DYNAMICS OF TELOMERE IN AGEING

Thesis submitted to

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Life science

SUBMITTED BY

AMIT CHATTERJEE

ROLL NO:-409LS2036

SUPERVISED BY

DR.BISMITANAYAK

ASST.PROFESSOR

DEPARTMENT OF LIFE SCIENCE

NATIONAL INSTITUTE OF TECHNOLOGY,

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DECLARATION

I hereby declare that the thesis entitled **"**DYNAMICS OF TELOMERE IN AGEING**"**, submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the Master Degree in Life Science is a faithful record of bonafied and original research work carried out by me under the guidance and supervision of Dr. (Miss) Bismita Nayak, Assistant Professor, Department of Life Science, National Institute of Technology, Rourkela. No part of this thesis has been submitted by any other research persons or any students.

Date:

AMIT CHATTERJEE

Place:

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DEPARTMENT OF LIFE SCIENCE
NATIONAL INSTITUTE OF TECHNOLOGY,
ROURKELA-769008

Dr. (Miss.) Bismita Nayak, M.Sc., Ph.D.
Assistant Professor.

Ref.No……
Date:.....

CERTIFICATE

*This is to certify that the thesis entitled "***DYNAMICS OF TELOMERE IN AGEING"** *submitted to National Institute of Technology; Rourkela for the partial fulfilment of the Master degree in Life science is a faithful record of bonafied and original research work carried out by AMIT CHATTERJEE (409ls2036) under my supervisions and guidance.*

Dr. (Miss.) B.Nayak
Advisor

Phone no.: +0661-2463682 Email:bismita.nayak@gmail.com

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At the end, I bow down my head to the almighty whose omnipresence has always guided me and made me energiesed to carry out such a project.

Date:

Amit Chatterjee

Place:

ABSTRACT

Telomere are composed of G rich nucleotide (TTAGGG)n typically 20 kb in length, are the regions of repetitive DNA at the end of the chromosomes. As the eukaryotic cells divide, the protective ends of the linear chromosomes, the telomeres, gradually shorten with each cell division. When a critical telomere length is reached, the cells are signaled into senescence, an irreversible state of quiescence. Thus, telomere length has emerged as replicative clock within each population of cells. Since telomere are also non coding sequence of DNA, as that of introns, there might be specific sites as of 5' splice site and 3'splice site, which might be the conserved regions in telomere and can possibility generate a signal for apoptosis after a certain time of cell division.

Since Telomere Length (TL) in the somatic cells determines the number of cell divisions, TL has been proposed as a marker for biological ageing and cancer. Under conditions of chronic oxidative stress, the rate of telomere shortening is increased. Since telomeres consist of the repetitive sequence TTAGGG, and have a high GC content, they are very vulnerable to base oxidation. Additionally, DNA strand breaks are less efficiently repaired at the telomeres when compared to the rest of our genome. This means that reactive oxygen species cause more damage to the telomeres and this damage is less well repaired. Genetics play an important role in determining TL, but environmental and lifestyle factors such as smoking, alcohol consumption, physical activity, body composition and coffee and tea consumption can affect oxidative stress levels, which may contribute significantly to accelerated telomere shortening. Since, buccal cells are under direct contact with smoke and various toxic chemical substances in people who are acutely habituated with taking tobacco and cigarettes. So in our work we have found the conserved region of the telomere and showed the prolonged effect of tobacco and cigarettes on these conserved regions of the telomere.

LIST OF TABLES AND FIGURES

1. INTRODUCTION:

James Watson and Francis Crick solved the mysterious structure of DNA in 1953 at Cambridge University and found that the two strands can be easily separated by a slightly high temperature. Marmur *et al* in 1960 showed that the two strands can regain their property of double helix when brought back to the room temperature. But the kinetics of renaturation curve in prokaryotic and mammalian DNA differed sharply (Britten and Kohne., 1965). These curve made the scientific community to think that the nucleotide sequence in eukaryotic DNA should differ markedly than that of prokaryotes. And these findings showed the presence of three classes of DNA sequence which reanneal at different rates in eukaryotic DNA during the process of reanneling. These are highly repeated fraction, moderately repeated fraction and non repeated fraction. These highly repeated fractionsof DNA comprises of centromere and telomere.

Telomere (from the Greek word "telos" meaning end "mere" meaning part) is a protective structure at the terminal end of the chromosome as described in **Fig 1**. They are composed of G rich nucleotide (TTAGGG)n typically 20 kb in length, terminating in a 3' single stranded DNA overhang **(**Allsopp *et al*.,1992). Due to the inability of the DNA polymerase to replicate at the 3' end of each DNA strand is incompletely copied during each round of cell division in a linear chromosome leading to attrition of the telomere sequences which Watson described as an "End replication problem". In humans, there are 46 chromosomes and 92 telomere ends that contain thousands of repeats of six nucleotide sequence, TTAGGG. Telomere length is maintained by a balance between the two processes that is lengthening of telomeres (telomerase activity) shortening of telomeres (the end replication problem)

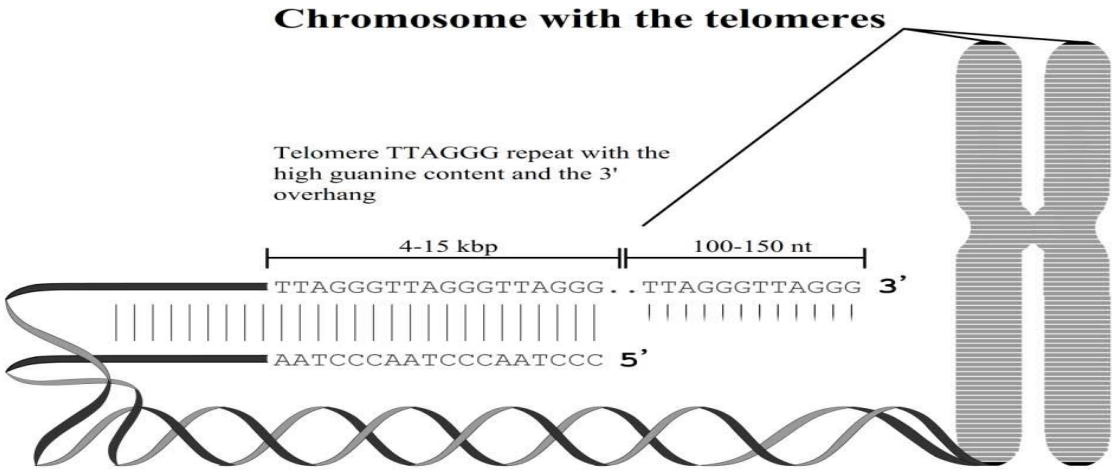


Fig 1 Structure of Telomere

Telomere has been considered as that part of chromosomal DNA where the organization is so compact that the protein involved in genome expression cannot access. Telomere are protected by the complex of six core proteins composed of TRF1, TRF2,RAP1,TIN2,TPP1 and POT 1 which altogether known as Sheltrin as in **Fig 2**. Sheltrin complex protein is only found at the end of the chromosome and its functions are limited to telomeric region due to the presence of TTAGGG recognition fold in the complex. This complex is responsible for the protection of telomeric DNA form DNA damage response by the formation of t- loop which resembles a strand invasion intermediate in homologous recombination (Griffith *et al*., 1999). However, formation of t- loops is conserved in telomere and plays a role in telomere protection as well as in regulation of telomerase. This protection is termed as capping and telomere become uncapped when it becomes too short to form t- loop or when certain sheltrin protein complex is defective.

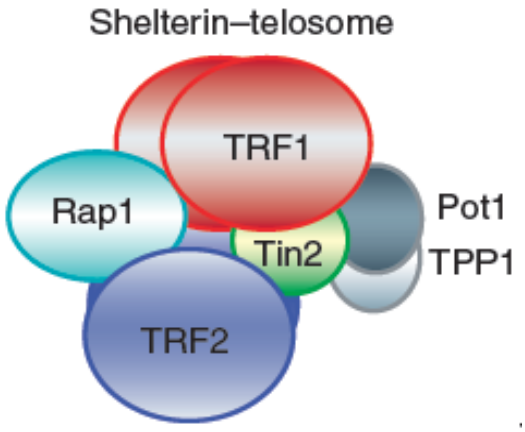


Fig 2 Components of Sheltrin protein

In addition to this six shelterin components, other additional proteins have been reported to be associated with mammalian telomeres listed in table1, which contribute to t-loop formation, maintenance and DNA damage signaling or repair. Examples of such factors include, Mre11 protein complex involved in detection of double-strand breaks and in the homologous recombination pathway of DNA repair; XPF/ERCC1, a component of the nucleotide-excision repair pathway; DNA- PKcs, a kinase involved in the non homologous end-joining (NHEJ) pathway; Ku70/80 also involved in the NHEJ pathway. The other Non Sheltrin protein complexes involved in telomere are presented in table 1.

Table 1- Non sheltrin proteins presented in human telomere

Protein Complex	Non telomeric function	Effects at telomere	Interaction
Mre11/Rad50/Nbs1	Recombinational repair, DNA damage sensor	t-loop formation	Associated with Sheltrin protein
ERCC1/XPF	NER, crosslink repair 3'flap endonuclease	Deficiency lead to formation of TDMs, implicated in overhang processing after TRF2 loss	Associated with Sheltrin protein
WRN helicase	Branch migration G4 DNA resolution	Deficiency leads to loss lagging strand	TRF2
BLM helicase	Branch migration, crossover repression	t-loop formation/resolution	TRF2
DNA -PK	NHEJ	deficiency lead to mild fusion phenotype	Associated with Sheltrin

1.1 Telomere and End replication problem

In most proliferating cells telomere length (TL) is dynamic, and with each cell division the lengths of telomeres in human somatic cells decrease gradually by 20 to 200 base pairs. Partially, this loss of base pairs is a consequence of so-called "end-replication problem". DNA polymerases are not able to replicate chromosomes completely, since one RNA primer remains on each daughter DNA strand. The last primers are removed by a 5'-3' exonuclease, but DNA polymerases cannot fill the gaps because there is no 3'-OH available to which a nucleotide can be added. As a consequence, the replication machinery has to leave a small region at the end (a piece of the telomere) uncopied. The end-replication problem leads to chromosome shortening with each round of cell division. Eventually, this will lead to elimination of the telomeres and, as a consequence, apoptosis or an irreversible growth arrest of the cells can takes place. These senescent cells are irreversibly arrested in G1 phase of the cell cycle. For this reason telomeres may serve as a biological clock of the cell. Both the end replication problem and the existence of an exonuclease only partially account for the loss of telomeres seen in cells, and therefore it was suggested that other mechanisms may be involved in accelerated telomere shortening.

1.2 Importance of Telomere length

Telomeres have been postulated to play two, apparently opposing, roles during tumor progression (Hahn., 2003). Telomere erosion in telomerase-negative cells can act as a cell division 'counter' that when coupled with speciesspecific telomere lengths, imposes a potent tumorsuppressivemechanism in long lived species. This has to be overcome to allow the cell division necessary to progress into malignancy. This is paralleled by the ability of shortdysfunctional telomeres to trigger genomic instability viachromosome fusion events. Therefore, dysfunctional telomereshave the capacity to promote the appropriate oncogenic chromosomal rearrangements that facilitate tumor progression (O'Hagan *et al*., 2002). In order to maintain tissue homeostasis in humans andother long lived species, cell division is required throughoutlife. In the absence of telomerase, this would result in anage-related loss of telomeric sequences. The corollary oftelomere-based tumor suppression in early life may

be anage related accumulation of senescent cells. In addition tothe obvious cessation of cell division, senescent cells havethe potential to disrupt tissue function in other ways.Senescent cells display altered gene expression profiles with a shift to a more catabolic and pro-inflammatory phenotype. The concept that telomere erosion may play a role in theageing phenotype is complicated by the fact that the stemcells of highly proliferative tissues express telomerase, assuch these cells have the potential to maintain telomerelength. However, despite of this telomerase activity, it is clear that many tissues appear to lose telomeric sequences as a function of age. Thus, telomere dynamics in the human body are likely to be complex andconfounded by differing replicative histories of the various cell lineages and by the presence or absence of telomeraseactivity. Nevertheless, because of the interest in telomerebiology from the standpoints of cancer, ageing and oxidative stress, a considerable amount of effort has been devoted to the analysis of telomere erosion in humans as a function of age.

1.3Technique to measure Telomere Length(TL)

Since the assessment of TL is important in understanding the relationship between oxidative stress, cancer, aging, stemness of a stem cell and TL regulation so different methods are used to analyze the Telomere length.

In recent reviews by Nakagawa *et al*., 2004 and by Lin *et al.*, *2004* the most important techniques to measure TL are discussed. The most widely used method is the telomere restriction fragment (TRF) assay with Southern blot analysis. In this method, average lengths of telomeres are measured by creating TRFs using specific restriction enzymes and hybridization with a radioactive or fluorescent probe. The main disadvantage of this assay is the subjectivity of the results, since TRF lengths can differ as much as 5% depending on which restriction enzyme is used. This means that sub-telomeric regions might be included in the determination of the TL. Other disadvantages of this method are the requirement of large amounts of DNA and the fact that the procedure is relatively time consuming.

A second important method used to measure TL is the fluorescence in situ hybridization (FISH), which can be subdivided in flow FISH and quantitative FISH. It allows the direct labeling of oligonucleotide probes to telomere sequences at the individual cell level. In this

method, fluorescent dyes are used to visualize telomeres with fluorescence microscopy or with a digital imaging system. Flow FISH uses fluorescence activated cell sorting (FACS) to sort different types of cells, while quantitative FISH provides measurements of telomere lengths of individual chromosomes. In contrast to quantitative FISH, flow FISH allows the analysis of telomeres in cycling and non-cycling cells instead of metaphase preparation and the procedure can be carried out in one day. A disadvantage of this method is that it uses whole cells, which limits its suitability in studies where only DNA is available.

A recently developed method is the single TL analysis STELA (Duncan M .Baird., 2005), which is a PCR based method that measures the lengths of telomeres at the individual chromosome level. First, a linker which consists of 7 repeats of TTAGGG followed by a 20 nucleotides containing non-complementary tail, is annealed to the telomeric G-rich strand 3'-overhang. In the second step the linker is ligated to the 5'end of the complementary strand of the chromosome. PCR can then be performed using a primer that is identical to this tail, together with a chromosome-specific upstream primer located in the sub-telomeric region, which can be made allele-specific by known polymorphisms in this region. The STELA was first developed for analysis of the human sex chromosome, the X and Y chromosomes, and it is suggested that this method can also be applied to other chromosomes.

Another PCR-based method is the quantitative polymerase chain reaction described by Cawthon., 2002. It avoids many of the problems encountered by TRF analysis by using primers that are specifically designed to amplify telomerichexamer repeats without generating primer dimer-derived products. In these primer template hybridizations, every sixth base is mismatched, whereas the last five bases at the 3'-end of the primers are perfectly matched to complementary bases in the template. This method measures relative telomere lengths by determining the factor by which a sample DNA differs from an arbitrary reference DNA in its ratio of telomere repeat copy number to single gene copy number. A disadvantage of this technique is that it does not provide absolute telomere lengths.

As described above, there are different methods to determine TL and it depends on the type and aim of the study which method should be used. Each method has its specific advantages and disadvantages that make it more or less useful for a specific purpose. But in this study we have tried a new method to determine the conserved region of telomereby using PCR.

2.Review of Literature:

The three main causes of telomere shortening are the incomplete replication of linear DNA the processing of the 5′ strand by an unknown exonuclease and unrepaired oxidative damage. The story of telomere begins with B. McClintok and H. Muller and whole history summarized in **Fig 3**

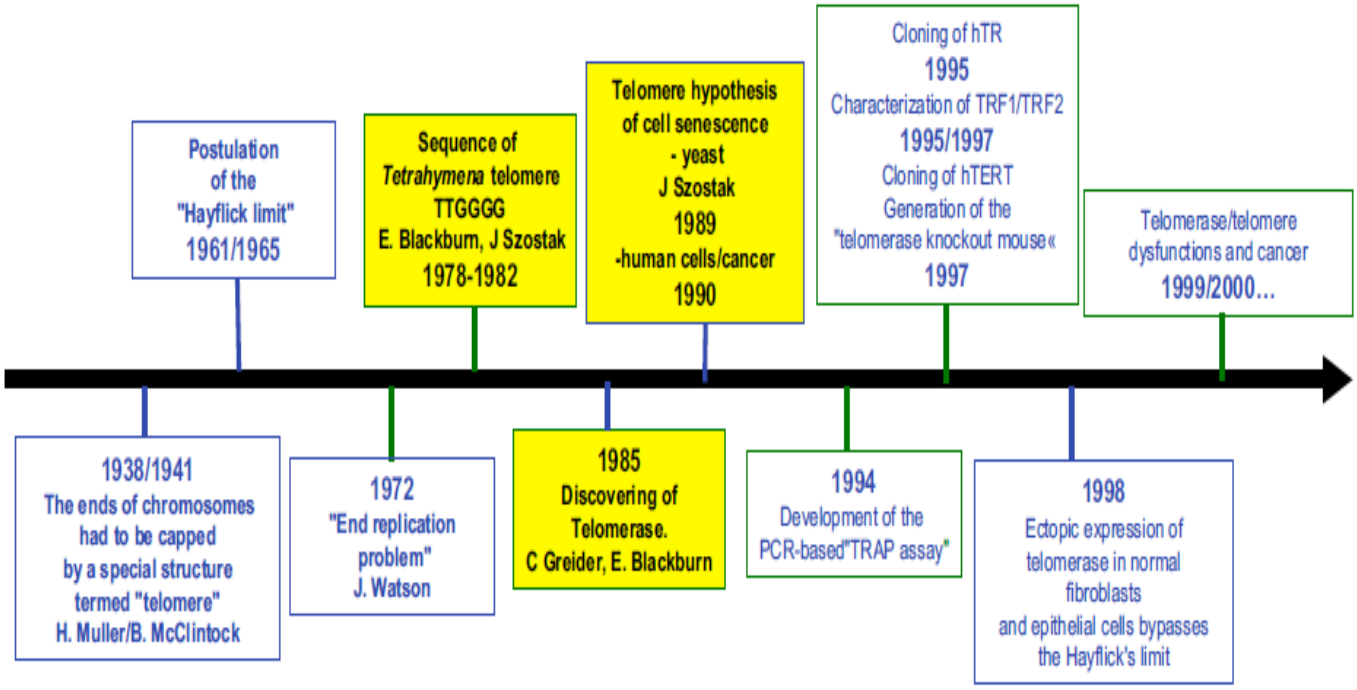


Fig 3 History of Telomere and Telomerase (Gilson. E, Benderdijan.S.,2010)

2.1 Telomere and DNA damage checkpoints

In all eukaryotic organisms, interruptions in duplex DNA molecules elicit a DNA damage response, which includes activation of DNA repair machineries and surveillance mechanisms, known as DNA damage checkpoints. Telomeres and double-strand breaks (DSBs) share the common feature of being physical ends of chromosomes. However, unlike DSBs, telomeres do not activate the DNA damage checkpoints and are usually protected

from end-to-end fusions and other processing events that normally promote repair of DNA breaks. This indicates that they are shielded from being recognized and processed as DSBs. On the other hand, chromosome ends resemble damaged DNA, as several factors required for DNA repair and checkpoint networks play important roles in telomere length maintenance. The sole purpose of these check points are to arrest cell cycle in response to DNA damage thereby coordinating cell cycle progression with DNA repair capacity (Weinert. T.A *et al*., 1994) and regulate transcription of DNA damage response genes, as well as activation and recruitment to damaged sites of various repair/recombination proteins that help cells to survive genotoxic stress (Viscardi.V *et al*., 2004) Hence ,these check points are considered as one of the main line of defense against genomic instability and failures in these checkpoints can lead to carcinogenesis as shown in **Fig 4.**

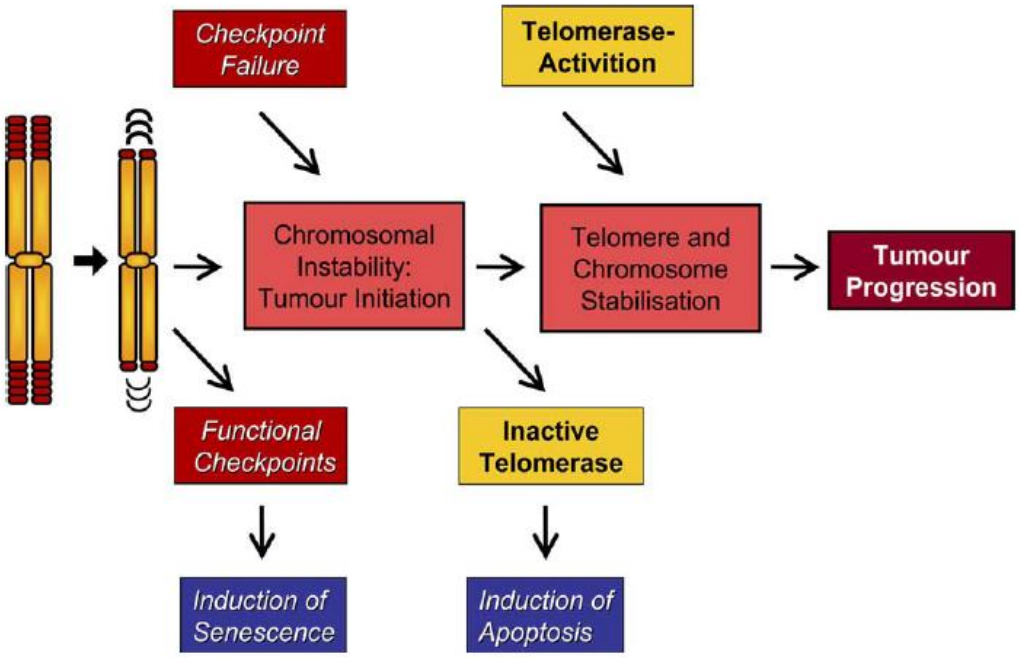


Fig 4: Showing the mechanism of checkpoint failure which co-operates with telomere dysfunction to induce chromosomal instability and cancer initiation. Initiated cancer cells have to stabilize telomeres and ongoing instability in order to survive and progress. Most human cancers achieve telomere stabilization by activating telomerase.

Besides regulating DNA repair and DNA damage checkpoint at telomeres, GT-rich specific telomere proteins play important roles in regulation of telomerase. The ds DNA telomere binding proteins are important for the negative regulation of telomerase, since disruption ofTRF1, Taz1 or Rap1 function causes massive telomerase-dependent telomere elongation (Smogorzewska A, De Lange T., 2004). G-tail binding proteins show dual roles in both positive and negative regulation oftelomerase action at telomeres. The negative regulation of telomerase by Pot1 in bothmammalian and fission yeast cells requires the function of several shelterin subunits that connect Pot1 to ds DNA telomere-binding proteins. In mammalian cells, TPP1 andTIN2 connect POT1 to TRF1 and TRF2 to inhibit telomere elongation (Loayza D, De Lange., 2003), but the POT1-TPP1 sub-complex has also been implicated in telomerase recruitment and enhancement of telomerase processivity.

2.2 Telomere as a non-coding sequence

Many non-coding DNA sequences have important biological functions as indicated by comparative genomics studies that report some regions of noncoding DNA that are highly conserved, sometimes on time-scales representing hundreds of millions of years, implying that these non-coding regions are under strong evolutionary pressure and positive selection (Ludwig M.Z., 2002). For example, in the genomes of humans and mice, which diverged from a common ancestor 65–75 million years ago, protein-coding DNA sequences account for only about 20% of conserved DNA, with the remaining majority of conserved DNA is represented in noncoding regions.Linkage mapping often identifies chromosomal regions associated with a disease with no evidence of functional coding variants of genes within the region, suggesting that disease-causing genetic variants lie in the noncoding DNA.According to a comparative study of over 300 prokaryotic and over 30 eukaryotic genomes, eukaryotes appear to require a minimum amount of non-coding DNA (Ahnert. S.E *et al*.,2008) this minimum amount can be predicted using a growth model for regulatory genetic networks, implying that it is required for regulatory purposes. In humans the predicted minimum is about 5% of the total genome.

Telomere are generally considered as a part of constitutive heterochromatin and have a unusual chromatin structure with extensive arraysof tightly packed nucleosomes that had a

shorter repeat size than bulk nucleosomes (Tommerup. H *et al*., 1994) and the N terminal tail of histone are subjected to post translational modifications such as methylation , acetylation , phosphorylation etc which can generate plethora of cellular responses (Jenuwein.T and Allis,C.D., 2001 and Lachner, M, O'Carroll. D. *et al*., 2001). This type of epigenetic modification at the chromatin region of the telomere or any other epigenetic error such as defect in Heterochromatin protein 1 (HP-1) family of proteins that mediate heterochromatin formation might disrupt the normal architecture of chromatin which may affect the telomere length regulation and telomere function (Maria A.Blasco., 2004). Similar to introns telomere are also non coding sequence at the end of the chromosome, but introns are spliced out by 2 trans-esterification reaction where the 5' splice site and 3' splice site act as a junction for the trans-esterification reaction (Crick.F.,1979) similarly there must be some junction in telomere still not reported which should exist and responsible for the telomere shortening and cellular senescence. Since replicative senescent cells express high levels of the cyclin-dependent kinase inhibitor p21(Taylor L.M, *et al*.,2004)and cyclin-dependent kinases are essential for thephosphorylation of retinoblastoma (pRb) protein, increased activation of p21 can prevent its phosphorylation. Hypophosphorylated pRb actively represses several genes required for S phase by sequestering the E2F transcription factors, thus inhibiting cellular proliferation (Haddad M.M, *et al*.,1999)

2.3 Telomerase and Cancer

A fundamental difference in behavior between normal versus tumor cell in culture is that normal cell divide for a limited number of times and exhibit cellular senescence whereas tumor cell have the ability to proliferate indefinitely and are immortal. As the cell divide telomere shortens due to end replication problem. Thus when telomere becomes sufficiently short, cells enter a irreversible growth arrest called cellular senescence. In most cases cells become senescence before they can accumulate enough mutation to become cancerous thus the growth arrest induced by short telomere may be a potent anti-cancermechanism.The most prominent hypothesis that maintenance of telomere stability is required for long term proliferation of tumor (Shay. J.W and Wright.W.E.,1996) Thus escape from cellular senescence and becoming immortal by activating telomere or alternative mechanism to

maintain telomere constitute an additional step in oncogenesis thus most tumor require for ongoing proliferation.

Telomerase is a ribo-nucleoprotein that maintains the length chromosome ends by synthesizing the telomere ends. Telomerase is a holoenzyme consisting of two components, telomerase RNA(TR) that serves as a template for the addition of deoxyribonucleoside at the end of the chromosome and a telomerase reverse transcriptase protein (TERT) that catalyzes the enzymatic reaction. Telomerase is expressed in somatic cells, germcells, embryonic stem cells and in vast majority of cancer cells, but the level of expression varies, from cell to cell. In normal somatic cells the level of expression is hardly detectable (Shay and Bacchetti., 1997) whereas in cancer cells there expression is so high that they can be used as a diagnostic marker for most of the cancer. Since the length of telomere in cancer cells are typically shorter than neighboring normal cells (Mokbel, K.,2000) and stem cells and the expression level is high in cancer cells they can be efficiently used as an anticancer target.

2.4 Telomere and Oxidative stress

Oxidative stress causes extensive damage to DNA, proteins, and lipids. This damage is a major contributor to aging and may afflict widely different degenerative diseases, such as cardiovascular diseases etc. The contribution to telomere loss by oxidative DNA damage was, in many cases, reported to overrate the contribution by the end-replication problem (Zglinicki.V T., 2002). Due to their high content of guanines, telomeres were demonstrated highly sensitive to damage by oxidative stress. Senescent cells were found to contain 30% more oxidatively modified guanines in their DNA and four times as many free 8-oxodG bases (Zglinicki.V *et al*., 2001). Furthermore, reactive oxygen species (ROS), especially hydroxyl radicals, produce single strand breaks, either directly or as an intermediate step in the repair of oxidative base modifications. In contrast to the majority of genomic DNA, telomeric DNA was reported to be deficient in the repair of single strand breaks (Petersen S *et al*.,1998). Consequently, telomeres appear to be especially sensitive for the accumulation of ROS-induced 8-oxodG DNA strand breaks. In addition to the direct effect of ROS on telomeric DNA, repair of oxidative lesions is less efficient in telomeres than in the rest of the genome. An increase in pro-inflammatory cytokines is often associated with oxidative stress, and part of the inflammatory condition (Floyd RA *et al*., 1999).Since telomerase activity was

found to be negatively correlated with the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α). TNF-α activates nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) transcription factors, and enhances the expression of pro-inflammatory genes, leading to an inflammatory response. Since oxidative modification and shortening of telomeres is induced by ROS, it is expected that antioxidants may be preventive (Furumoto K *et al*., 1998).

2.5. Telomere and Ageing

The adaptive and innate immune response undergoes dramatic changes with age. For the immune system to be effective the utmost important phenomena is self-renewal which is highly dependent on telomere maintenance. The cells and organs are the main player of the immune system and all the cells arise from the hematopoietic stem cells .In hematopoietic system stem cells are the telomerase expressing cells (Hiyama *et al.,* 1995, Morrission *et al*., 1996). Stem cells isolated from adult bone marrow showed shorter telomere than a similar subset isolated from fetal liver or cord blood cells suggesting that progressive shortening of telomere length with age occurs in Hsc cells (Vaziri *et al*., 1993). Hence age related loss in telomeric DNA suggest that telomerase activity in insufficient to completely prevent the loss of telomeric DNA. So it might be assumed that telomere shortening have a negative impact on renewal potential of hematopoietic lineage but more research is required to proof this assumption.

However the immune response is most strong during the adulthood but during the childhood and as the age progresses the immune response becomes weaker which night be due to decrease in humoral and cell mediated immune response. The humoral and cell mediated immune responses are dependent on B cell and T cells of the body and in a study of telomere shortening in T cells among children and adult it was reported that telomere shortening occur at a rapid rate of (>1 kb per year) in peripheral blood cells of the young children then at reduced rate between the age of 4 and 20 years and at constant and intermediate rate through the remainder of the adult life(Frenck *et al*.,1998). However, recent research showed that there is a decrease in the ratio of naïve and memory T cell with aging and telomere restriction fragment length of naïve T cell was greater than memory cell hence telomere shortening occur during differentiation of T cell (Franceschi and Cossarizza.,1995) . But in the case of

naïve and memory B cell, it does not differ in telomere length and they lose telomere at similar rate with age (Son Huiping *et al*., 2002). So this report might suggest a possible reason that why the immune response is constant at the middle of the age. And since, thymus is the site of T-cell maturation and development and thymic function is known to decline with the age, thymus reaches the maximum size at puberty and then atrophies with significant decrease in both cortex and medullary cells and increase in the total fat content of the organ. Weng et al., 1996, showed that thymus have a high degree of telomerase activity. Telomerase is expressed at a high level in thymocyte sub-populations, at an intermediate level in tonsil T lymphocyte, and at a low to undetectable level peripheral T lymphocytes (Weng *et al*., 1996).

Studies in patient with rheumatoid arthritis (Koetz *et al*.,2000 and Schonland *et al*.,2003),scleroderma (Artlett *et al*.,1996), Wegener's granulomatosus (Vogt *et al*., 2003), insulin-dependent diabetes mellitus (Jeanclos *et al*.,1998), psoriasis (Weng *et al*., 2000), and atopic dermatitis (Weng *et al*.,2000) have shown telomeric erosion in peripheral blood mononuclear cells from patients compared to controls. These reports shoes that there are multiple factors responsible for telomere attrition.

3. Objective:

Telomere is the regions of repetitive DNA at the end of the chromosomes. As the eukaryotic cells divide, the protective ends of the linear chromosomes, the telomeres, gradually shorten with each cell division. When a critical telomere length is reached, the cells are signaled into senescence, an irreversible state of quiescence. Thus, telomere length has emerged as replicative clock within each population of cells.Since telomere are also non coding sequence of DNA, as that of introns, there might bespecific sites as of 5' splice site and 3'splice site, which might be the conserved regions in telomere and can possibility generate a signal for apoptosis after a certain time of cell division.

Since TL in the somatic cells determines the number of cell divisions, TL has been proposed as a marker for biological ageing and cancer. Under conditions of chronic oxidative stress, the rate of telomere shortening is increased. Since telomeres consist of the repetitive sequence TTAGGG, and have a high GC content, they are very vulnerable to base oxidation. Additionally, DNA strand breaks are less efficiently repaired at the telomeres when compared to the rest of our genome. This means that reactive oxygen species cause more damage to the telomeres and this damage is less well repaired. Genetics play an important role in determining TL, but environmental and lifestyle factors such as smoking, alcohol consumption, physical activity, body composition and coffee and tea consumption can affect oxidative stress levels, which may contribute significantly to accelerated telomere shortening. Since, buccal cells are under direct contact with smoke and various toxic chemical substances in people who are acutely habituated with taking tobacco and cigarettes, we are interested to know if prolonged period of intake of these substances have an effect in teleomere shortening which may lead to development of other complicacies like development of cancer and directly help in ageing that can be compared with teleomere shortening of normal individuals with different age groups.

The objectives of this thesis are

- To find the conserved sequence in telomere of human chromosome.
- To measure the length of the conserved region with the help of Polymerase chain reaction.
- To find the impact of smoking on the length of the conserved region of telomere inbuccal cells of people having a record of prolonged smoking and to that of normal individuals of different age groups.

3.1 Plan of work

Human telomere sequence were obtained from gene bank

↓

Alignment of sequences were done to find the conserved region

↓

To design a primer for conserved region

↓

Collection of buccal cell samples from different age group of smoker and non-smoker.

↓

Isolation of DNA from buccal cells

↓

PCR amplification of the conserved region.

↓

Amplicon size was analyzed using Agarose Gel Electrophoresis

↓

Comparison and result analysis

4. Materials:

- NCBI Gen Bank (http://www.ncbi.nlm.nih.gov/genbank/)
- Clustal X 2.0.11
- BioEdit 7.0.9
- TreeGraph 2.0.45
- Cotton/Dracon swab
- DNA isolation kit (Qiagen)
- Primer (Sigma)

  Forward –CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT

  Reverse- GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT
- Taq Polymerase(Sigma)
- PCR (Kit)
- Agarose (Himedia)
- Ethidium Bromide
- Mili Q water
- DNA ladder 100bp,1 kb(Sigma)
- 6X DNA dye (Sigma)

Equipments

- Autoclave
- Laminar Air Flow
- Centrifuge (Remi)
- Thermocycler(BioRad)
- Electrophoretic Gel Apparatus(BioRad)
- Power pack (BioRad)
- Gel documentation(BioRad)

5. Methods:

Obtaining Human telomeric sequence and its Alignment

Human Telomeric sequence of chromosome 4q (U74496), 7q (AF027390) ,6p (101185) and 3p (5658205) were obtained from NCBI Gen Bank (**http://www.ncbi.nlm.nih.gov/genbank/**) and RNA and Protein sequence of Telomerase were obtained from Telomerase database (**http://telomerase.asu.edu/**) and by using the software **Clustal X 2.0.11**,and **BioEdit v7.0.9** multiple sequence alignment was done. The Phylogenetic tree was plotted using the software TreeGraph 2.0.45

Multiple sequence alignment was performed using software Clustal X 2.0.11 .Alignment parameter were as follows

A- RESET NEW GAPS BEFORE ALIGNMENT

B -MULTIPLE ALIGNMENT PARAMETERS- include two gap penalties and the scores for various identical/ non-indentical residues.

C-Matrice- BLOSUM (Henikoff) 80

D-Output-The output file which was obtained was in CLUTAL format

Primer Designing

The forward and reverse telomere specific primer was designed using a free online software Primer-BLAST (**www.ncbi.nlm.nih.gov/tools/primer-blast**).Primer was ordered of the following sequence from Sigma.

Forward primer- CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT

Reverse primer- GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT

Sample collection

Buccal cells were obtained with the help of sterile cotton swab/Scrapper or dracon and were washed in PBS buffer after collection,the donor was advised vigorously rinse his/her mouth with water for about 15 sec to remove food particles and not to have any food,prior half an hour before donation of the sample.

Isolation of DNA: DNA was isolated using DNA isolation kit from Qiagen and was isolated using the Manufacturer's protocol.

- 20µl of proteinase K was added in the sample
- 200 µl of Buffer AL was added to the sample and was mixed by pulse-vortexing for 15s.In order to ensure efficient lysis.
- Incubate at 56°C for 10 min to maximize the DNA yield.
- (96–100%) of 200 µl ethanol was added in the sample, and mixed again by pulse-vortexing for 15 s.
- The mixture was transferred to QI Aamp Mini spin column (in a 2 ml collection tube) without wetting the rim and centrifuged at 6000 x g (8000 rpm) for 1 min and was placed in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
- AW1 and AW2 buffer was added to the sample and was centrifuged at (8000 rpm) for 5 min and then was placed in a new collection tube
- 50 µl Buffer AE or distilled water was added and incubated at room temperature (15–25°C) for 5 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min to obtain the good quality of DNA

Estimation and quantification of DNA

DNA was quantified using UV Spectrophotometer since purines and pyrmidines in nucleic acid show absorption maxima around 260nm if the DNA sample is pure without significant contamination from proteins or organic solvents. The ratio of OD_{260}/OD_{280} was determined to assess the purity of the sample. Sample was diluted 100 times in autoclave Mili Q water and concentration was calculated using the following formulae

$$\text{DNA concentration } (\mu g/ml) = \frac{OD_{260} \times 100 \text{ (dilution factor)} \times 50\ \mu g/ml}{1000}$$

1 A260 of ds DNA = 50 μg/ml

Isolated DNA was analyzed in 0.8% Agarose gel Electrophoresis, Ethidium bromide was used as fluorescent dye, which intercalates between the stacked bases. 2μl of 6X gel loading dye was added to 8μl of each DNA sample before loading the wells of the gel and was run in 70 volt until the dye has migrated two –third of the gel.

PCR analysis-

Conserved region in Telomere sequences were amplified using the following conditions

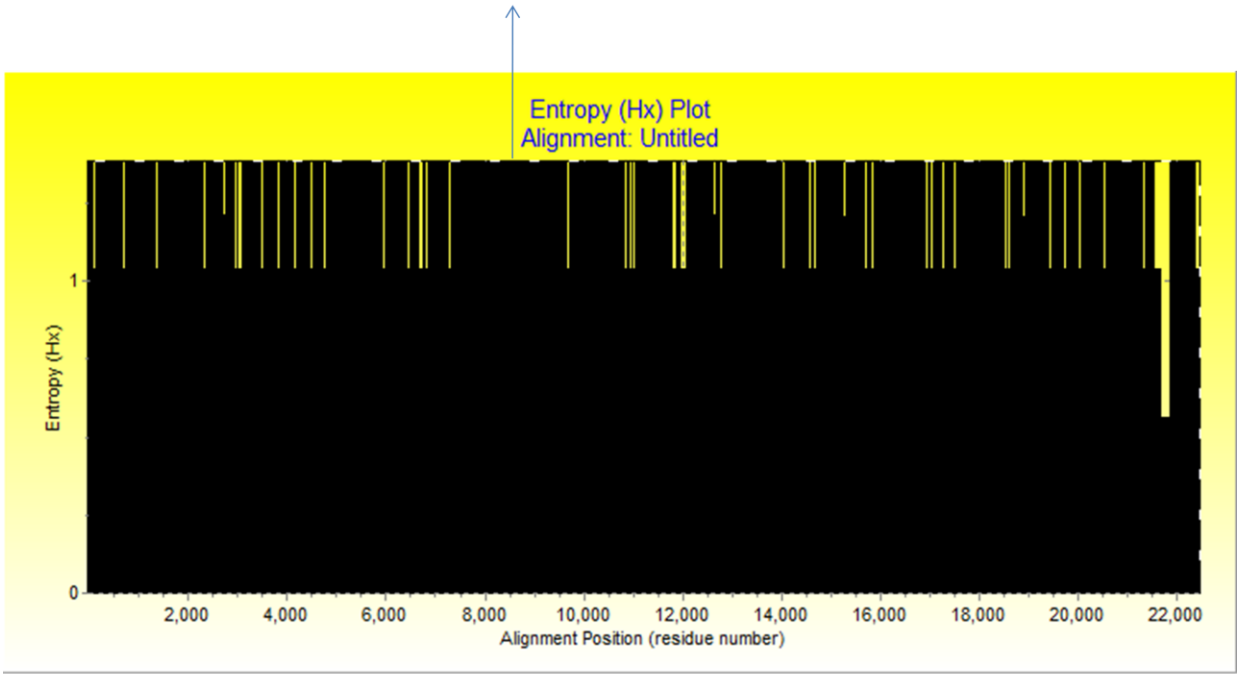
- 95°C for 3 min - For activation of Taq Polymerase and then for 30 cycles each at
- 94°C for 45sec - Denaturation of DNA strand
- 50°C for 45 sec -Primer annealing
- 72°C for 1 min -Extension
- 72°C for 1 min -Final extension

Amplicon size analysis- PCR product was run on 1.2 % agarose gel with 100bp ladder and 1000 bp ladder to analyze the size of the conserved region of the agarose gel

6. Results and Discussion:

Screening for the conserved region of telomere- Telomere sequence of 4q, 3p, 7q, 6p were obtained from GENE bank and multiple sequence alignment were performed. The Entropy Vs alignment graph was plotted using BioEdit which showed in **Fig 5**, the existence of a highly conserved region of telomere in this chromosome sequence.

Highly conserved region

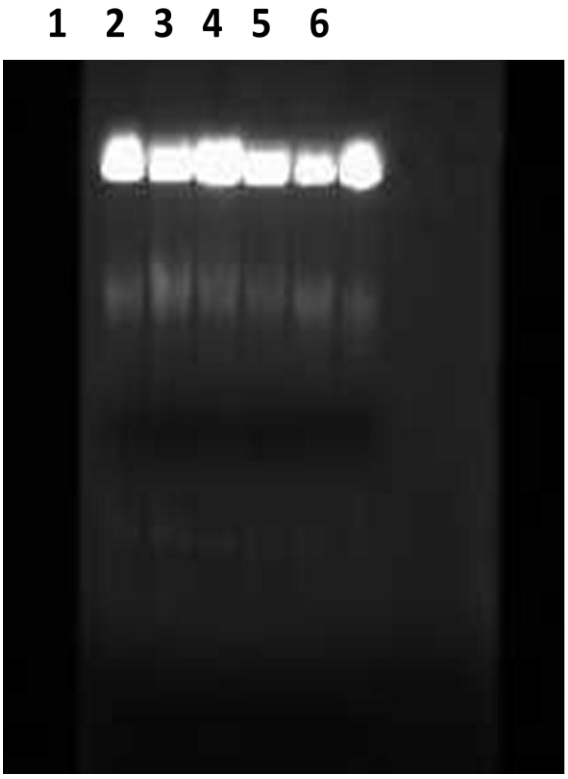


Entropy vs Alignment graph

Fig 5 Entropy Vs Alignment graph which showed a highly conserved region in the telomere.

The region of the plot which showed minimum Entropy has the highest conserved sequence and the primer was designed according to the sequence.

Isolation of DNA- DNA isolated from the buccal cells from different donors of same and different age group usingDNA isolation kit and were analyzed in 0.8% Agarose gel electrophoresis**, Fig 6**



Lane1-22 years of Age

Lane2--25 years of Age

Lane3--27 years of Age

Lane4--28years of Age

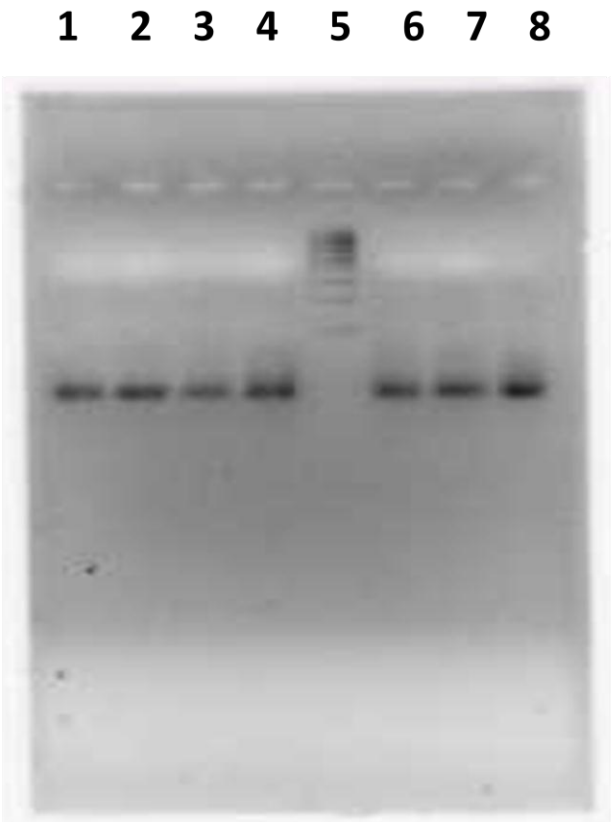
Lane5--29 years of Age

Lane6--30 years of Age

Fig 6-Shows the isolated DNA from Buccal cells

Genomic DNA of buccal cells were isolated and the gel shows isolated DNA are of good quality

PCR analysis- Buccal cell DNA was isolated from smoker andnon-smoker of same and different age group, and PCR was performed to find whether the smoke and Reactive oxygen species (ROS) have any effect on the conserved region of the telomere of buccal cells with age since these cells are in direct threat among the smokers.



Lane1- 22 years of age

Lane 2- 22 years of age

Lane3-22 years of age

Lane4- 22 years of age

Lane 5- 1 Kb marker

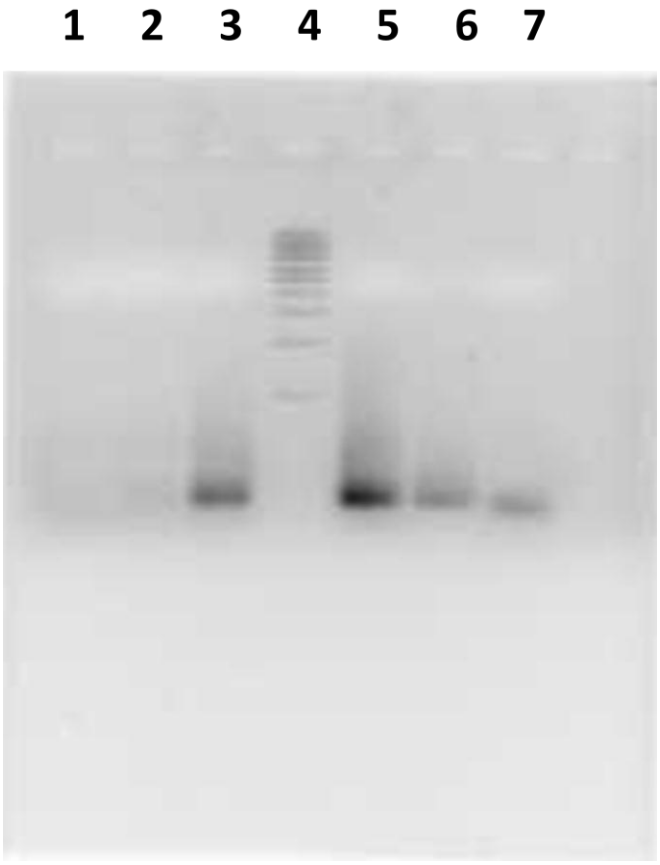
Lane6-22 years of age

Lane7-22 years of age

Lane8- 23 years of age

Fig 7- Shows the conserved region of the telomere does not change in the same age of the individual

The PCR result shows that, the length of conservedregion in telomere which has been identified by different bands does not change within the same age group, and remains constant in normal individuals.



Lane 1- Negative control (Without Template)

Lane 2- Negative control (Without Primer)

Lane 3- 22 Years of age

Lane 4- 1 Kb marker

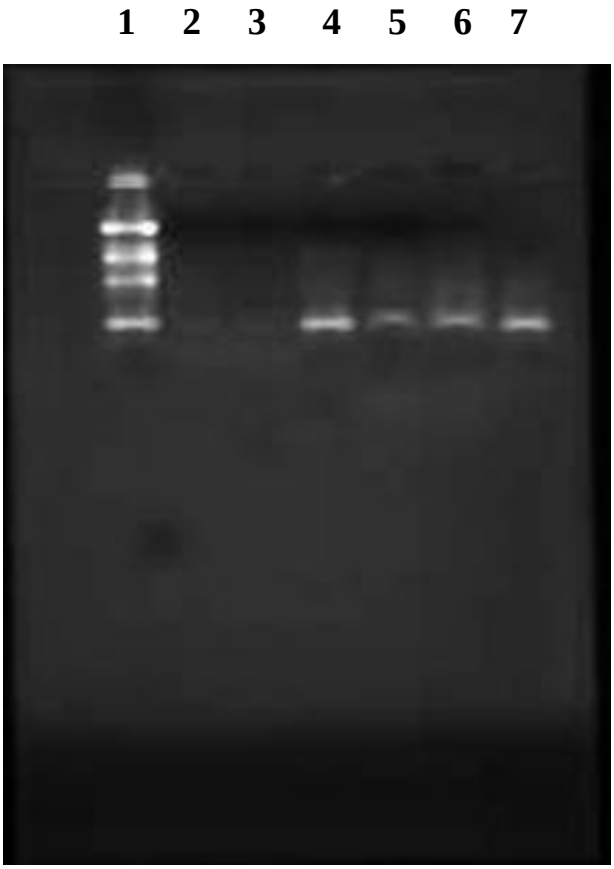
Lane 5- 27 Years of age

Lane 6- 25 Years of age

Lane 7- 40 Years of age

Fig 8 shows the length of the conserved region changes with the higher age group

The PCR result shows that, the length of conserved region in telomere which has been identified by different bands changes with higher age group in normal individual. As the negative control does not shows any band, we confirm that the primer is amplifying the right conserved region.



Lane 1- 100 bp marker

Lane 2- Negative control (Without Template)

Lane 3-Negative control (Without Primer)

Lane 4- 30 Years of age , (Non Smoker)

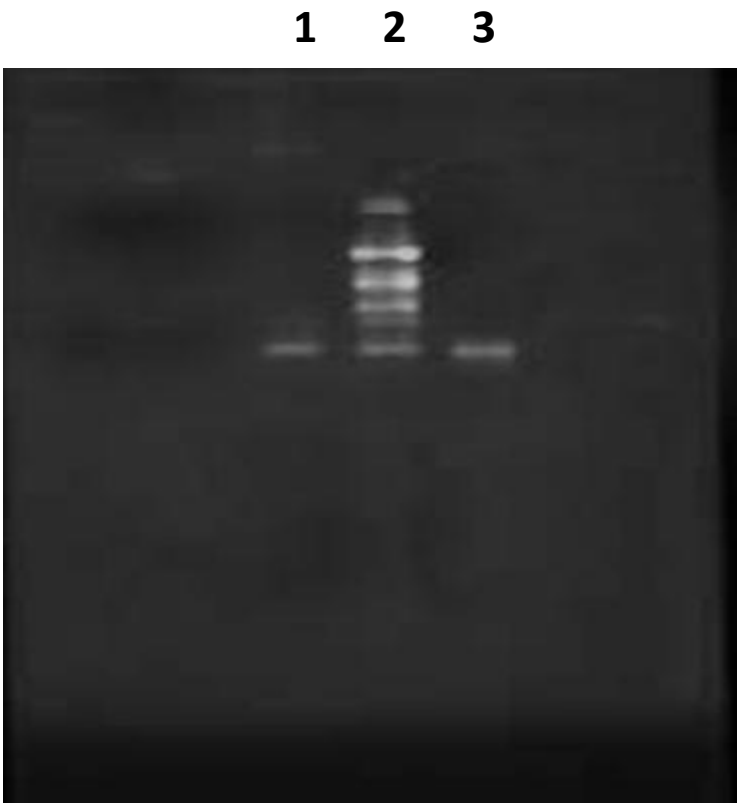
Lane 5- 28 Years of age, (Smoking history of 5 years)

Lane 6- 29 Years of age (Smoking history of 7 years)

Lane 7- 30 Years of age (Smoking history of 9 years)

Fig 9shows that the conserved region does not change within the same age group between smoker and non-smokers

The PCR result shows that, thecigarette smoke has no effect on shortening of the conserved region of the telomere, since the band patterns are same within the same age group of acute smokers and non-smokers.



Lane1- 30 Years of age (Non smoker)

Lane 2- 100 bp marker

Lane 3- 30 Years of age with smoking history of 9 years)

Fig 10 Shows that the conserved region remains the same within the normal and a smoker of same age when extension

The PCR result shows that, the cigarette smoke have no effect on shortening of the conserved region of the telomere, since the band patterns are same within the same age group of acute smokers and nonsmokers even when the extension time of the PCR was increased up to 2 mins. By increasing the extension time, it confirms that our primer was amplifying the whole of the conserved region which could be nearly of 100 bp sequence.

7. Conclusions:

According to free radical theory of ageing intercellular ROS can trigger stress induce premature senescence or apoptosis depending on the DNA damage but from these data we can say that cigarette smoking have no effect on the conserved region of the telomere which might be due to following reasons.

- The conserved region of the telomere might be protected by some proteins other than the Sheltrin/Non sheltrin protein due to which the length of the conserved part is not shortening but in comparatively higher age group it is shortened hence these region might act as a regulator in cell cycle .
- The buccal cells which were isolated might be metabolically very active and are in quiescentstage so thatcigarette smoke has no effect on the telomeric DNA and hence the length of the conserved region is maintained.
- Smoke induce oxidative stress might induce some mutation in this conserved region of the telomere which cannot be detected by PCR, Agarose Gel Electrophoresis analysis. Sequencing of this PCR product might reveal the secret of telomere attrition.
- According to Population mixture model (Itzkovitz *et al*.,2008) that translate telomere length changes, divides stem cells into 2 population.

  Cells with longer telomere- known as repopulating pool.

  Cells with shorter telomere- known as derived pool comprising fully differentiated cell

  So there might be two scenarios:

1- Depletion of derived pool under stress might be compensated by an increased repopulation rate so repopulating cells have longer telomere length.Hence, overall effect on shortening of the conserved teleomere region/length is null.

2- Compensation of the depleted derived pool by increasing the rate of cell division, thus predicting a certain degree of telomere attrition.

But the results from the current study suggest the former possibility.

8. Future Prospective:

- Cancer cell have a shorter telomere length as compared to normal somatic cells so a comparison of oral cancer cell, non-smoker and smoker might give us the true story behind the secret of telomere attrition
- Further sequence analysis of PCR products to identify other sub domain in the telomeric region and possible interaction between them.
- Analysis of the conserved region to figure out whether there is any protein which is interacting with the conserved sequence which is yet to be identified
- To determine whether this conserved region plays any role in apoptosis or in other cell cycle regulatory mechanism.

References:

1. Allsopp, R.C, Vaziri, H., Patterson, C., Goldstein, S., Yougli, E.V. and Futcher, A.B., 1992. Telomere length predicts replicative capacity of human fibroblasts. Proc. Natl. Acad. Sci. USA 89:101148**.**

2. Britten, R.I. and Kohne, F.E., 1968. Repeated sequences in DNA.

3. Grifith, J.D., Comeau, L., Rosenield, S., Stansel, R.M., Bianchi, A., Moss, H. and de Lange, T., 1999. Mammalian telomeres end in a large duplex loop. Cell, 97:503514**.**

4. Weinert, T.A., Kiser, G.L., Hartwell, L.H., 1994 .Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair, Genes Dev. 8 ;652–665.

5. Nyberg, K.A., Michelson, R.J., Putnam, C.W., Weinert, T.A., 2002 Toward maintaining the genome: DNA damage and replication checkpoints, Annu. Rev. Genet. 36; 617–656.

6. Viscardi, V., Clerici, M Telomeres and DNA damage checkpoints, Hugo Cartagena-Lirola, Maria PiaLonghese,Department of Biotechnology, Università di Milano-Bicocca,Piazza dellaScienza 2, 20126 Milan, Italy.

7. Ludwig, M.Z., 2002. "Functional evolution of noncoding DNA". *Curr. Opin. Genet.Dev.* 12 (6): 634–9.

8. Ahnert, S.E. Fink, T.M.A. and Zinovyev, A., 2008.. "How much non-coding DNA do eukaryotes require?" *J.Theor. Biol.* 252 (4): 587–592.

9. Shay, J.W. and Wright, W.E., 1996. Telomerase activity in human cancer Curr. Opin .Oncology 8, 66-71.

10. Haddad, M.M., Xu. W., Schwahn, D.J., Liao, F., Medrano, E.E., 1999. Activation of a cAMP pathway and induction of melanogenesis correlate with association of p16(INK4) and p27(KIP1) to CDKs, loss of E2F-binding activity, and premature senescence of human melanocytes. Exp Cell Res.,253(2):561–72.

11. Hodes, R.J., 1999. Telomere length, aging, and somatic cell turnover. J. Exp. Med., 153/156**.**

12. Hiyama, K., Hirai, Y., Kyoizumi, S., Akiyama, M., Hiyama, E., Piatyszek, M.A., Shay, J.W., 1995. Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *J.Immunol.* 155: 3711–3715.

13. Morrison, S.J., Prowse, K.R., Ho, P., Weismann, I.L., 1996. Telomerase activity in hematopoietic cells is associated with self-renewal potential. *Immunity* 5: 207–216.

14. Corey, D.R., 2002. Telomerase inhibition, oligonucleotides, and clinical trials. Oncogene 21, 631–637**.**

15. Shay,J.W.,andBacchetti, S., 1997. A survey of telomerase activity in human cancer. Eur. J. Cancer., 3 :787–791.

16. Son, N.H., Joyce, B., Hieatt, A., Francis, J.,Chrest, J. Y. and Weng, N., 2003. Stable telomere length and telomerase expression from naıve to memory B-lymphocyte differentiation .Mech. and Ag. of Dev. 427-432.

17. Frenck, R.W., Blackburn, E.H. and Shannon, K.M., 1998. The rate of telomere sequence loss in human leukocytes varies with age. *Proc. Natl. Acad. Sci. USA.* 95:5607–5610.

18. Tommerup, H., Dousmanis,A. and de Lange,T., (1994). Unusual chromatin in human telomeres. Mol. Cell. Biol., 14, 5777--5785.

19. Jenuwein,T. and Allis,C.D., 2001. Translating the histone code. Science, 293, 1074-1080.

20. Koetz, K., Bryl, E., Spickschen, K., O'Fallon, W.M., Goronzy, J.J. and Weyand, C.M., 2000. Tcell homeostasis in patients with rheumatoid arthritis. Proc. Natl Acad. Sci. USA 97, 9203–9208.

21. Vogt, S., Iking-Konert, C., Hug, F., Andrassy, K., Hansch, G.M., 2003. Shortening oftelomeres: evidence for replicative senescence of T cells derived from patients with Wegener'sgranulomatosis. Kidney Int. 63, 2144–2151.

22. Jeanclos, E., Krolewski, A., Skurnick, J., Kimura, M., Aviv, H., Warram, J.H., Aviv, A., 1998. Shortened telomere length in white blood cells of patients with IDDM. Diabetes 47, 482–486.

23. Schonland, S.O., Lopez, C., Widmann, T., Zimmer, J., Bryl, E., Goronzy, J.J., Weyand, C.M., 2003. Premature telomeric loss in rheumatoid arthritis isgeneticallydetermined and involves both myeloid and lymphoid cell lineages. Proc. Natl. Acad. Sci. USA100, 13471–13476.

24. Itzkovitz, S., Shlush, L.I., Gluck, D., Skorecki, K., 2008. Population mixture model for nonlinear telomere dynamics. Phys. Rev. E78 060902-1–060902-4.

25. Gilson, E. andSégal-Bendirdjian, E.,2010. "The telomere story or the triumph of an open- minded research" Biochimie.321-326

26. Nakagawa, S., Gemmell, N.J., Burke, T.,2004. Measuring vertebrate telomeres: applications and limitations.2004. Mol. Ecol.13:2523-2533.
27. Duncan, M., Baird, M., 2005."New developments in telomere length analysis" Experimental Gerontology 40 363–368.

28. Richard, M.C., 2002 "Telomere measurement by quantitative PCR" Nucleic Acid. Res.Vol30 , No 10e47.

29. Hahn, W.C., 2003. Role of telomeres and telomerase in the pathogenesis ofhuman cancer. J. Clin. Oncol. 21, 2034–2043.

30. O'Hagan, R.C., Chang, S., Maser, R.S., Mohan, R., Artandi, S.E., Chin, L., DePinho, R.A., 2002. Telomere dysfunction provokes regional amplification and deletion in cancer genomes. Cancer Cell 2, 149–155.

31. Smogorzewska, A, de Lange, T., 2004. Regulation of telomerase by telomeric proteins. Annu Rev, Biochem., 73:177–208.

32. Loayza, D., De Lange, T., 2003 POT1 as a terminal transducer of TRF1 telomere length control. Nature,423(6943):1013–8.

33. Von Zglinicki, T., 2002. Oxidative stress shortens telomeres. Trends Biochem. Sci. 27:339-344.

34. Von Zglinicki, T., Burkle, A., Kirkwood, T.B., 2001. Stress, DNA damage and ageing -- an integrative approach. ExpGerontol., 36:1049-1062.

35. Petersen, S., Saretzki, G. .andvonZglinicki, T.,1998.Preferential accumulation of single-stranded regions in telomeres of human fibroblasts. Exp. Cell Res.239:152-160.

36. Floyd, R.A., Hensley, K., Jaffery, F., Maidt,L., Robinson, K., Pye, Q., Stewart, C.,1999. Increased oxidative stress brought on by pro-inflammatory cytokines in neurodegenerative processes and the protective role of nitrone-based free radical traps. Life Sci.,65, 1893-1899.

37. Furumoto, K., Inoue, E., Nagao, N., Hiyama, E., Miwa, N., 1998. Age-dependent telomere shortening is slowed down by enrichment of intracellular vitamin C via suppression of oxidative stress. Life Sci., 63:935-948.