeres have been observed in control group.

7.2 Universal STELA

Universal STELA is used to measure the load of ultra-short telomeres that are thought to trigger replicative senescence due to the presence of few or groups of ultra-short telomeres. Both TRF and U-STELA technique was also employed to analyze and correlate the results obtained from these studies. In previous studies tissue culture senescence was found to be induced with a mean telomere length much longer than what would be expected to induce the mechanism of cellular senescence (Bendix, Horn, Jensen, Rubelj, & Kolvraa, 2010a). Based on these previous studies, a number of groups have proposed that telomere related cellular senescence may instead be caused by a single or group of few ultra-short telomeres (Hemann, Strong, Hao, & Greider, 2001) (Abdallah et al., 2009) and not certainly by a global decrease in mean telomere length. The reason for adopting both of these telomere length measurement techniques in this study is to get better insight between the role of ultra-short telomeres or global mean telomere length in causing idiopathic recurrent pregnancy loss (iRPL). The data obtained from the U-STELA was difficult to analyze because the signal intensity of the blot was very low on the southern blot image. Gel electrophoresis produced gel image with large smears as expected after digestion with the restriction enzymes ***Fig (11)***. After southern blotting and chemillumiscence hybridization smears with relatively long telomere appeared on the blot, but critically short telomeres ≤ 1300bp were not clearly visible. The reason of low signal intensity could be because of the improper or low hybridization of telomeric probe or reagents may be contaminated or any other unspecified reason. In this study the results obtained from the TRF analysis and gene expression study indicate the possibility of the presence of few ultra-short telomeres and complements with the findings of the results generated from TRF analysis and gene expression study of the shelterin protein complex. The down regulation of two important of TRF2 and TPP1 leads to an inappropriate binding of shelterin protein, which could be the reason behind extensive telomere attrition and may lead to the formation of critically short telomeres (≤1300 bp). As shown below (Fig.12) the presence few ultra-short telomeres ≤1300 bp are slightly visible, which helped in concluding our research. The results obtained from normal control group revealed longer mean telomere length, but the presence of critically short telomere has not been observed due to very low chemillumiscence signals or improper binding of the telomeric probe to the ultra-short telomeres.

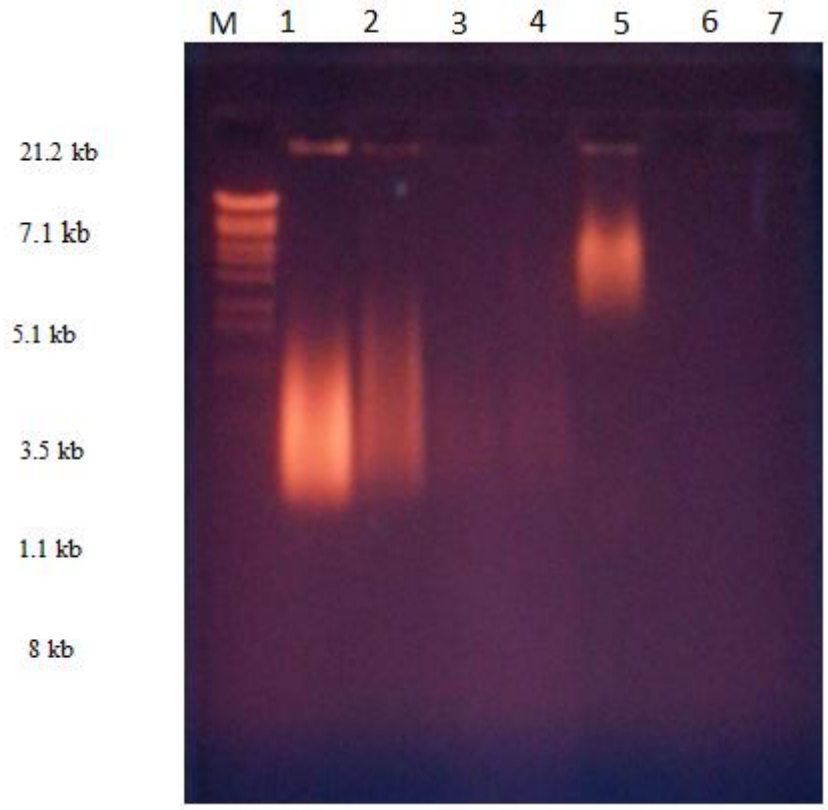


Figure 10: Universal STELA: Gel electrophoresis image after restriction digestion. The digestion seems successful as clear smears formed after DNA digestion.

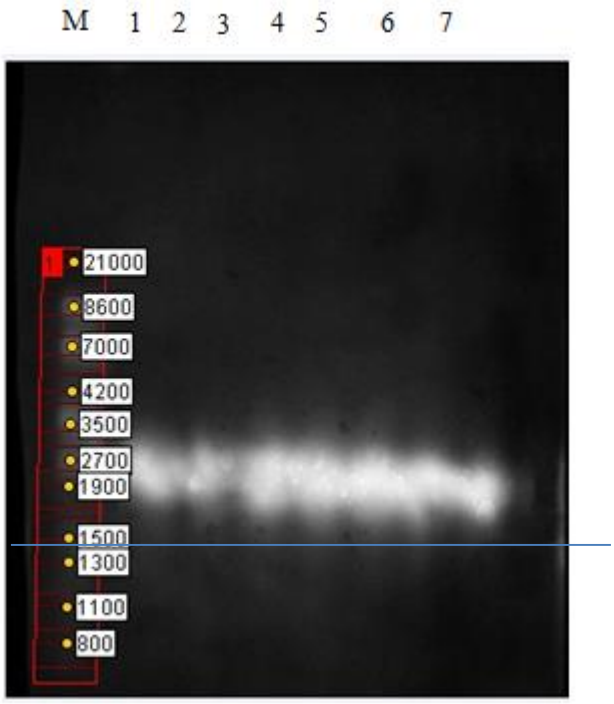


Figure 11: Southern blot including samples from (A) iRPL Patients and (B) Normal control group

7.3 Gene expression of Shelterin protein complex

Telomeres are associated with shelterin, which is a complex associated with the protection of DNA ends from being recognized as a double stranded break that would induces DNA damage response. Understanding the gene expression pattern of shelterin protein complex in patients suffering from idiopathic recurrent miscarriage will be significant in further elucidating the complex relation between telomere length dynamics in recurrent miscarriage patients.

The expression levels of mRNA were measured in tissues from abortion material in patients with RPL and compared with adjacent normal control group using ΔΔct method. The data obtained from the expression study indicates the down regulation of two important shelterin proteins TRF2 and TPP1 whereas up-regulation in TRF1 and POT1 has also been observed as shown in fig (**B**)

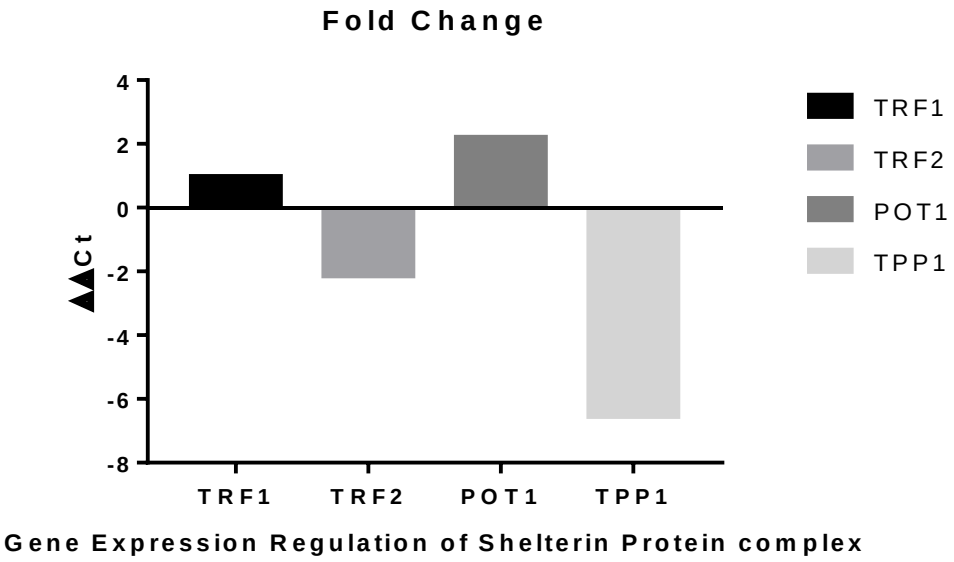


Figure 12: A bar graph showing the rates of up-regulated and down-regulated expression of shelterin protein in relation to the control group.

As shown in **Figure 3.2**, TRF2 is down-regulated in patients VS control group, whereas TPP1 showed significant down-regulation relative to the control group. TRF1 is up-regulated (1 fold) and POT1 also showed up-regulation in the study (2 fold). The two-tailed P value is 0.0004, considered extremely significant.

7.4 Discussion

Telomeres play a significant role in the maintenance of chromosomal integrity, and guide proper alignment of chromosomes during meiosis and mitosis (Thilagavathi et al., 2013b). The shortening of telomeres has been linked to many diseases such as cancer (Calado & Young, 2012). The diseases caused by the defects in telomeres are referred to as telomeropathies (Holohan, Wright, & Shay, 2014). The role of telomere shortening in various reproductive disorders such as recurrent miscarriage has not been well studied.

Maintaining constant telomere length is significant in providing a suitable environment for normal fetal development as it has been observed that oxidative stress during fetal development can lead to anomalies such as rupture in amniotic membrane and premature birth (Menon et al., 2012). Normal pregnancies require a constant maintenance of chromosomes during utero life and variations in telomere length may lead to reproductive complications.

In this study we investigate the potential role of telomere homeostasis in idiopathic miscarriage for that purpose TRF and U-STELA was used to asses mean telomere length and ultra-short telomeres. RT-qPCR was used for gene expression analysis of important shelterin protein complex (TRF1, TRF2, TPP1 and POT1). The purpose of adapting U-STELA and TRF analysis was to understand the relationship between the ultra-short telomeres and overall mean telomere length and their collective role in inducing cellular senescence. Cellular senescence has been observed in tissue culture with relatively longer mean telomere length and can be correlated with the presence of few or group of ultra-short telomeres, however in this study overall decline in mean telomere length has been observed. The gene expression analysis of shelterin protein complex is significant to understand the role of telomere binding protein in overall telomere length maintenance, little is known about any potential role of all this telomere biology related factors in Idiopathic recurrent pregnancy loss.

The shortened telomere length observed in the abortion material of females with recurrent miscarriage is the first such study to our knowledge, though shorter telomere have been reported in female partner experiencing recurrent miscarriage (Thilagavathi et al., 2013b).

The accumulation of free radicles has been reported to cause telomere shortening(Z. Wang et al., 2010). Short telomere fails to protect DNA ends that in return triggers DNA damage pathways (ATM, ATR) and cause apoptosis or cellular senescence (Titia de Lange, 2002).

The study demonstrated a clear decline in mean telomere length ≤ 1300 bp threshold. The results obtained from U-STELA were difficult to interpret because of low signal detection on the blot due to which it was difficult to see critically short telomeres. Although it is reasonable to speculate the presence of ultra-short telomeres due to the down regulation of two important components of shelterin protein (*TRF2* and *TPP1*) which induces telomere uncapping due to which the chromosomes ends are exposed to oxidative stress causing extensive telomere shortening. Telomere shortening leads to improper meiotic segregation in germ cells that have a potential to induce aneuploidy that may result into a recurrent miscarriage. Telomere shortening induces aneuploidy which is usually found in human embryos and female germ cells with various reproductive disorder particularly recurrent pregnancy losses.

To understand the complex regulatory network, the gene expression analysis provides a better insight into to the characterization of genes involves in the development of disease and other important biological processes. The RT-qPCR technique has been adapted due to its high reproducibility, accuracy, sensitivity and potential to produce high throughput data (Aithal & Rajeswari, 2015). The reference gene selected for this study is *β actin* due to its high stability and low expression variability (Rebouças et al., 2013).

Telomeric repeat binding factor 1 (TRF1) plays a significant role in the maintenance of telomere function and ensures the normal homeostasis of the cell. Both TRF1 and TRF2 are important member in this regard as the stable interaction of other shelterin protein chiefly depends on the proper binding of these proteins (TRF1, TRF2) (Galati et al., 2015). The variation in the normal expression levels of these shelterin proteins will induce anomalies in telomere homeostasis resulting into various reproductive and age related disorders such as recurrent miscarriage. Decreased expression of *TRF2* may negatively impact the structural organization of double stranded DNA as TRF2 binds to double/single stranded DNA junction (R M Stansel et al., 2001), and facilitates the invasion of 3’ G-rich overhang (Amiard et al., 2007). TRF2 is not only restricted to telomeres but also accumulates at the site of double strand breaks at the non-telomeric region of the DNA (Bradshaw et al., 2005). *TRF2* down-regulation may inhibit the

accumulation of TRF2 at the end of chromosomes resulting into an activation Ataxia telangiectasia mutated (ATM) and ATR pathways that may cause apoptosis or cellular senescence in developing embryo and also induce DNA damage in the non telomeric region by facilitating the early stages of homologous recombination (Mao, Seluanov, Jiang, &Gorbunova, 2007). Thus TRF2 provides chromosomal stability and protection by maintaining the telomere structure as aberration in chromosomes may be the possible etiologic genetic cause of recurrent miscarriage (Bobadilla-Morales et al., 2009).

The study showed two-fold up-regulation in *POT1*, whereas *TPP1* is significantly down-regulated. Both POT1 and TPP1 are strongly associated with each other as shown in previous studies (Hockemeyer et al., 2007b)(Hwang et al., 2012). POT1 is a single stranded DNA binding protein; the stable interaction of POT1- TPP1 ensures the binding of POT1 to the duplex telomeric DNA. Control of telomere length is significantly dependent on the proper binding of POT1- TPP1, but still the dependency of POT1 on TPP1 in providing the protection to chromosomal ends is largely unknown (Hockemeyer et al., 2007b). The complex of POT1-TPP1 plays an important role in inhibiting the DNA damaging signal by removing sensors, such as RPA at the telomeric ends (Kendellen, Barrientos, & Counter, 2009b). Improper binding of POT1-TPP1 complex may help cell's DNA repair machinery to recognize chromosomal ends as double stranded breaks resulting into cell cycles arrest or apoptosis which may harm the normal fetal development by inducing cellular senesce in developing embryo. TPP1 binds to TIN2 and POT1 that enables the binding of six proteins together. An interaction of all these six proteins leads to an association between single and double stranded proteins of the telomeric t-loop.

Short telomeres induce disruption in recombination of homologous chromosomes and nuclear reorganization resulting into aneuploidy or meiotic arrest. Short telomeres characterized to induce abnormal embryonic development and decreased rate of blastocyte development (Lee et al., 1998). Combination of various factors involves in telomere shortening out of which the inappropriate binding of shelterin complex is of tremendous significant as suggested by this study. Other possible mechanisms such as inhibition of normal telomerase activity due to the mutation in telomerase coding gene cannot be ruled out.

Studies related to telomere length and reproductive aging revealed variable contradictory results in which telomere length dynamics is both positively and negatively related with different

measures of reproductive aging(Aydos et al., 2005)(Dorland et al., 1998)**.** The data obtained in this study clearly indicates the relationship between short telomeres and recurrent miscarriage and are consistent with previous studies where short telomeres have been linked in producing disease like symptoms (Artandi & Attardi, 2005)(Turner, Wong, Rai, & Hartshorne, 2010b)(Thilagavathi et al., 2013d).

In conclusion, All Abort material showed down-regulation of 2 major shelterin proteins (TRF2, TPP1), which both of these proteins contribute to incomplete capping known to have led to chromosomal instability through Breakage-fusion-bridge (BFB) cycle. Short telomeres limit the replicative capacity of embryo or blastocyst thus leads to developmental delay or embryo arrest.

Variation in shelterin complex homeostasis may also induce unwanted DNA damage repair pathway resulting into cell cycle arrest or apoptosis. This area of research is still in its infancy and is open to further research as limited data is available on directly analyzing the telomere length from the abortion material in patients suffering from idiopathic recurrent miscarriage. Future research into this area will be of significant importance and can be used as a biomarker of reproductive aging.

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