Introduction & Review of Literature
Replication of DNA involves copying of existing sequences in a template and primer dependant action of DNA polymerase. This leaves a part of the 3' terminus of lagging strands unreplicated. Thus all eukaryotic chromosomes need the assistance of a special machinery to add terminal sequences to DNA to prevent attrition through successive replication cycles.

The chromosome ends have specialized repeat arrays of hexa- or octa nucleotides making the telomeres. Telomere is important element in chromosomes apart from centromeres and origins of replication. It prevents end to end fusion, aberrant recombination and in general it provides stability to genome. The telomere sequences are maintained by a special reverse transcriptase called telomerase which is RNA dependent DNA polymerase (TERT), having integral RNA sequence as template (TR or hTR in Humans).

In general, normal cells lack expression of telomerase (hTERT) even when there is detectable RNA component. As a result, there is progressive loss of telomeres at every round of replication eventually leading to cellular senescence. This is commonly referred to as end replication problem, which renders the ends of chromosome to get shortened at every cell division. So in normal cells telomere length reduces at every cell division, whereas in cancer cells telomerase is over expressed and telomere length is always maintained. Telomere length defines the life span of cells as the cells with longer telomeres enjoy extended population doublings. So telomeres indirectly provide proliferative potential to cells.

Cancer is a disease of uncontrollable cell divisions and has deregulated signaling and gene networks. As a disease, cancer falls next to cardiovascular diseases in causing human mortality the world over. According to estimates published in “Global Cancer Report 2007” released by American Cancer Society, more than 12 million new cases and 7.6 million deaths from the disease world wide were predicted to occur during 2007. Majority of cancer incidence, and deaths would have occurred in developing nations while 5.4 million cases and 2.9 million deaths would have occurred in economically developed countries. The three common male cancers in developed nations are prostrate, lung and colorectal (Bowel), while in women three most common cancers are breast, colorectal and lung cancer. In developing countries in male, most common are lung, stomach, liver cancer and in women, it is breast, cervicel and stomach cancer (Global Cancer Facts and Figures, 2007).
Early diagnosis and effective targeted therapy are the main priorities in cancer treatment. This is obliterated by acquired multiple mutations underlying deregulatory events and indistinguishable morphologic characters. One of the therapeutic strategies is to restrict cancer cell doublings and induce apoptosis. As telomeres provide proliferative advantage to the cells, restricting the telomere synthesis by various means could limit the replicative potential of cells and induce senescence or cell death programmes. One of the targets selected is telomerase RNA (hTR) which acts as template in telomere synthesis, while hTERT protein can also be used as target. Gene knockdown of hTR will make telomerase as defective enzyme that would fail to maintain telomere length. Shortened telomeres could result in induction of apoptotic programme as critically reduced lengths of telomeres could be recognized as DNA damage in cellular systems.

Many molecules to date have been emerged as probable therapeutic molecule and one of among them is targeted ribozyme (catalytic RNA molecule) as a tool to knockdown the undesirable gene transcripts in cancer. Ribozymes are naturally occurring catalytic molecules that can be engineered to work against chosen targets. Among those, hammerhead ribozymes have been well characterized and used for many therapeutic interventions. Hammerhead ribozyme has conserved catalytic sequences flanked by arm of complementary to the target RNA. Ribozymes have also proven to work in vivo in many cellular and animal studies and offer as good therapeutic molecule for study. The present study involves a) developing ribozyme against telomerase RNA in HeLa cells, b) to observe the effect of ribozyme c) to evaluate hTR as suitable target and d) therapeutic efficacy of expressed ribozymes in HeLa cells.

I.1. Biology of Telomeres and Telomerase

I.1.1. Historical Background

The term telomere was coined by Herman J. Muller, who used to work with flies and observed that the ends of chromosomes were different from other part of genome when irradiated with X rays. Later on Barabara McClintok, while working with Zea Maize, described the rupture of chromosome resulting in fusion of their ends, with consequent formation of dicentric chromosomes and concluded that the telomeres play a crucial role in integrity of chromosome and prevent rupture fusion bridge
cycles which are catastrophic to cellular survival. The telomere research was
reinitiated again 30 years later when JD Watson raised the question of replication of
3’ end of the template. At the same time Russian geneticist Alexsei Olovnikov, found
link between the problem of end replication and cellular senescence (Hayflick and

Leonard Hayflick in 1958 started culturing cells in vitro and found the population of
normal human fibroblast in culture, doubled a finite number of times after which the
cells stopped dividing and entered Phase III phenomenon as per Hayflick’s term.
According to Hayflick, the primary culture is phase I and the phase at which cells
actively dividing and grows for 10 months or so, termed as Phase II, subsequently
cells enter non dividing Phase III (Shay and Wright 2000). Later on, Hayflick also
proved the existence of some counting device referred it as “replicometer” to
designate putative molecular event counter and such replicometer is located in
nucleus, and subsequently proved telomeres as counting device (Wright and Hayflick
1975). It was the time when the convergence of end replication problem and
senescence programme got impetus in scientific field (Shay and Wright 2000).

For Olovnikov, the problem in terminal replications was the cause of the progressive
shortening of the telomere that acts as internal clock to determine the number of
division that cell could experience throughout its life and could control the process of
aging (Olovnikov 1973).

Joseph Gall and Elizabeth Blackburn studying the extra-chromosomal elements of
ciliated protozoan Tetrahymena thermophilia. It has micronucleus with normal
chromosomes and macronucleus with fragmented chromosome and found the ends of
which have repetitive sequence CCCCACA. Blackburn along with her graduate student
identified there is special enzyme for maintaining such repetitive ends and referred the
enzyme as telomere specific “Terminal transferase”, later on called as telomerase
which could add telomere sequence in artificially introduced telomere like sequence
in the extracts of Tetrahymena (Greider and Blackburn 1987).

**I.1.2. Telomeres**

The chromosome ends have specialized repeat arrays of hexa- or octa nucleotides
making the telomeres. The telomeres are composed with a set of proteins which
protect the telomeres from end to end fusion and recombination events. The telomere
sequence is maintained by a special reverse transcriptase called telomerase which is RNA dependant DNA polymerase (TERT), having integral RNA sequence as template (TR or hTR in Humans). The Telomerase complex extends short stretch of TTAGGG sequence. Human chromosome ends bear 3-18kb of double stranded DNA of TTAGGG repeats which are necessary for telomere functions in somatic cells (Farr et al. 1991, Hanish et al. 1994). The termini of human telomeres carry 100 to 280 base protrusions of single stranded TTAGGG repeats (Markov et al. 1997, McElligott and Wellinger 1997) which are effective substrate for telomerase in vitro.

Absence of telomerase complex leads to progressive loss of telomeres at every round of replication eventually leading to cellular senescence. This is commonly referred to as end replication problem, a replication related mechanism which renders the ends of chromosome to get shortened at every cell division. Telomere length predicts the replicative capacity of human cell as proved by Harley group in early 1990’s who first time provided a model converging Hayflick limit hypothesis and later on several groups modified the model based on the status of stress the cells encountered during carcinogenesis process (Allsopp et al. 1992). Their postulation was that the loss of telomeres due to incomplete DNA replication and absence of telomerase provides mitotic clock, which thrives on signals for cell cycle exit, and limiting replicative capacity of cells. In cells with active telomerase the loss at each replication and cell division is counterbalanced with addition of telomeres.

**I.1.3. End Replication Problem**

As the replication fork reaches the 3’ end of the (Lagging strand) template strand, a stage is reached when primer cannot be synthesized anymore. This leaves a part of the terminus unreplicated. In linear replicons, there is no DNA beyond the end of chromosome to serve as a template for an RNA priming event, the gap between the final lagging strand (Okazaki) and the end of chromosome cannot be filled in. So the 5’ end of lagging strand will lose some nucleotides every time cell replicates its DNA. In the early 1970s it was first suggested that the lagging strand synthesis of linear strand DNA templates would be incomplete for two reasons. First there is no known mechanism that ensures priming of the most distal Okazaki fragment synthesis from the very end of the template molecule.
The Newly synthesized Daughter strands (in red), loss its terminal end in subsequent replication cycle, due to primer removal at 5' end which results in reducing telomere length at every division of cell cycle.

Fig I. 1: The model depicts the “End replication Problem” at chromosome ends (From Dr. Jerry Shay’s Web page).

Accordingly the template sequence between the end and the most distal okazaki fragment would not be replicated. Second there is no known mechanism for the most distal RNA primer to be replaced by DNA. Consequently the sequence of the most distal RNA primer would be lost in the daughter strand. In contrast to lagging strand synthesis, leading strand synthesis is thought to continue to the very end of the template molecule (Fig I. 1) (Ohki et al. 2001).

Thus the daughter strands are always found recessed and have C rich 5’ ends. The reason behind existence of about 150-200 base single stranded overhang of G rich strand could be due to special nuclease which are C strand specific may be activated or may be due to removal of RNA primer and inability of polymerase to complete the very end of chromosome (Kenneth et al. 2000, Ohki. et al. 2001).

I.1.4. Processivity of Telomerase.

Carol greider in early 90s proved that telomerase elongation rate is 74nt/min in vitro. The E.coli polymerase I and reverse transcriptase have turnover rates of about 10nt/second, and E.coli Pol III has turnover of 100nt/second, considering telomerase
is very slow in elongation. The enzyme’s processivity adds many d (TTGGGG) repeats rather than disassociating after each nucleotide or each hexanucleotide. The processive character of the enzyme was proved with *Tetrahymena* which synthesized (TTGGGG)n repeats. Telomerase enzyme recognizes the characteristic telomeric repeat (TTGGGG) and elongates the 3’ end adding TTG. After this step the enzyme complex translocates and positions the telomerase RNA template i.e. 3’-AACCCCAAC-5’ to the newly synthesized TTG and base pairing is through 3’-AAC-5’ of template to the extended telomeric repeat TTG. Now the translocated telomerase enzyme complex elongates further to complete the full tract of 5’-TTGGGGTTG-3’ of the telomere. The processivity of telomerase in human telomerase also worked out and same is referred in Fig (I. 18).

### I.1.5. Telomerase Accessory Factors.

Telomerase holoenzyme apart from hTERT and hTR has several accessory factors associated with it, which includes Est1A/B and Dyskerin (Fig I. 2). The functional constituents of telomerase are conserved across species from yeast to human. Human genome contains atleast three EST1 orthologs (*Ever Shorter Telomeres in yeast is an accessory factor*), having role in telomerase regulation.

![Human telomerase](image)

Fig I. 2: A model on telomerase provides information on the interaction of telomerase enzyme with RNA component and telomere and other proteins associated with the complex (Adapted from smogorzewska and deLange 2004, with permission).
Human telomerase protein interacts with another RNA binding protein i.e. dyskerin, a putative pseudouridine synthase functioning in ribosomal processing as it binds with small nucleolar RNAs (snoRNA). Very recently (Venteicher et al. 2008) it was proposed that in addition to above-mentioned complex, telomerase also associated with ATPase pontin and reptin as components for its assembly. But Cohen et al. (2007) with mass spectrometric analysis found only two proteins i.e. hTERT and Dyskerin at catalytically active telomerase complex.

I.1.6. Telomere maintenance proteins

The mammalian telomere with TTAGGG repeats are bound by multi protein complex known as telosome or shelterin. Three shelterin subunits TRF1, TRF2 (TTAGGG repeat factor 1, 2) and Pot1 (Protection of Telomeres 1) directly recognize TTAGGG repeats. They are interconnected by three additional shelterin protein TIN2, TPP1 and Rap1 forming a complex that allows cells to distinguish telomeres from sites of damage.

Without protective activity of shelterin, telomeres are no longer hidden from DNA damage surveillance and chromosome ends are inappropriately processed by DNA repair pathways.

The TIN2 that tethers TPP1 and POT1 to TRF1 and TRF2, also connects TRF1 to TRF2 and this link contributes to the stabilization of TRF2 on telomeres. This shelterin complex is abundant at chromosome ends and not elsewhere, and it is present in telomeres throughout the cell cycle. The shelterin protects telomeres, by making it assume a special structure of telomeric DNA and is also implicated in the generation of T loops and in controlling the telomere synthesis by protecting telomere from accessibility for telomerase (Fig I. 3) (Smogorzewska and deLange 2004).
I. 1. 6. 1. Telomere Length control by shelterin (TRF1 and POT1)

TRF1 binds with duplex telomeric repeat (TTAGGGTTAG) and the number of TRF1 present in telomeric tract is correlated with length of repeat array (Vansteensel et al. 1997, Smogorzewska et al. 2000). TRF1 negatively regulates telomere length by occupying duplex telomeric tract and increasing the accessibility and recruiting of POT1 in single stranded region to block telomerase from accessing telomeric ends (Fig I. 4). Overexpression of TRF1 shortens telomere length and dominant negative mutant of TRF1 resulted in extended telomeric ends proving the role of shelterin complex in telomere length (Bauman and Cech 2001, Lei et al. 2002, Loayza and De Lange 2003).

![Fig I. 4: Model of POT1 regulation of Telomere Length (reproduced from Smogorzewska and De Lange 2004 with permission).](image)

I.1.6.2. Telomere length homeostasis

In cells that express telomerase, the length of duplex telomeric repeat array is kept within a species and cell type specific narrow range. In humans, the average length varies between 5 and 15 kb (Smogorzewska and De Lange 2004). The primary cause of telomere shortening is incomplete DNA replication. (Huffman et al. 2000; Makarov et al. 1997; McEligott and Wellinger 1997, 1996; Wright et al. 1997). The telomerase lengthening and shortening provides a control towards activity of telomerase.

One of the model envisages telomere length dependent changes in chromatin structure influencing the productive association of telomerase with telomeres 3’ ends. A long telomere would have lower probability to be in telomerase extendible state than short
telomere. In this model a telomere could shorten over several rounds of DNA replication without being elongated by telomerase, before its chromatin structure would switch and become competent for telomerase-mediated elongation. Thus association rather than activity of telomerase would be regulated element. Recently it has been argued that the homeostasis mechanism of telomeres is achieved by a switch between telomerase extendible and non-extendible states (Gael Cristofari and Joachim Lingner 2006).

I.2. Telomeres, Telomerase and Cancer.


Cancer is a disease accompanying deregulation of cell division. According to a WHO bulletin “Cancer is a generic term for a group of over 100 chronic diseases, which can affect any part of the body. A defining feature of cancer is the rapid creation of abnormal cells, which grow beyond their usual boundary and can invade adjoining parts of the body. The cells may also spread to other organs, a process referred to as metastasis”. As cancer cells are morphologically and behaviorally indistinguishable at early stages, it is very difficult to predict the transformed status of cell in the niche of normal cells.

I.2.2. Biological Aspects of Cancer.

Cancer is a disease with unresolved ‘puzzles’ of complex circuitry of interrelated molecular events which enable the cell survive and proliferate in an uncontrollable way as a result of accumulated mutation and overall, generation of genomic instability. Cancer cells arise as a result of continuous genetic evolution and natural selection of mutant cells. Genetically deviant cells are generated as a result of environmental insult or normal errors in replication. One of historical hypotheses on tumor evolution is that, tumor initiation occurs in single normal cells by induced change which makes it neoplastic and provides it with selective growth advantage over adjacent normal cells. From time to time as a result of genetic instability in the expanding tumor population, mutant cells are continuously produced (Nowell 1976). Through multiple rounds of proliferation, mutation and selection, a neoplastic variant
evolves to cancer. Ultimately the fully developed malignancy as it appears clinically has a new genetic constitution e.g., unique aneuploid karyotype associated with aberrant metabolic behavior and specific antigenic properties.

Tumorigenesis thus is a multistep process, which involves genetic alteration at different steps of development, resulting in permissive transformation of normal in to highly malignant derivatives (Hanahan and Weinberg 2000).

Knudson’s two hit hypothesis gave impetus to identification of genes responsible for and genetic changes that lead to cancer. Discovery of tumor suppressor genes and oncogenes has helped to visualize the nature of changes underlying tumorigenesis. Loss of gene function (tumor suppressor gene) and gain of gene function (oncogene) through mutation, deletion and other genetic events are associated with cancer. There are hundreds of cancer types and subtypes of tumors within specific organs, each having its own distinct abnormal regulatory circuits. It is primary question as to what are the regulatory circuits within each type of target cell to be altered to become cancerous cell. Each subset seems to have its own distinct pattern of genetic changes, reflecting on complexities of molecular events to become cancerous (Knudson 1971).

The vast range of alterations resulting in cancer cells have been catalogued by Hanahan and Robert Weinberg (Hanahan and Weinberg 2000) into six essential alterations in molecular physiology that collectively dictate malignant growth. And those are as follows

1) Self sufficiency in growth signals
2) Insensitivity to growth inhibitory signal
3) Evasion of programmed cell death (apoptosis)
4) Limitless replicative potential
5) Sustained angiogenesis
6) Tissue invasion and metastasis

These six capabilities are shared by most of the known type of cancer. Among those, the limitless replicative potential is of our interest for study and therapeutic intervention. Understanding the basic cellular physiology with such an aberrational event would be helpful to devise possible therapeutic intervention. Still failure at the front of cancer treatment is attributed to lack of early detection and indistinguishable morphological features of cancers from normal cells. The six most possible genetic
events catalogued by Weinberg provide a window at each step for therapeutic intervention. One such effort is to intervene into the potential of cells with their acquired capability of limitless replicative potential using catalytic RNAs. Before exploring the possible interventional modalities, it would be appropriate to understand the developing concepts and molecular players that provide the "limitless replicative potential" to the cells.

I.2.3. Cellular Controls Over its Life span

At cellular level each cell tries to protect its genome integrity by different means and the changed one is evicted by its own planned programmes (Apoptosis). Cells in higher organisms, have defined life time (population doubling time) and DNA maintenance mechanisms i.e. DNA repair machineries, gate keepers and molecular policing (P53 and pRB and other tumor suppressor genes) and apoptotic molecules (Bcl family and Caspases etc), to ensure the proper cellular functioning and the integrity of genome. The variant cells at certain times may be favored for propagation. Realizing the possible changes and certainty of evolution in favoring such changes, cells must have wired in such a way to limit their divisions, and genome size, life time etc to protect themselves from the possible stress induced changes.

Tumorigenic process involves acquiring multiple mutations during its course of propagation, and those ensuring their proliferation are selected. Each mutation probably requires 20-30 divisions. It is expected that such mutated cells must expand to, about 1 million cells before a second mutation to occur. As mutations are commonly recessive in nature they must be propagated either by recombination events or by clonal expansion in such a way as to eliminate the wild type alleles. Limiting the number of cell divisions would prevent the possibility of emergence of such mutated cells or preventing mutated cells from progression. So one obvious strategy to prevent cancers is to have limited cell divisions (Shay and Wright 2005).

Most cells in primary culture divide over a limited number of cycles. After a period of rapid cell proliferation, their division rate slows down, and cells ultimately cease to divide. Such cells become unresponsive to mitogenic stimuli but remain viable for extended period of time. Hayflick and Moorhead (1961) found that cells have definite life span and human cell limit is around 52, and fetal cells go around 50 ±10. This phenomenon is called as Hayflick limit or phase III phenomenon (Hayflick and
Senescence is defined as a state of permanent growth arrest in which cells enter the G0/G1 phase of the cell cycle, assume an enlarged flattened morphology and exhibit altered pattern of gene expression and metabolic activity (Artandi and Depinho 2000, Campisi 2000, Sitte et al. 2000, Narita et al. 2003). This form of senescence, which follows extended period of propagation in vitro, is termed replicative senescence (Ittai and Weinberg 2004).

Subsequent to Hayflick's discovery various studies proved different form of senescence that normal cells can enter senescence rapidly in response to various physiological stresses, often referred to as “Stress-induced Senescence”. So senescence programme can be activated by the cells when such physiological stress is encountered. Mutation in p53, pRB, p16/INK 4A molecular activators of senescence make the mouse more tumor prone, suggesting senescence to be a tumor suppressing mechanism (Artandi and Depinho et al. 2000).

The finite replicative potential of cells is linked not to chronological age of culture but to the number of cell divisions and to telomeres. The telomere shortens at every cell division due to end replication problem and the shortening is wired in tumor suppressor net work. Telomere length can be extended or maintained by telomerase and doing so it can extend life time of the cells.

Cultured human cells express two distinct barriers to control the indefinite proliferation. In epithelial cells the first barrier appears to be mediated by stress induced cyclin kinase inhibitors which protect RB from inactivation through phosphorylation. Cells at this first barrier are under stasis, (a viable G1 arrest with normal karyotypes) and cannot be rescued by introduction of telomerase. This stasis is what is referred to as mortality stage I (M1). Senescence barrier or premature senescence represents this Rb mediated barrier (Fig I. 5). The second proliferative barrier is associated with shortened telomeres. This barrier is stringent in long lived animals and with strict repression and may have evolved as a mechanism of tumor suppression. Continuous telomere erosion in cells which overcome the first barrier leads to unprotected telomeric ends that may fuse with other chromosomes and cause wide spread genomic instability.

Cells lacking functional p53 exhibit crisis: A high L1 (DNA synthesis index) and massive cell death. Cells retaining functional p53 exhibit largely viable growth arrest
in all phases of cycle and show moderate L1. In both situations prior introduction of hTERT will immortalize the population. Human carcinoma development requires overcoming these senescence barriers and reactivating telomerase activity. Many of derangements observed in human cancers reflect alterations in molecules that enforce stasis and telomerase repression.

Fig I. 5: In the absence of telomerase, telomeres shorten with each cell division. Cellular senescence (Mortality stage -M1) begins when there are on average length of telomeric repeats remaining. In fibroblast cultures, M1 requires p53 and pRb activity. If p53 or pRB mutated cells continue to divide, and telomeres get shortened to an extent to prevent further replication (Mortality stage-M2). Expression of telomerase can bypass this stage and induction of limitless replicative potential could be achieved (Wright and Shay 1995, Redrawn with permission from Shay)

1.2.4. Telomere Dependent Proliferative Barrier: Agonescence or Crisis.

The human cells in culture that overcome the Rb mediated barrier still express a finite life span, and proliferation in absence of telomerase leads to erosion of telomeres and causes genomic instability and telomere dependent proliferation barrier. Where p53 function is absent or abrogated this barrier presents a crisis and cell death. (Rheinwald et al. 2002, Shay et al. 1993). When wild type p53 is present, this barrier termed as “Agonescence”, produces most viable growth arrested cells (Fig I. 6) (Stampfer and Yaswen 2003).
In most of human cellular immortalization, overcoming the RB mediated barrier has been achieved utilizing viral oncogenes that obligately inactivate p53 in addition to pRB. Overcoming the second barrier relied on conditions that produce inactive p53. Human epithelial cells transduced with SV 40 large T antigen, or post stasis cells lacking functional p53 typically cease growth during a period of crisis with rare immortalization (Shay et al. 1995, Gollahon et al. 1996, Gao et al. 1996, Van Der Haegen and Shay 1993, Bartek et al. 1991). Telomerase activity was not found in these populations, while wide-spread genomic instability is present. Cells in crisis maintain proliferation and ectopic expression of hTERT can efficiently immortalize post stasis epithelial cells by escaping the second barrier (Dickson et al. 2000, Rheinwald et al. 2002, Stampfer et al. 2001, Kiyono et al. 1998). These results indicate that loss of p53 by itself or in combination with RB inactivation, is not sufficient to produce immortal transformation, whereas introduction of telomerase activity is sufficient in such (p53-/-, pRb-/-) genetic background.

![Diagram of immortalization process](image)

Fig I. 6: Model for Immortalization of human mammary epithelial cells cultured *In vitro* (Adapted from Stampfer and Yaswen 2003, reproduced with permission from Yaswan)

### I.2.5. Overcoming the Telomere-Dependent Barrier and Turning on Telomerase

The cells cultured from human tissues do not spontaneously overcome telomere dependent barrier, and emergence of immortal cells is very rare even in p53-/-, pRB-/-
status. Telomerase introduction can overcome this barrier. The mechanism enforcing telomerase repression has not been studied. Some of the clues that human dermal tissues and cultured keratinocytes express a low level of telomerase activity correlate with the presence of stem cells within the population. The telomere dependent proliferative barrier might be possible in such telomerase expressing cells by preventing differentiation of the telomerase expressing stem cells (Bringold and Serrano, 2000).

1.2.6. Role of Telomeres in Suppressing and Facilitating Carcinogenesis.

There are different views on the role of telomeres in carcinogenesis, which can vary with different genetic backgrounds and species. Progressive telomere shortening during primary human fibroblast division in vitro leads to activation of senescence programme at a time point coinciding with Hayflick limit i.e. up to 60-80 population doublings. The telomere shortening is sensed as DNA damage and the shortened telomere signal is transduced to tumor suppressor pathways controlled by p53 and pRb. Inactivation of p53 and pRb by viral proteins leads to extended replicative life span, and prevents senescence.

![Diagram of senescence effector programme](image)

Fig I. 7: The effector programme for senescence is illustrated in centre box. The p53 is activated by ATM or by p19 ARF leads to transcriptional activation of p21, inhibition of CDK4/6 and decreased Rb phosphorylation. Activation of p16 INK4A inhibits CDK4/6 and blocks Rb phosphorylation (redrawn from Steven artandi and Ronald depinho 2000).
The INK 4A locus encodes two tumor suppressor genes, p16INK4A and p19 ARF, that activates Rb and p53 respectively, and both are involved in senescence. Human foreskin keratinocytes and human mammary epithelial cells senesce earlier than fibroblasts after 20-30 population doublings. These epithelial cells arrest prematurely as a result of accumulation of p16INK4A. Epithelial cells escape this check point either spontaneously via repression of p16 IN4A promoter methylation or experimentally through inactivation of pRB by viral protein HPV E7 protein. Bypassing such checkpoint allows the epithelial cells to directly encounter true telomere based replicative senescence at which point they are likely to be immortalized by hTERT. Expression of activated oncogenes H-RAS in human cells, activates the p19ARF which binds to MDM2, a regulator of p53. MDM2 (or HDM2) acts as an ubiquitin ligase for p53, targeting ubiquitin mediated degradation of p53 (Kubbutat et al. 1997, Haupt et al. 1997). Stimulation of p19ARF therefore blocks MDM2 mediated p53 ubiquitinylation, resulting in p53 activation (Pomerantz et al. 1998, Zhang et al. 1998, Kamijo et al. 1998). The p19ARF-p53 axis served to integrate a variety of growth and oncogene signals that enforce a number of responses including G1 cell cycle arrest and senescence depending on the specific signal and cellular context (Fig I. 7).

I.2.7. Telomere Shortening may Impede Tumorigenesis.

Telomere hypothesis was formulated to explain its role in senescence. Telomerase is reactivated in 90 % of cancers, and telomere lengths in tumor cell lines are often shorter than primary normal cells. The end replication problem poses threat to the cell’s life as a result of attrition of telomere length. A drastic reduction in telomere length would be sensed as DNA damage, and DNA damage activates p53 which could cause cell cycle arrest.

It was proved that there is clear genetic link between the p53 and telomere, as shortened telomere activates the p53 and removing TRF2 from telomere causes chromosomal fusions and cell death in an ATM and p53 dependent manner (Fig I. 8). The functional links were provided with mouse studies with mTR/INK4A compound mutant mice and mTR/p53 compound mutant.

The INK4A /ARF deficiency inactivates only p19 ARF arm of p53 pathway, leaving the ATM-p53 /DNA damage arm that is activated by telomere dysfunction. The tumor
size reduced significantly in mTR-/- INK4A-/- mice with severe telomere dysfunction compared to INK4A-/- mice with intact telomere function.

Double stranded DNA Damage

Fig I. 8: The ATM telomere pathway (redrawn with permission from Tej K Pandita from Tej K.Pandita 2002).

I. 2. 8. Telomere Crisis Promotes Tumorigenesis

Studies with mouse embryonic fibroblasts from late generation mTR -/-, p53-/- mice show enhanced transformation by telomere dysfunction in p53-/- background in contrast to the reduced transformation as a result of telomere dysfunction in INK4A-/- background (Greenberg et al. 1999).

This suggests that telomere crisis is not a tumor suppressor mechanism; instead it is a period of extreme chromosomal and genetic instability that results in cell death or cellular transformation depending on stochastic genetic changes and activation of adaptive mechanisms. In human cells the final cell division that precedes replicative senescence can be thought of as “early crisis”. Senescence is dynamic programme in which the rate of cell growth slows gradually and gene expression profiles are altered. Despite telomere shortening the genome remains stable until few cell divisions at
which point the frequency of dicentric chromosomes and chromosomal fragments increases dramatically. These observations indicate that in human cells early crisis actually begins at the very end of senescence as telomere dysfunction deteriorates and chromosomal integrity is compromised. At this critical point cell growth is checked by p53 and pRb activation. So the telomere hypothesis and its role in promoting or preventing tumorigenesis is based on status of p53 and pRb pathway proteins. The modified model of telomere hypothesis and cancer, aging is represented in Fig I. 9.

Fig I. 9: Modified view of Telomere Hypothesis. Telomere shortening in primary Human cells leads to replicative senescence and cell death (M1) dependant on status of p53 and pRb. Mutation in p53/pRb allows cells continued cell division and enter telomere-mediated crisis (M2). Expression of telomerase can bypass replicative senescence or telomere crisis lead to immortalization (dashed lines). Similarly reactivation of telomerase with such genetic catastrophe makes cells tumorigenic (Redrawn from Artandi and Depinho 2000).

I.3. Biology of Human Telomerase RNA (hTR)

I.3.1. hTR Locus

Human Telomerase RNA (hTR) is encoded by a single copy gene localized to chromosome 3, and locus 3q26.3. This locus is found to be frequently amplified in certain types of solid tumors, cancer of cervix, lung and squamous cell carcinoma of the head and neck (SCC-HN). Increased copy number of hTR was found in comparison to normal tissues of cervix and SCCHN (Soder et al. 1997). The catalytic protein
component hTERT maps to chromosome 5, at 5q15.33, region which is frequently amplified in cancer with increased copy number of transcripts too.

A 3 to 10 fold increase in steady state levels of hTR has been observed in variety of immortal cells (Yi et al. 1999, Avilion et al. 1996) indicating that some regulation of hTR is associated with acquisition of telomerase activity. In situ hybridization experiments show that the relative levels of hTR in human cells are significantly different from those in adjacent tissues to be clinically useful in diagnosis of cancer (Morales et al. 1998, Morales et al. 1998, Yashima et al. 1997, Yashima et al. 1997).

In normal human diploid cells, there is lack of mRNA for the catalytic subunit hTERT and presence of integral RNA component of telomerase hTR, whereas in cancers both the components are found to be present or elevated (Meyerson et al. 1997, Miller & Rosman 1989, Morales et al. 1999, Morgenstern and Land 1990, Nagano and Kelly 1994, Nakamura et al. 1997, Kilian et al. 1997, Soder et al. 1997).

### I.3.2. Telomerase RNA Structure

Human telomerase RNA (hTR) is about 451 bases long, is rich in GC, and has conserved secondary structures and is transcribed by RNA polymerase II (Feng et al. 1995). The prediction of telomerase RNA structure was determined by aligning the 35 vertebrate telomerase RNA sequence using conserved sequence as anchor points. The conserved region (CR) was numbered from 5' to 3' and designated the CR1 through CR 8. The first conserved region (CR1) represents the template region and longest conserved region found to be CR2 and CR5 (Fig I. 10). The 5' end of human RNA is 45 nucleotides upstream of template (CR1) sequence, the 3' end of human telomerase RNA has been mapped to 3 nucleotides down stream of CR8 (ACA) motif. There are 10 conserved helical regions seem to be universally present in vertebrate telomerase RNA structure. These helices constitute 4 distinct structural domains. Paired region is referred as 'P' and single stranded or junction regions as 'J' in the following section (Fig I. 10) (Chen et al. 2000).

1) Pseudo knot domain.
2) CR4- CR5 domain
3) The box H/ACA domain
4) CR7 domain.
Junctions are regions between the two successive helices. The region between helices p2b and p3 is named as j2b/3. The unstructured template region will be discussed prior to structured domains.

a) Template Region

The first conserved region (CR1) consensus sequence 5'-CUAACCCU-3' represents the template region of telomerase RNA that specifies the synthesis of the 5' TTAGGG-3' telomere repeats in vertebrate. The sequence upstream of the template region pairs with the sequence located at nucleotide 187-208 to form helix P1.

In human telomerase RNA (hTR) helix P1 is the only conserved structural element located upstream of template and was good candidate for defining template boundary. The helix P1 is divided into two base paired region helix P1a and P1b which are separated by an internal loop.

b) Pseudoknot Domain:

The pseudoknot domain contains CR2 and CR3 sequences and is stabilized by helices P2A, P2b and P3. In addition to base pairs that co-vary, putative base pairing was included that extends the helix P3 and allows a coaxial stacking between P2b and P3. Such coaxial stacking between two helical regions would significantly stabilize this type of pseudoknot structure. The pseudoknot region after the base 165 contains an unpaired stretch of 'A's and target site GUC and partially paired region which was taken for ribozyme designing and used as target site in this study. The j2b/3 region is conserved not only in sequence but also in length. This length conservation is also important for maintaining a stable conformation of this pseudoknot element (Fig I. 11).

c) CR4-CR5 Domain:

The CR4-CR5 domain lies downstream of pseudoknot domain and consists of stem loop structure established by helices p5 and p6. The intervening loop contains the conserved CR4 and CR5 sequence elements. The pseudoknot and CR4-CR5 domain can independently assemble with TERT protein and are essential for telomerase activity in vitro. This CR4-CR5 domain is designated as activation domain of telomerase. Recently the CR4-CR5 domain was modified to have internal loop, which is evident from enzyme activating structures of telomerase RNA studies (Tesmer et al. 1999, Mitchell and Collins 2000).
Fig I. 10: Structure proposed by Chen et al. (2000) by aligning different phylogenies of hTR (Adapted from Chen et al. (2000) with permission from Carol Greider).

Fig I. 11: Pseudoknot region which includes the target region selected (180) for ribozyme targeting in the present study.

d) The Box H/ACA Domain:

The box H/ACA domain includes p4, Box H, P7a, P7b and box ACA, which form a conserved structure similar to the structure found in the box H/ACA family of Sno RNAs (Gannot et al. 1997, Mitchell et al. 1999a). The conserved sequence of box H
(CR6) and Box ACA (CR8) motifs are both single stranded as seen in small nucleolar RNAs. The box H/ACA Sno RNAs also terminates 3 nucleotide down stream of a consensus ACA motif.

e) The CR7 Domain

CR7 domain defined by a highly conserved sequence of helices P8a, P8b and the loop L8. The sequence and length conservation of CR7 region suggests that the structure is specific to vertebrate telomerase RNA that functions in localization and processing events of hTR.

I.3.3. Comprehensive Structure of hTR

Though the four domains seem to contribute to telomerase function in vivo, telomerase catalytic activity in vitro only requires the core (pseudoknot) and CR4-CR5 domains each of which binds independently to the TERT proteins (Mitchell and Collins 2000).

The deduced structure for the core domain encompasses five short helically paired regions designated P1, P2a.1, P2a, P2b, P3 and some single stranded regions (J). Three of the paired sequences (P2a.1, P2a, P2b) together form stem of hairpin, a portion of which can base pair with sequences down stream to form the P3 helix, creating potential pseudo knot region. The P3 base pairing in pseudo knot domain is important, as the deletion in the region disrupting the P3 base pairing reduces or abolishes telomerase activity. Apart from core and CR4-CR5 region, an extensive mutagenesis study in other regions provides the comprehensive hTR structure (Fig I. 12). Residues 18-37 are postulated to form base pairs in P1. Deleting residue 33 or beyond drastically reduced telomerase activity, but the residues 1-32 are dispensable for telomerase activity which indicates that at least some base pairing in the P1 region is required for optimal function. The previously predicted stem of base paired regions P2a.1 has extended stem structure while the primary sequence of P2b and P3 stems are relevant in functional reconstitution of the human telomerase complex. The accessibility mapping analysis of hTR has failed to confirm the stem formation in hTR, suggesting that either the P3 base pairing doesn’t occur or it may be dynamic. Other studies are supportive of this evidence that P3 may have dynamic structure; adopting different conformations during various stages of telomere repeat synthesis or in different physiologic states of telomere repeat synthesis.
Fig I.12: The schematic view of core sequence consisting of 210 base of the 5' of hTR. The template region has been indicated in numbers 44-54, and base pairing nucleotides in the region of nt 62-147 and 33-205. These regions were shown to contribute to optimal telomerase function. The P2a.1 helix is extended proximally by 3 bp (Adapted from Hinh ly et al 2003 with permission).

The P3 facilitates the homodimerization of hTR both in vivo and in vitro, and such P3 dependent dimerization is essential for ribonuclear protein complex (Mitchell and Collins. 2000). To assess the importance of helix P1 structure in telomerase function, variety of RNA truncation mutation with this 5’ end at positions of 1, 15, 26, 32, 44 and end position 212, as base pairing nucleotides falls in 5’ of template and 3’ of pseudoknot regions. These mutants which lack 3’ half of RNA can reconstitute telomerase activity provided CR4-CR5 RNA fragment are present. RNA mutants hTR 1-212, 15-212, 26-212 and 32-122 reconstituted telomerase activity and showed typical 6 nucleotide repeat patterns of elongation products (Fig I.13 and I.14).

Fig I.13: The P1 helix is shown as separate P1a and P1b helices, formed of template and 3’ of pseudoknot region (Adapted from Chen and Greider 2003 with permission).
Removal of first 31 residues in RNA resulted in increased overall telomerase activity. But mutants lacking P1a and P1b in the 44-212 region resulted in additional bands at position +6, +12, +18 and +24, because the 5' end of hTR 44-212 RNA located only 2 residues upstream of template. This suggests that mutant truncated RNAs can fold differently and alter the function of telomerase. Changing the P1b helix 38-uuuuuu-43 to 38-AAAAAA-43 resulted in base paired structure and overall reduction in activity and abnormal pattern of telomere products (Fig I. 14) (Chen and Greider 2003).

I.3.4. RNA-RNA Interaction of hTR

The hTR itself participates in intramolecular interactions and the CR4/CR5 region, which contained the P6.1 loop involved in an RNA-RNA interaction with template region intramolecularly. Mitchell and Collins (2000) showed that deletion of the residues of 303-315 (p6.1) results in improper interaction between hTR and hTERT and abrogate telomerase activity (Fig I. 15).
The loop bases of P6.1 (U307, G308, and G309) are inaccessible in vivo, but accessible in vitro (Antal et al. 2002) suggestive of hTERT mediated protection, due to interaction of hTERT with these nucleotides. The P6.1 hairpin and template region has highly conserved bases, and such interaction would have some biological significance. Altering the nucleotides in the regions altered the telomerase activity.

The new model of RNA secondary structure argues the possibility of close proximity of template region with that of P6.1 of CR4/CR5. The hairpin might be having interaction at multiple sites in the template domain. If this interaction is dynamic then the hairpin may be involved in mechanism of translocation of the template during reverse transcription (Fig I. 16) (Lai et al 2003).

The conserved hairpin in the CR4/CR5 domain may play similar role in processivity of human telomerase. P6.1 may prepare the template domain by either stabilizing the template or positioning it for proper reverse transcription by the protein.

1.3.5. hTR Dimer Model

The purified human telomerase is found to have dimer of RNA template and most probably of two protein components. The sequences in the hTR pseudoknot P3 helix mediate TR dimerization. The P1b helix of the hTR pseudoknot template domain
contributes to 5' template boundary definition. Based on the observations the
interaction of hTERT and hTR contributes to both DNA synthesis and processivity
function of telomerase. The hTR-hTR interactions at the P3 helix interface could
stabilize the hTR dimer structure, permitting one to allosterically influence the
function or conformation of the other telomerase RNA molecule (Fig I. 17).

The N terminus of hTERT contains stable integrated and structurally complex
template RNA. The interactions of hTERT domains RID1 and RID 2 with hTR
sequences are essential for processivity and DNA synthesis respectively, which
facilitates dynamic hTR arrangement i.e. alternative use of the two potential active
sites of telomerase that are likely involved in telomere synthesis. An alternative model
for the function of telomerase dimerization is that it facilitates the simultaneous
extension of two DNA substrates (Moriatry et al. 2004).

Fig I. 17: Schematic diagram showing RNA-RNA and RNA-Protein interaction viz... hTR-hTR and
hTR-hTERT, hTERT -hTERT. RID 1 is connected to core hTERT domain RID2, RT, C terminus. The
RID2-P6.1 interaction site is likely essential for DNA synthesis. RID 1 pseudoknot template and RID 1
C terminus interaction may regulate repeat addition processivity. RID1 and C terminus hTERT may
interact with different hTR molecule. The P3 in hTR molecule contribute in dimerization and
processivity and regulate allosteric regulation of other hTR monomer (Adapted from Moriatry et al.
2004 with permission)
1.3.6. Template region of hTR processivity- Model

The template region of human telomerase consist of 11nt sequence viz., 3'-CAAUCCCCAUC-5' complementary to nearly two telomeric repeats d (GGTTAG). The template sequences of other vertebrates vary in length ranging from 8-11nt suggesting 8-nucleotide may be sufficient for telomerase function. Out of the 8 nt, six form universally conserved elongation domain (3'CCAAUC-5') which codes for single telomeric repeat. The alignment domain contiguous to the 3' end of the elongation chain consists of additional nucleotides complementary to the telomeric sequence (Fig I. 18).

![Diagram of Telomerase Processivity](image)

Fig I. 18: A)Human telomerase RNA (hTR) template with hTERT in a complex ,and B)annealing of primer base pairs with template region of hTR C) Primer extension of telomerase until the 5’ end is reached. D) Translocation and realignment of primer terminus to the 3’ end of the template for next round of extension (Redrawn from gavory G et al. 2003 with permission from Balsubramaniam)

The alignment domain functions to reposition the primer substrate by base pairing during processive synthesis. Unlike elongation domain, the alignment domain of telomerase is not completely conserved throughout vertebrates and varies in length from 2nt (3'UC-5') for rodents to maximum of 5 nt (3'-CAAUCC-5') for humans. This strongly suggests that not all nucleotides in the alignment domain of human telomerase are required for function.
Altering the sequence of the alignment domain of human telomerase at the first two positions from the 3’ end (56C and 56CA55 respectively) influence some aspects of telomerase activity. Deletion of the first 3’ residue (56C) of the alignment domain of the templatae sequence marginally reduced overall activity to 84 ± 8% whilst deletion of the sequence 56CA55 had more pronounced effect. So the most 3’ terminal residues 56C and 55A of the alignment domain of human telomerase do contribute to optimal function of telomerase but are not essential for catalytic activity in vitro. At least 3 nt of the DNA substrate with RNA template is required for efficient realignment and processive synthesis.

I.3.7. hTERT Interaction with hTR

In most telomerase studied, the 5’ template boundary definition is regulated by template adjacent TR stem structures and intervening template linker sequences, which have been proposed to constrain movement of the template in the active site. In hTR, this structure is the P1b helix (Fig I. 19) (Autexier and Greider 1995, Prescott and Blackburn 1997, Chen and Greider 2003, Seto et al. 2003). hTERT contains reverse transcriptase motifs that are relatively well conserved with other RTs and flanked by specific N and C terminal sequences. The C terminus may constitute the polymerase thumb of TERT and is important for nucleotide addition (Peng et al. 2001, Huard et al. 2004). The hTERT C and N termini may associate with DNA substrates and also regulate telomerase specific property of repeat additions. Two regions in hTERT- N terminus are involved in hTR interaction viz., RNA interaction domain 1 and 2 (RID1 and RID 2) that are separated by non conserved catalytically inessential linker (Xia et al. 2000).

P1b Helix

Template Linker

56 Template seq 46 ——— UGGCC

3’-CAAUCCCAUCC UGUUUUUUACC GG-5’

Fig I. 19: The sequence of hTR that are involved in interaction with hTERT (redrawn from Moriarty et al 2005).
RID 2 likely regulate telomerase assembly via interactions with the hTR pseudoknot/template domain, possibly transiently or with low affinity. This is essential for repeat addition processivity, and contributes anchor site-specific catalytic functions. A sub region of RID1 referred to as N-DAT (dissociative activities of telomerase) is not required for specificity and affinity of telomerase DNA interactions and telomere maintenance in vivo (Armbruster et al. 2001, Lee et al. 2003). A short C-DAT region has also been identified at the extreme C terminus of hTERT though C-DAT sequences contribute to human telomerase catalytic function (Banik et al. 2002, Huard et al. 2004, Moriarty et al. 2005).

I.3.8. Transcriptional and Post-transcriptional Regulation of hTR

Elevated hTR levels may be due to increased transcription rate and an increased half life as a result of the association of hTR with catalytic protein subunit or other regulatory modifications or combinations of the factors. It seems as if there is coordinated program for the derepression of telomerase during tumor formation (involving regulation of both hTR and hTERT) rather than just focal activation of hTERT.

There is an increased steady state level of hTR, due to up-regulation of hTR transcription and it is not due to presence of hTERT protein. Since increased hTR transcription is not present in cells expressing exogenous hTERT but occurs only in cells in which endogenous gene has been activated by process of in vitro Immortalization or by viral oncogenes on in vivo tumor formation. The increased transcription rate of hTR only in immortal cell types expressing their endogenous hTERT suggests that changes or mutations in immortalization process affect a coordinated telomerase reactivation program that may up regulate hTR expression. Thus increased transcription partly accounts for the elevated steady state hTR levels in cells expressing their endogenous hTERT. Association of telomerase RNAs with hTERT may also contribute its increased half-life.

Expression of telomerase catalytic subunit alone may cause moderate increase in the steady state hTR levels by increasing half-life without affecting its transcription rate. The half-life of hTR ranged from 4.4 to 32 days from different cell types. The half life of hTR in H1299 cells is the longest half-life reported for any eukaryotic RNA.
Dramatically elevated hTR levels in a wide variety of human tumors (Morales C.P 1998, Yashima et al. 1997) are attributed to hTERT protection by binding to hTR.

I.3.9. Transcriptional Regulation of hTR

A number of transcription factor play a role in transcription regulation. The hTR promoter contains the consensus TATA box and CCAAT box in close proximity to the transcriptional start site, and is transcribed by RNA polymerase II. Some of the putative sites correspond to transcription factors AP1 and ETs and response elements for gluco corticoid, progesterone and androgen steroid hormone. The minimum promoter activity was found with 272 bp upstream of the transcriptional start site, and contains DNase I protected site, which encompass the 4 consensus binding site for the Sp1 family of transcription factors in addition to the CCAAT box (Zhao et al. 2003).

![Diagram of the sequence of the wild-type hTR core promoter (-107/+69)](image)

Fig I. 20: The sequence of the wild-type hTR core promoter (-107/+69) is shown above. The number on either side of the sequence is related to the transcriptional start site. The hTR template boundary region is indicated in bold. The respective transcription factor binding sites are shown above the sequence.

Binding of both Sp1 and Sp3 has a regulatory role in hTR expression. The Sp1 induces the HTR expression whereas Sp3 suppresses the hTR expression (Fig I. 20 and I. 21).

So the upstream of CCAAT box conferred a positive regulation on the promoter and 3' sites down stream of the CCAAT Box appeared to be repressive, and the site immediately down stream of CCAAT mediates the strong repressive effect, due to its positioning in relation to NF-Y binding (Zhao et al. 2003). Mutation in Sp1 site upstream of CCAAT box from patients with aplastic anemia and lack of sp1 sites downstream of the transcriptional start site has positive effect on promoter activity, which implies a negative regulatory role for the upstream site (Keith et al. 2004).
Fig I. 21: Schematic illustration of the hTR gene promoter. Regulatory sequences (GC box and CCAAT box sites) and their cognate binding factors are shown. The Sp1 and NF-Y binding nucleotide sequences are indicated (Copied from Zhao et al. 2003 reproduced with permission from Nicole Keith).

The Sp1 binding site has ability to bind Sp1 and Sp3, so it is possibly, cooperation among other transcription factor binding the promoter that determines whether these Sp1 sites act in a positive or negative manner. The tumor suppressor and cell cycle regulator pRB induces hTR promoter activity and pRb doesn’t bind directly to DNA and instead mediates its effects through recruitment of additional transcriptional regulators, and increasing the affinity of Sp1 and NF-Y binding to hTR.

The MDM2 a master regulator of p53 activity also binds with Sp1 and represses the functional activity of transcription. The retinoblastoma protein has ability to relieve the repression by displacing the Sp1 from MDM2 and activate transcription through Sp1 (Johnson-Pais et al. 2001). Studies have shown association of MDM2 with Sp1 and over-expression of MDM2 represses hTR transcription (Zhao et al. 2005).

**I.3.10. Regulation of hTR by Cell Signaling pathways.**

The mitogen activated protein Kinase (MAPK) signaling cascades modulate transcription of hTERT through numerous effector binding sites within core promoter, including the c-Myc, Ap1, Ets transcription factors (Cong et al. 2002, Kyo and Inoue 2002). MAPK also has a role in HTR regulation. Co-expression of constitutive active domain of MEKK1, the major MAP3K for the JNK pathway, causes repression of hTR (Billsland et al. 2006).

Hypoxia response element (HRE) sites with in the core promoters of HTERT and hTR offers binding site for hypoxia inducible factor-1 (HIF-1), indicating that hTR may be regulated by HIF-1. Expression of hTR and hTERT is repressed by co
repressor C terminal binding protein (CtBP) that binds with various histone modifying enzymes (Glaspool et al. 2005). This suggests a role for chromatin remodeling in regulation of telomerase.

I.3.11. Epigenetic Regulation of hTR

Lack of hTR expression was associated with acetylated histones H3 and H4 and acetylated lysines 9 of H3, a modification generally associated with active gene expression, and hypermethylation of lysine 9 of H3 facilitates formation of heterochromatin and is generally associated with gene repression.

The 1765 bp encompassing the HTR gene and both 5' and 3' flank is located within a CpG island. This suggests a positive role of DNA methylation in hTR regulation. However, hTR expression levels and DNA methylation patterns didn’t correlate in cell lines and tumor tissue though there is strong correlation in DNA hypermethylation with hTR levels in ALT cell lines. Some of the studies have proven the role of chromatin remodeling in hTR transcription. Such studies were undertaken with ALT cell lines SUSM-1, KMST6 and W138-SV40 (Atkinson et al. 2005) and human mesenchymal stem cell (Serakinci S.F et al. 2006). In such cell lines the lack of hTR was associated with reduced levels of acetylated histones H3 and H4 and acetylated lysine 9 of H3. Conversely, the hypermethylation of Lysine 9 of H3 facilitates formation of heterochromatin generally associated with gene repression consistent with hTR repression in those cell lines. In contrast, the telomerase positive cell lines 5637, A2780, C33a and Alt cells SKLU, GM847, which express high levels of hTR, had elevated association of acetylated histones H3 and H4, with hypomethylation at Lysine 9 of H3 (Atkinson et al 2005).

I.3.12. Telomerase RNA Localizations

The human telomerase RNA travels through different cellular compartment, and by FISH studies it was evident that hTR accumulates within intra nuclear foci called Cajal bodies (CB). It was found in many tumor cells, but not in primary and ALT cells (where hTERT is minimal). Cajal Bodies are evolutionarily conserved domains present in nuclei in animal and plant cells (Gall 2003). They are enriched with RNA processing enzymes, RNP assembly chaperones, and other protein with structural
roles. Some of RNAs also found in Cajal bodies called small Cajal body RNAs (scaRNAs) which can guide sequence specific modifications of small nuclear RNAs (snRNAs) during their assembly into splicesosomal snRNPs (Jady et al. 2003).

The human telomerase RNA contains H/ACA motif shared by many scaRNAs and large family of small nucleolar RNAs (snoRNAs) (Mitchell et al. 1999a, Henras et al. 2004). The H/ACA motif recruits proteins, including pseudouridine synthase cbf5p/Dyskerin, forming an RNP that can recognize and modify specific sites of target RNA molecule (Henras et al. 2004).

1.3.13. hTR Biogenesis

The human hTR primary transcript is synthesized by RNA polymerase II, capped with tri methyl guanosine at its 5' end, internally modified, and its 3' end is processed to make a mature hTR transcript. Unlike RNA POL II based transcripts, hTR ends in Poly A independent fashion. The proper processing is dependent on H/ACA motif in hTR, which is at 3' end of the molecule. This H/ACA motif is also present in non coding RNAs which guides in post transcriptional modifications. Such non coding RNAs undergo co transcriptional processing by ribonucleoprotein assembly (RNP). This H/ACA motif RNP assembly have a co transcriptional binding of proteins including Dyskerin (Cbf5in yeast) NHP2, NOP10 and GAR1. Human hTR also has these four proteins. The H/ACA motif alone may not be sufficient for TER accumulation. The loops of H/ACA motif 3’ hairpin is proposed to interact with unknown RNP biogenesis factor in case of human telomerase RNA (Fig I.23) (Dragon et al. 2000, Dragony and Collins 2003).

It was expected that the endogenous transcription continues beyond the mature 3’ end, and hTR transcripts extending beyond 500 base were also detected in RT PCR, but not by northern blotting (Wong et al. 2002), as they exist at very low steady state level. Transcription for more than 500 nt downstream of the hTR 3’ end could provide a kinetic delay in 3’ end processing that enhances RNP assembly. The 3’ loop has two separable roles viz., its involvement in a) RNP biogenesis and b) in RNP enrichment in Cajal bodies.

The CR 7 region which contains this H/ACA, a box that is common in snoRNAs, mostly functions as guide RNA in the site specific pseudo-uridinylation of rRNAs in the nucleolus. There are small Cajal bodies specific RNAs (Sca RNAs) in CB which
carries conserved sequence elements as localization signal for Cajal bodies. Conserved sequence motifs are called Cajal Body Box (CAB) and studies have shown that hTR also contains such elements. This CAB boxes are cis acting localization signals with conserved nucleotides of 411-UGAG 414, which directs for CB specific accumulation. The Cab box in hTR shown in Box (Fig I. 22) (Beata Jady et al. 2004).

Fig I. 22: The sequences comprising Cajal Body Box (CAB).

The modification of hTR i.e. its trimethyl cap is expected to happened in CBs, as some methylase activity also associated with CBs. Moreover the hTR is not shuttling to cytoplasm for its modification, so most probably the modification takes place in CBs.

Fig I. 23: self explanatory model of hTR RNP assembly and Processing steps. The Proteins (green) are processing proteins of RNP and in orange is TERT (Reproduced with modification from Collins 2006).
1.3.14. Cell Cycle Regulated Trafficking of Telomerase RNA and TERT.

Telomere is synthesized during S phase in human cells (Ten Hagen et al., 1990). Redistribution of components of telomerase has been observed during S phase. Wong et al. (2002) showed by linking hTERT with GFP, that the fusion protein signaled predominantly nucleolar to nucleoplasmic, as cell progressed to the S phase. The recruitment of telomerase to telomeres is restricted to S phase, i.e. during the timing of telomere elongation. Telomerase accumulates at only a subset of telomeres in given cell at any given time. Telomerase may not act on every telomere during every cell cycle as demonstrated in yeast cells, where only small fraction of telomeres are extended within given cycle (Teixeira et al. 2004). Studies in yeast and human cells show the telomeres extends preferentially short telomeres in a population (Teixeira et al. 2004) Chromosomes replicate at different rates during S phase (Woodfine et al. 2004); their timing of telomere replication may vary with replication induced changes in chromatin structures and telomere accessibility at individual chromosomes.

During G1 phase, hTR and hTERT are observed in separate intra nuclear structures; hTR is present in Cajal bodies (Jady et al. 2004) and hTERT accumulates in distinct nucleoplasmic foci. Specifically during S phase, hTR and hTERT exhibit dynamic redistribution and become targeted to common intranuclear sites.

![Diagram](image)

Fig I. 24: The model depicts the phase specific localization of hTR and hTERT during A) G1 phase B) early S phase C) Mid S phase and arrows are possible trafficking constructed based on observation by Terns group (reproduced with permission from Dr. Terns)
In early S phase both hTR and hTERT can be found associated with nucleoli, but not in shared compartment. hTR is present in Cajal bodies that seem to reside around the periphery of the nucleolus, whereas hTERT seems to be distributed throughout the interior of the nucleolus (Fig 1. 24).

Movement of Cajal bodies to and from nucleoli has been documented previously (Paltani et al. 2000) and may account for the appearance of hTR at nucleoli in S phase (Tomlinson et al. 2006). The hTERT and hTR localization to Cajal bodies associated foci preceded localization to telomeres and suggested that Cajal bodies with compartmentalized cargo of hTR and hTERT deliver telomerase to individual telomeres throughout the cell (Tomlinson et al. 2006).

I.3.15. Functions of hTR

The telomerase RNA is known for its contribution in telomere synthesis. But still steady state meager presence of hTR and its over expression prompted to look for extra telomeric functions of hTR. Still at infancy, hTR is found to have some roles in regulation of certain functional genes in normal metabolism. The hTERC (hTR) presence increases wound healing, and tumorigenic property of overexpressed telomerase (hTERT) component. In mouse models, hTERT exerts inhibitory effect on wound healing and tumorigenesis process in absence of hTR (Cayuela et al. 2005). Inhibition of hTR expression triggers a rapid, telomerase independent growth arrest associated with p53 and CHK1 activation and hTR effects are mediated through ATR, and it is sufficient to impair the ATR mediated DNA damage repair pathways (Kedde M et al. 2007). Still more informations are needed to assign extra telomeric functions to hTR.

I.3.16. Disorders of Telomerase hTERC Variants

Inherited mutations in hTERC underlie one form of rare human disorder, dyskeratosis congenita (DC) that involves haematopoietic failure characterized by abnormal pigmentation in skin, dystrophy in nail, oral lueokoplakia, and immunodeficiency and bone marrow failure. Autosomal dominant form of DC results from germ line inheritance of mutations in hTERC and most of them are heterozygous carrying one
normal allele too. Lymphocytes from DC patients show the abnormal expressions of hTERC, reduced levels of telomerase activity and reduced telomere length.

Mutation in C116T, A117C, C 204G, G304A of hTERC have shorter telomere length of about 3.7 to 4.6 kb when compared to age matched health individuals who possess 8-12 kb and patients bearing these variants showed no telomerase activity in the primary cells from the patients in reconstituted in vivo telomerase assays (Fig I. 25) (Hinh Ly et al. 2005).

Patients heterozygous for these alleles showed lower level of telomerase activity. Variants G228A and G45A show same telomere length as the normal cells and without any effect on telomerase activity (Hinh Ly et al. 2005).

The table I.1 below shows the variants of hTR and associated clinical abnormalities.

Fig I. 25: Mutations in hTR region found in different diseased phenotypes, Mutations of DC given in red color and with aplastic anemia and MDS or PNH are in green. Thick lines indicate nucleotide deletions, whereas boxed regions show a large deletion that completely removes the sequences of the box H/ACA and CR7 domains (reproduced from Hinh Ly et al. 2005).
Table I.1. hTERC variants and associated manifestations in terms of telomerase activity, telomere length and clinical symptoms.

<table>
<thead>
<tr>
<th>hTERC sequence Variant</th>
<th>Clinical diagnosis</th>
<th>Telomere length</th>
<th>Telomerase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core Domain</td>
<td></td>
<td></td>
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<tr>
<td>C116T</td>
<td>Aplastic Anemia (severe pancytopenia)</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>C204G</td>
<td>Aplastic Anemia (Moderate Pancytopenia)</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>A117C</td>
<td>Aplastic Anemia (Severe Pancytopenia)</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>G143A</td>
<td>Dyskeratosis Congenita</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>Δ96-97</td>
<td>Dyskeratosis Congenita</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>CR4-CR5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G305A</td>
<td>Aplastic Anemia (moderate)</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>G322A</td>
<td>Myelo Dysplastic Syndrome</td>
<td>Not determined</td>
<td>+</td>
</tr>
<tr>
<td>H/ACA domain</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Δ389-390</td>
<td>Essential Thrombocytopenia</td>
<td>Not determined</td>
<td>_</td>
</tr>
<tr>
<td>C408G</td>
<td>Dyskeratosis Congenita</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>G450A</td>
<td>Aplastic Anemia (Severe)</td>
<td>+ +</td>
<td>+++</td>
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<td>CR7 domain</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Δ378-451</td>
<td>Dyskeratosis Congenita</td>
<td>+ +</td>
<td>_</td>
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<tr>
<td>Hyper variable Region</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G228A</td>
<td>Aplastic Anemia (moderate) or healthy</td>
<td>+ +</td>
<td>+++</td>
</tr>
</tbody>
</table>

+++ With in reference range,  ++ 2-3kb less than reference range
+ 3-6kb less than reference range.
1.4. Ribozyme Biology

The RNA molecules having intrinsic ability to break and form covalent bonds are called ribozymes (Cech et al. 1981, Kruger et al. 1982, Gurrier Takada and Sydney Altman 1983). Ribozymes were discovered in the group I intervening sequences (IVS) in the pre rRNA of *Tetrahymena thermophila*, by Thomas Cech and his colleagues and from RNA component of *Escherichia coli* bacterial RNAse P by Sydney Altman and his colleagues (Cech et al. 1981, Gurrier Takada et al. 1983). Tom Cech and Altman together shared Nobel prize for the discovery of catalytic RNA.

These RNA molecules are typically small and can catalyze a chemical cleavage reaction or formation of phosphor-ester bond in the absence of protein. Ribozymes are found in nature to work in cis and through studies these cis-acting sequences have been engineered to give a targeted ribozymes in trans. This modification prompted their application to broader areas in gene regulation especially in gene therapy field.

Six types of catalytic motifs are known viz. Group I introns, RNase P, hammerhead ribozyme, hairpin ribozyme, and axe head ribozyme of HDV and RNA transcripts of the mitochondrial DNA plasmid of *Neurospora* (Symons 1987, Foster and Symons 1987, Kijima et al. 1995). The catalytic activity of ribozyme has been demonstrated to occur through the rearrangement of phosphodiester bonds (Von Tol et al. 1990, Kumar and Ellington 1995).

The ribozymes are classified according to their size as large and small ribozymes. The large ribozyme consist of Group I and Group II ribozyme, and RNA component of RNAse P. The class of small ribozyme includes hammerhead ribozyme, hairpin ribozyme, hepatitis delta ribozymes and varkud satellite (VS) RNA and other artificially selected ribonucleic acids (Schubert and Kurreck J 2004).

All ribozymes were believed to be metalloenzymes requiring Mg$^{2+}$or other divalent cations for both folding and catalysis. The two metal ion mechanisms were proposed in which hydrated Mg$^{2+}$ ions played a role in general acid base catalysis. This prediction appears to be correct in Group I introns, but general acid base catalysis appears to be catalytic strategy and in many cases RNA itself rather than passive scaffold for metal ion binding is an active participant in acid base catalysis, in the sense that nucleotide functional groups rather than metal complexes often mimic the roles of amino acids that play crucial role in the active sites of protein enzymes. For
the present study, we used hammerhead ribozyme targeted against telomerase RNA component.

1.4.1. The Hammerhead Ribozyme

The hammerhead ribozyme was discovered in the plant viroid RNAs as satellite RNAs. These satellite RNA of tobacco ring spot virus RNA replicate only in tissues infected with tobacco ring spot virus (TRSV). It becomes encapsidated in TRSV and reduces the accumulation of TRSV and decreased the severity of symptoms as compared to infection by TRSV alone. Thus sTRSV RNA is a molecular parasite of TRSV and potentially may act as antiviral agent (Haseloff et al. 1988). The self cleavage of both (+) and (-) sTRSV was discovered by Bruening and his colleagues who showed that the cleavage products contain 5' hydroxyl and 2', 3' cyclic phosphate termini (Symons et al. 1987). The ribozyme consists of 3 base paired helices I-III connected by 2 single stranded regions and a bulged nucleotide which gives wish bone structure (Fig I. 26).

The core catalytic domains of hammerhead ribozyme has been reduced to 11 nucleotides high lighted in fig 26 (a). Stem I does not have any conserved nucleotides (Basically stem I and III are complementary arms of target) and stem II has two conserved nucleotides (10.1 and 11.1), which usually form base pair adjacent to catalytic core.

Fig I. 26: The numbering and stems of catalytic hammerhead ribozyme shown with its cleavage site. The fig a) is in cis Rz, found in nature and b) right is in Trans, engineered to modulate gene expression. The nucleotides highlighted (between stem I and III) represent core-catalytic domain of the ribozyme.
Ribozyme bearing shorter length of stem II (less than two nucleotide) become less active when compared with ribozymes bearing stem II length of more than two nucleotide.

The most commonly found cleavage triplet in nature is GUC (GUA, AUA have also been observed). The cleavage site in general is said to be NUX and by experiments the preferred cleavage site with in terms of Km are GUC, AUC, GUA, AUA, CUC, UUC, GUU, UUA, AUU, CUA, UUU, CUU while in terms of Kcat they are GUC, CUC, UUC, GUU, AUA, AUC, GUA, UUU, UUA, CUA, AUU, CUU.

Self cleaving reactions have been observed in satellite RNA of TRSV (Buzayan et al. 1986) the avocado sunblotch viroid (Hutchins et al. 1986) and virusoid Lucerne transient streak virus (Foster and Symons 1987). The human hepatitis delta virus (HDV) (Branch and Robertson 1991) and the ribosomal RNA (Symons 1992) have been reported to possess catalytic activities as ribozymes.

I.4.2. Sequence Requirements of Hammerhead Ribozyme

By mutational analysis, minimum nucleotide requirement, which is actually required for catalysis was determined. With exception of position 7 the substitution of any other nucleotide in the conserved single stranded region of central core destroys catalytic activity. At position 7 there is still dependence of particular nucleotide, highest cleavage rates observed with U, followed by G, A, C which have 60%, 50%, 20% activity respectively (Ruffner et al. 1990). In Stem I & II no conserved nucleotides have been found but global structure of ribozyme is affected by nucleotide sequence directly adjacent to the core (Amri and Hegermann 1996).

I.4.3. Catalytic Mechanism

The presence of divalent metal ions is essential for hammerhead catalysis. The metal ion promotes proper folding of the RNA to form catalytic core, and acts as catalytic cofactor (Dahm and Ulhenbeck 1991). Once the ribozyme cleaves its substrate it targets multiple targets as like enzymatic property of turnover.
Fig I. 27: Proposed mechanism of Hammerhead ribozyme mediated phospo-diester bond cleavage.

The mechanism of cleavage proposed was the deprotonation of the 2’ hydroxyl group by magnesium aqua hydroxy complex bound by the pro-R oxygen at the phosphate cleavage site followed by nucleophilic attack of the resultant 2’alkoide on the scissile phosphate, forming penta co-ordinate phosphate intermediate. The 5’ leaving group departs yielding the 2’ 3’ cyclic phosphate with inverted configuration (fig. I. 27).

I.4.4. Ribozyme at Clinical Trials

Three ribozymes have been completed with their Phase II trials angiozyme, a ribozyme targeting VEGFR-1(Flt-1) is the receptor for vascular endothelial growth factor (flt-1), expression of which gets induced in hypoxic condition and it helps in matrix degradation, proliferation, migration and tube formation of endothelial cells. The receptor (Flt1) also gets induced by hypoxic conditions, and decrease in this receptor nullifies the effect of VEGF and prevents the formation of new blood vessels (Angiogenesis) and hypoxia induced injury of tumor cells leads to cell death.

Targeted ribozyme against Flt-1 could ensure the induction of cell death of tumor tissues (Cunningham.C 2002, Weng et al. 2005). A phase II trial for another ribozyme OZ1, against HIV-1 is underway. In another study, involves the stem cells transduced with ribozyme containing virus and then injected to the patients with non- Hodgkin’s lymphoma in HIV patients (Michinzie et al. 2003, Ngok et al. 2004). Other clinical
trials are Herzyme against Her/neu oncogene, and ribozyme against Hepatitis C virus (HCV) that is Heptazyme LY 466700 has also passed through clinical trials. Novel tools like siRNA have taken over ribozyme therapeutics as they offer more efficacy and mechanism of action is independent of target structure.

I.5. Telomerase as a Therapeutic Target

Telomerase expression is regained in cancerous foci compared to adjacent or normal tissue, and it provides the proliferative advantage by maintaining telomere length. Hence telomerase seems to be one of the most commonly associated and the promising drug target.

I.5.1. How Telomerase Therapy Affect Cell Proliferation

Terminal DNA gets shortened by about 50-150 bp at every cell division in the absence of telomerase. After certain generations the telomere length becomes minimal and attains the critical length and it may further extend to the sub telomeric region, which will activate the DNA damage-signaling programmes. In presence of p53, telomere mediated induction of apoptotic programs are activated, in absence, p53 independent pathways may result in induction of apoptosis or differentiation programmes in cancers.

I.5.2. Strategies to Target Telomerase

Telomerase is a unique multicomponent RNA protein complex with a reverse transcriptase catalytic subunit (hTERT) along with an RNA molecule (hTR) as core components while other regulatory proteins hsp90, p23, TEP1 and dyskerin etc, are associated with this core. In view of the regulation of telomerase at transcriptional, post transcriptional and posttranslational level, different strategies have been proposed to devise appropriate treatment modalities to attenuate or suppress telomerase activity or reduce telomere length (Table I. 2)
Table I.2: Telomerase based Therapeutics

<table>
<thead>
<tr>
<th>Target molecule</th>
<th>Inhibitors</th>
<th>Comments</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>I) Pre transcriptional Promoter regulation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sp1 (+ regulator of hTR promoter)</td>
<td>Retinoids, Tamoxifen NY Mutants</td>
<td>Signal Transduction Inhibitors,</td>
<td>Poole et al. 2001</td>
</tr>
<tr>
<td>Sp3 ('-' regulator of hTR promoter)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRb (+ regulator of hTR promoter)</td>
<td></td>
<td>Transcription factor inhibition</td>
<td>Xu et al. 2000</td>
</tr>
<tr>
<td>P53, E2F1 ('-' regulator of hTR promoter)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein Kinase C Alpha</td>
<td>bis indole maleimide</td>
<td>Phosphorylation cannot be achieved and results in reduction or abolition of telomerase activity</td>
<td></td>
</tr>
</tbody>
</table>
I.5.3. Limitations.

The anti-telomerase therapeutics poses a problem that inhibition of telomerase will pronounce their effect after multiple generations as erosion of telomeres is gradual. Telomeres in human tumor cells are generally shorter than those in normal cells and ranges from 100 to few thousand bases long. And to observe the effect of anti telomerase, one needs longer period and in such time span lag, cells or human system may develop a drug resistance, or exposure of harmful effect of drug toxicity due to prolonged exposure (stopping treatment would result in re-growing of telomeres) or bystander effect (chemotherapy) and in such period it is also anticipated that additional mutational events may take care of telomerase independent telomere length regulation (Alternative Lengthening of Telomere). But such a time gap may be useful to the terminally ill patients if the drug is non-toxic and effective.

As most cancer cells possess a high proliferative index compared with normal stem or germ line cells anti telomerase therapy is expected to have minimal side effects such as bone marrow toxicity and this would be much safer for patients than conventional chemotherapy (Blasco et al. 1997). However, telomerase negative tumors have been identified and alternative mechanism of maintenance of telomere (ALT) exists in some tumors. So anti-telomerase therapy can’t be regarded as a fool-proof way of treating all human cancers. Though it will deprive tumor cells of a growth advantage over surrounding normal cells which could result in a significant reduction in malignant progression of tumor (Shay and Bacchetti 1997).
Telomerase targeting is not limited only to the telomerase core complex. In addition the disruption of telomerase maintenance could be achieved through targeting components other than telomerase that are involved in telomere maintenance mechanism e.g., targeting TRF1 and TRF2 and Pot I proteins found in telomere maintenance. The simultaneous downregulation of telomerase and disruption of telomere maintenance mechanism by various means might be expected to produce even greater detrimental effects on cancer cell viability.

The development of anti telomerase therapy, drug developments and clinical studies have been undertaken in rapid succession and have been completed to different extents. It is likely that telomerase inhibitors will have most impact in minimal disease states such as maintenance therapy after tumor debulking by chemotherapy or in combination with cytotoxic chemotherapy. Possibilities may exist to use telomerase inhibitors as adjuvant therapy in early disease. Present scenarios have improved the field of telomerase-based therapeutics, as the assumption that telomerase has role only in maintaining telomeres is not quite valid. As extra-curricular functions of telomere including its role in carcinogenesis and other cellular events open up new avenues and promises around telomerase as therapeutic target. The strategies and tools used for targeting telomerase and telomeres are given in Table. I. 2

The usage of existing technologies and recent advancements in assays and innovative trial design would facilitate to understand the basic biology of human telomerase to strive for betterment of human health.

I.6. Objectives of the Present Study.

Telomerase RNA molecule is selected in our study to evaluate its suitability as a good target in cancers. As the copy number and transcript levels of hTR is elevated in most of the cancer, by reducing the hTR level, it was expected to alter telomerase activity, restrict telomere synthesis and induce apoptosis in cancer cells. The present study involves a) to develop and to evaluate the designed ribozyme in vitro and in vivo b) to study the alterations in telomerase associated gene expression in ribozyme expressing cells. The following objectives were charted in the present study.
• Designing of hammerhead ribozyme against telomerase RNA.

• Cloning of human telomerase RNA.

• Cloning of ribozyme (active and mutant Rz) in pStu I vector.

• *In vitro* evaluation of cleavage reaction.

• Cloning of ribozyme (active and mutant Rz) in mammalian expression vector pCi-NEO.

• Expression of constructs in HeLa cells.
  
  • Analysis of ribozyme expression.

  • Effect of ribozyme against hTR.

  • Effect on telomere and telomerase activity.

  • Effect on cell cycle.

  • Effect on morphology of cells.

• Analysis of Global gene transcript profiling by Differential display.

• Proteomic profiling to identify the modulation of gene expression at translational level.

• *In silico* Analysis of differentially expressed genes.