Excessive Telomere Shortening and Accumulation of DNA Damage Signals May be to Blame for Latent Cancer Diagnoses in Pedriatric Survivors

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Disclaimer: I did use AI in my paper, but only to find scholarly articles that mentioned «The Trinity at the Heart of the DNA Damage Response.» I had previously heard about the «holy trinity» of DNA damage markers in several other papers discussing telomere damage, but no specific proteins were discussed. This led me to conduct a general Google search for «holy trinity of DNA damage to telomeres». The first results came from the AI overview, which listed ATM, ATR, and DNA-PK as the primary regulators of damage responses. This overview provided the sources from which the information was pulled, and the aforementioned article was included. After reading this article, I gained a general understanding of DNA damage markers and, more specifically, the functions of each protein kinase within the «holy trinity». The Google AI overview was the only source of artificial intelligence I consulted. Still, I found it helpful for summarizing the article and the major points I needed to understand regarding DNA damage signals.

The majority of cancers arise from mechanisms within cells that attempt to prevent DNA damage. In pediatric cancers, the mechanisms underlying cell immortalization remain under investigation. However, in the past 10 years, there has been an increased observation that pediatric cancer survivors experience a secondary cancer diagnosis later in life. While the cancer treatment methods of radiation and chemotherapy can target immediate cancer cells in pediatric patients, they are also indirectly affecting normal cells and excessively shortening normal telomeres. This research on transgenic mouse models utilizes Telomere Restriction Fragment analysis (TRF) to assess telomere length before and after cancer treatments. Along with FISH-flow to identify the presence of «holy trinity» DNA damage markers after cancer treatments.

Telomeres and Telomerase

Imagine you are Juan Ponce de León in search of the infamous Fountain of Youth, and, knowing that this search could be the remedy for aging and immortality, your thirst for it drives you. You finally find the fountain itself and drink from its magical waters, obtaining something other people have only dreamed of. Your body has been restored to full youth, and you will never have to spend a day of your life scared by the contraction of a potential illness or disease. In fact, the natural aging of your body has ceased altogether. However, because of your immortality, you are stuck watching the world around you change, and the people you love wither away. You find yourself isolated in a world you no longer recognize or feel you have a place in. Immortality isn›t always what you expect, and it comes with a cost.

The Fountain of Youth is a myth that has been told for centuries, but its message about the consequences of immortality and human desires remains prevalent. In the real world, there are no springs of youth; rather, the immortality we may experience results in a painful and deadly disease. Cancer results when cells in the body are at significant risk of damage, and response systems activate to «save» the cell, inducing immortality. Immortality in cells leads to continued, uncontrolled cell division without the proper death of other cells in that area. The ultimate result is the formation of an abnormal mass of cells within a tissue; this is called a tumor. The uncontrolled cell division leading to tumor formation can also create a greater opportunity for DNA damage and mutation within these cells. If a cell population harbors mutations in its DNA that have the potential to induce disease, these mutations can lead to cancerous cells that form malignant tumors. The diseases that arise from these malignant tumors are cancer, which can present in varying tissues, organs, and cell types. To better understand how to cure cancer and prevent it altogether, it is essential to examine what happens within a cell for it to become immortal and develop damage.

A fundamental process of development throughout a person›s lifetime is cellular replication, which occurs through an evolutionarily conserved, highly regulated process called the cell cycle. The cell cycle is also part of another important process, mitosis, which results in the cellular division of a parent cell into two identical daughter cells. During mitosis, the entire DNA genome is replicated and divided between the two daughter cells. This DNA replication process is highly conserved across all organisms and is essential for proper cell function, which in turn supports tissue and organ function. The cell cycle is constantly occurring in the human body to regenerate dying cell populations and support growing ones. Although the cell cycle is a conserved, foundational process, DNA becomes shorter with each division. This is referred to as «the end replication problem,» in which a small end portion of the DNA sequence is lost with each cycle (Ackermann). The final sequence of DNA codes for the removal of proteins necessary for mitosis to occur, so these end sequences are not actually replicated, meaning the daughter cells receive a shorter DNA sequence.

No important DNA is lost during cellular replication because the ends are capped by telomeres, which are repetitive DNA sequences that do not encode proteins. Simply put, telomeres are protective caps at the ends of chromosomes that prevent end-to-end fusion with other chromosomes or the degradation of the DNA sequence (Ackermann). Since cell division is continuous throughout an organism›s life, telomere length serves as a biological marker for chronological age. The average length of a telomere in an adult human›s somatic cell is between 4-15 kilobase pairs in comparison to the 50,000-300,000 kilobase pairs of a single chromosome (Gorenjak). With every cell cycle, it is reasoned that telomeres shorten by 50-200 base pairs, and newly formed daughter cells inherit shorter telomeres, which is how telomeres serve as a mechanism for determining biological age. The protein complex that binds to the DNA sequence is called telomerase, which creates the initial telomere of the parent cell, which is then replicated in the daughter cells, ensuring that every chromosome in every cell is properly capped with a telomere.

Telomerase solves «the end replication problem» by adding repetitive DNA sequences that do not code for proteins but are solely used to form telomeres. Telomerase is an active enzyme during embryonic development, when cell growth and division are high. However, the enzyme is terminated in somatic cells of an organism once cell differentiation begins. During differentiation, cells lose their potential to become any cell type necessary for development and become specialized to support specific tissue regions. The overall function of telomerase and its termination are regulated by several genes that ensure the cell›s proliferative needs are met, allowing it to function (Gorenjak). As mentioned earlier, telomerase is an enzyme composed of protein subunits, *TERT* and *TERC*, giving it a multifaceted function. *TERC* initiates telomere formation by introducing telomere RNA that interacts with the DNA promoter region to facilitate extension. Then the compound *TERT*, also known as reverse transcriptase, adds nucleotides to the ends of telomeres during cell division, ultimately extending telomeres. Telomerase is essential during cellular division, as it can initiate telomere extension during replication if signals indicate that the telomere is at risk of being excessively shortened, leading to DNA damage. However, overactivation of telomerase or damage signals in the cell can cause excessive telomere lengthening, which becomes a triggered response in all future daughter cells. Mechanisms such as telomerase reactivation and alternative lengthening of telomeres can produce an immortal cell population that often leads to diseases such as cancer.

Telomere Damage and Cancer

A cancerous cell is definitively immortal and experiences no biological aging due to a telomere alteration, making it excessively long and prone to DNA mutations that lead to disease. Telomere lengthening can be triggered by aging factors, which is typically why cancers arise in older populations, as cells have continued to divide and approach the Hayflick limit. The Hayflick limit is the number of times a chromosome

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can be divided and replicated before its telomeres become short and no longer protect against DNA damage. The limit is estimated at around 60 division cycles, and once reached, a cell enters senescence, in which it no longer divides but remains actively involved, serving as structural support for the surrounding tissue. Senescence is a way to prevent DNA damage from excessive telomere shortening; however, rather than signaling a cell to enter senescence, it may signal the need to reactivate telomere lengthening. Telomere extension can occur in two ways: reactivation of telomerase or the alternative lengthening of telomeres (ALT) mechanism.

There is not much currently understood about the mechanisms contributing to ALT. However, extensive research worldwide has been conducted on the mechanisms that reactivate telomerase and the overactive function it acquires. Cancer cells can maintain immortality by activating telomerase, which extends telomere length indefinitely. An upregulation of telomerase can induce cell division leading to tumor formation. At the same time, impaired telomerase function can cause excessively short telomeres, leading to chromosomal instability that may recruit alternative lengthening mechanisms, which also lead to cancer. The occurrence of cancerous diseases is usually not observed until later in life, when cells are more likely to enter senescence and mutations in telomerase or the activation of ALTs occur.

Telomere length is highly variable at birth due to genetic predispositions and environmental teratogens to which a fetus is exposed. As development continues in childhood, telomere lengths begin to normalize and average out as biological processes are increasingly regulated internally and less rapid, major environmental changes occur. Previous studies have shown that gender, race, paternal age, smoking status, physical activity, traumatic events, obesity, and oxidative stress can all impact telomere length and can cause a fetus a predisposition for telomere length abnormalities (Gorenjak). Intrinsic risk factors include maternal age, for which research has observed a 6-15% increase in risk for every 5-year increase in maternal age. Another impacting factor is that structural birth defects consistently increase the risk of childhood cancer, potentially due to cell populations already experiencing replicative stress. Genetic factors, such as germline DNA mutations, chromosomal aneuploidy, and epigenetic disorders, account for 5-10% of childhood cancers (Spector). These genetic determinants of childhood cancer have been heavily researched using genome-wide association studies, which have identified common variants associated with cancer. While researchers can more fully understand the underlying genetic abnormalities contributing to several cancers, there remain over 90% of pediatric cancer types with unidentified or unspecified mechanisms.

As outlined above, telomere shortening is associated with a mechanism promoting cancer, but may also be induced by cancer treatments themselves. Parental exposure or even fetal exposure to high-dose ionizing radiation or chemotherapy increases the risk of pediatric cancers since these treatments are directly purposed to induce cell stress that will lead to cancerous cell death (Spector). When these treatment methods are utilized in such young patients, there is an increased risk of latent disease development because their biological processes are so vulnerable. Development in the first years of infancy occurs at an unprecedented rate, and many biological processes continue to be regulated during these years. The first years of growth are characterized by high cellular turnover, leading to rapid telomere shortening, which later stabilizes in early adulthood (Gorenjak). This is why an average telomere length is difficult to determine in childhood because there is great variability observed across cells. It is not until early adulthood that an average telomere length can be observed.

Telomerase activity, which induces this longer pediatric telomere length, can lead to the occurrence of mutations in *TERT* and *TERC* complexes. A mutation in one of these two foundational complexes is likely to occur in genetic cancers that cause an upregulation of telomerase, which continues to extend telomere length. There is also the potential that in-utero teratogens and environmental stressors induce replicative stress during development, causing early recruitment of cell damage signals such as POT1, ATM, ATR, and DNA-PK. POT1 is directly responsible for recognizing telomere length abnormalities (Richard). The other three singles mentioned are considered the «holy trinity» of DNA damage signaling. ATM is responsible for identifying double-stranded DNA breaks, ATR recognizes replication stress and single-stranded DNA breaks, and DNA-PK repairs the double-stranded breaks through non-homologous end joining (Blackford). Telomeres in pediatric cancer patients are often longer than in healthy individuals because they have less exposure to environmental stressors and decreased aging. However, the nature of chemotherapy treatments is to induce oxidative stress in immortal and proliferating cells to stop cell division. The treatments induce oxidative stress directed towards creating breaks in telomeres that then trigger cell death or senescence (Gorenjak). However, radiation and chemotherapy are not cell-specific treatments, and thus all surrounding cells and tissue receive the same oxidative stress-induction, leading to an accumulation of damage signals in noncancerous cells as well.

Treatments that expose individuals to oxidative stress cause regulated DNA damage to immortal cells in attempts to prevent further proliferation and trigger the DNA damage response of senescence. These are the rationales and mechanisms behind radiation and chemotherapy when treating cancer cells. However, this exposure to oxidative stress using these treatments may induce DNA damage in healthy cell populations as well, putting pediatric cancer survivors at risk for telomere dysfunction leading to subsequent malignant neoplasms (Richard). As mentioned earlier, there is great variability in childhood telomere length due to developmental processes still being regulated. Introducing further reproductive stress to such young patients is putting healthy tissue populations at risk for future dysfunction.

Research in the last ten years has seen a higher prevalence of latent cancer diagnosis in childhood cancer patients, potentially due to the treatment methods. Researchers Richard and Man have investigated that the subsequent tumor formations are more likely to occur in the regions where the previous treatment was directed, such as children who received radiation in the head, throat, and neck regions had greater chances of developing thyroid subsequent malignant neoplasm. Other researchers have shown a higher risk of subsequent malignant tumors occurring with specific radiation treatments rather than chemotherapy due to the ionizing oxidative stress X-rays (Gramatgers). Further, they observed that there was accelerated shortening of telomeres in pediatric survivors compared to control participants in two separate studies by two separate researchers, Aalbers and Gramatges. The research that put the necessity of finding alternative treatments for pediatric patients into perspective was a study conducted on a St. Jude Lifetime Cohort population. This cohort was formed with the initial purpose of checking in on patients post-treatment and monitoring their health. However, after noticing that quite a few survivors were receiving a secondary diagnosis while only in their thirties, researchers began looking at telomere length impacted by treatment methods (Song). These previous studies have shaped the goals and methods that will be proposed in the following section for the purpose of looking at how treatment modalities of radiation and chemotherapy affect telomere length and whether there is an increase in DNA damage signals as a result.

Research Proposal

This research will work to address two specific aims utilizing two different methods of analyzing telomeres. The first aim will be to observe how telomere length is affected before and after cancer treatments, more specifically, chemotherapy and radiation treatments. The second aim is to identify DNA damage markers before and after the above treatments in both cancer and normal cells. There has been an increase in research within recent years on pediatric cancer survivors and the long-term effects experienced from cancer treatments. The aims stated above will aid in addressing why pediatric cancer survivors have a greater chance of developing other forms of cancer later in life. The most common treatments utilized in both adult and pediatric cancers are chemotherapy and radiation therapy, which target telomere lengthening mechanisms. These mechanisms, either telomerase or alternative lengthening mechanisms, become inactivated to prevent the immortality of cancer cells. However, these treatments are inducing oxidative stress in all cells within their exposure range,

both cancer and healthy cells. Oxidative stress induces an accumulation of DNA damage markers within cells to trigger DNA recombination and repair. This research will work to observe both telomere length and DNA damage markers after cancer treatments to determine if these are mechanisms of higher cancer risk later in the life of pediatric patients.

This research will be conducted in juvenile mice models utilizing telomere restriction fragment (TRF) analysis to look at changes in telomere length and fluorescence in situ hybridization (FISH) to observe DNA damage markers. In response to aim 1, I hypothesize that there will be a significant decrease in telomere length for co-treatment mice models due to both chemical agents and ionizing radiation targeting varying telomere lengthening mechanisms. I also expect there to be a greater number of DNA damage markers in co-treatment mice due to multiple mechanisms of DNA replication being targeted via varying treatments.

Cancer mice models will be created by upregulating the activity of *TERT* in telomerase. To accomplish this, a gene will be introduced into the mices genome, which will encode for an upregulation of transcription factors for *TERT*, ultimately causing increased activity of *TERT* and telomerase. A DNA sequence of *TERT* with an upregulated promoter region will be introduced to the nucleus of fertilized mouse eggs using a glass micropipette. Once within the nucleus, this newly introduced DNA will be integrated into the genome during the first several cell divisions. The transgenic mice eggs will be implanted in adult female mice to carry during development and birth (Sharing Laboratory Resources, 1994). Transgenic offspring using an upregulation of *TERT* transcription factors require several generations to produce. Although this method is highly feasible, it will require a longer time to produce the desired homologous genome for upregulation of *TERT*. From these mice offspring, those that express cancer development, malignant or not, in the first several weeks after birth will be used within the experimental conditions. A mice model has been chosen for this experiment because it will provide an understanding of telomere length before and after specific treatment options that are controlled. The majority of research highlighting the latent effects of cancer treatments done on pediatric patients is either specific to the type of initial cancer diagnosis or secondary diagnosis and is thus limited in the extent of cancers they discuss. Research conducted in 2020 by Nan Song was my first insight into the latent adverse effects of radiation and chemotherapy treatments. These researchers utilized the St. Jude Cohort study population to look at how specific treatment types and locations may have induced chronic health concerns later in life. The population addressed by Song was pediatric-specific primary diagnosis, and they observed a significant decrease in leukocyte telomere length when DNA samples were taken almost 20 years later. The cancer types observed were varying, but overwhelming; their results suggested an increase in aging for survivors by 11.4 years (Song, 2020). If these researchers were observing an increase in aging among survivors, then it can be inferred that DNA damage markers were increased in these patients› cells, leading to senescence and telomere lengthening.

These previous results and others lead me to build my current research. To look at telomere length in the mice models before and after varying cancer treatments I will be utilizing a telomere restriction fragment analysis (TRF). Experimental mice are the transgenic mice with increased *TERT* activity, whereas the control mice were non-altered mice born from wild-type parents. Somatic cells will be extracted from the mice one week after birth. If cancer or tumor formation is already prevalent at this time, then cells will be taken from that specific site. DNA will be extracted using a DNeasy Tissue and Blood Kit. For more accurate readings, it is recommended that a cell pellet of about $1x10^{\wedge 6}$ cells be used, which will then be quantified using a spectrophotometer such as Nanodrop. Next, the DNA must be digested using a combination of reaction enzymes and separated on an agarose gel, which was loaded with radiolabeled TRF marker and Gel Red. The radiolabeled TRF marker can be visualized after hybridization with a telomere-specific probe, indicating specific telomere length, while the remaining genomic DNA is visualized with Gel Red. Next, the DNA undergoes hybridization and denaturation, during which the C-rich probe is introduced to identify telomere repeat sequences. The gel undergoes a series of washes and then is prepared for scanning. The gel is wrapped in the cassette, and a screen is placed on top. Exposure is for 4 hours or overnight using an imaging software such as Typhoon PhosphorImager. The TRF lengths are then calculated (Mender & Shay, 2015). The end product of TRF analysis is a Southern Blot with DNA appearing as a «smear» on the gel, which is what is quantified. The Southern Blot is also run with a DNA molecular weight ladder in between every 10 lanes. Previous research has shown that TRF length analysis of Leukocyte telomere length is typically performed over a range of 3-20 kilobases, so the DNA ladder will span this entire range to observe the varying telomere lengths. The controls of this TRF will include a positive control of the wild-type mice and a negative control where no DNA sample is added to a well to ensure there is no contamination of the samples or gel. Following the controls on the Southern blot would be DNA samples from all experimental mice prior to cancer treatment, allowing for initial telomere length determination.

After determining telomere lengths prior to treatment, the experimental groups would be separated and receive varying treatments between 3-4 weeks of age, when they are still in the juvenile stage. As of now, the estimated number of mice per condition is 5. Conditions would include five mice that undergo chemotherapy, five that undergo radiation treatment, and five co-treatment mice that experience both treatments. The procedures described above for TRF analysis would be repeated when mice are approximately 3 months old to determine the latent effects of treatments on telomere length. The calculation of telomere length would indicate whether telomere shortening occurs and, if so, to what extent, depending on the treatment type. These results would then be compared to control mice to see whether the treatment caused increased shortening outside of natural shortening from cellular division and aging. I predict that mice that underwent radiotherapy will have shorter telomere length than chemotherapy- or control-treated mice, because radiotherapy can infiltrate a larger tissue region that may include healthy cells. I also expect that co-treatment will result in the greatest telomere shortening compared to all other conditions.

Since these mice are being studied in their juvenile stages, we would also look for any markers that could suggest latent development of cancer during adulthood. The three major DNA damage markers looked at in this research are ATM, ATR, and DNA-PK because these three kinases are the major regulators of damage response mechanisms (Blackford & Jackson, 2017). To identify these markers in post-treatment cells, FISH-Flow analysis will be utilized to visualize their mRNA presence in the cell. Somatic cells from all mice are obtained, cancer treatment mice cells are collected from the region of treatment, and placed in a cell wash device that standardizes the preparation of all cells for analysis. These cells are suspended and fixed, to permeabilize the cells that are resuspended in 70% ethanol so that when probes are introduced, they can hybridize. To introduce the probes, the cells are rehybridized with a buffer containing the specific FISH probes coding for ATM, ATR, and DNA-PK mRNA. The cells are transferred to a plate and sealed, then incubated in a dark space in a static position. After a minimum of six hours, the plates are removed, and the cells are washed to remove excessive FISH probes. The cells are then resuspended in DAPI solution and left to incubate once again. Then, using flow cytometry, the FISH probes can be visualized along with the DAPI stain, and analysis is run using software such as FloJo (Antony et al., 2023). The final result of the FISH-flow analysis is both a visual display of DAPI and FISH probes within the cell, and will be run with a no-serum control in which no probes were introduced, which will identify potential background staining from binding to non-specific sites. A visual result occurs along with a graph from software analysis, suggesting the quantity gradient of DAPI and probes present in the cell.

From these results, I would predict that these DNA damage markers would have a greater quantity in mice that received cancer treatments than in the control mice. Previous research has highlighted that the therapies involved in cancer treatment utilize some of the direct triggers of DNA damage, such as UV light and platinum chemotherapies (Mouw et al., 2017). Also, pediatric patients are receiving treatment when key

biological processes are still being regulated and may impair normal DNA repair capacities. Even in research conducted on pediatric cancer survivors, those who received radiation treatments near the head, throat, and chest regions or received alkylating chemotherapy had an increased likelihood of developing thyroid cancer or subsequent malignant neoplasms five or more years after their initial treatment and diagnosis (Richard et al., 2020). From these previous findings of latent tumor formation post-cancer treatments in human patients, I would expect to see an increase in potentially all three DNA damage markers in comparison to the control, as these could serve as the potential triggers for the reactivation of telomere extension.

Conclusion

The two major mechanisms inducing cell immortality include telomerase reactivation and alternative lengthening of telomeres. Both processes induce cell proliferation and accumulation, leading to tumor formation that can be benign or malignant, with malignant formations being the production of cancer. The most commonly used cancer treatments are radiation therapy and chemotherapy, which target the identified cancerous cell population through tissue layers to induce DNA replication damage, preventing further cellular division and marking the cancer cells for degradation. In order to achieve a cancer-free state, treatments must be continuous and repetitive, and often occur in tandem to increase stress on the cancer cells. However, while inducing oxidative stress in the cancerous cells, the surrounding non-cancer cells are also experiencing this stress dysregulation in normal processes.

The cancer treatments used for oxidative stress remain quite similar across cancer and patient types. While it is rare to develop cancer before the age of 20, it is still possible and is the sad reality of pediatric cancer, with the most common therapies including surgery, radiation, and chemotherapy. During any cancer treatment, the amount of exposure is calibrated to the amount of targeted cancerous cells; however, even with treatments regulated for patient type, these methods put the overall body under increased stress. Children are extremely vulnerable to the side effects of these treatments because their biological processes are still undergoing regulation and could experience lifelong dysregulation if damaged.

The methods behind radiation and chemotherapy target mitotically active cells, cells that continue to proliferate. To stop further cell division, the cell›s immortality is targeted, which will allow cancer cells to be prone to degradation during treatments. The key mechanism allowing the cell its immortality is the maintenance of excessively long telomeres, so treatments aim to shorten telomeres by targeting and terminating the regulatory mechanism of immortality. The use of TRF analysis in the mice model explained above allows the somatic telomere length before and after treatment to be determined alongside a control to observe the true extent of telomere shortening that occurs. Cancer survivors tend to have shorter telomere lengths in general compared to non-cancer individuals, potentially making these patients more prone to DNA damage. Pediatric survivors are especially prone to increased DNA damage while also having a longer aging period remaining post-treatment, in which latent malignant tumor formation may occur. Should telomere shortening occur in pediatric patients after treatment, they may exhibit increased DNA damage signals in cells within the previous treatment area, which could trigger a second cancer diagnosis in later years.

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