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Telomere-mediated Genomic Instability in Cells from Ataxia Telangiectasia Patients

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Ataxia Telangiectasia Mutated protein (ATM) is one of the first DNA damage sensors and is involved in telomere repair. Telomeres help maintain the stability of our chromosomes by protecting their ends from degradation. AT patients lacking the gene ATM are susceptible to acquiring chromosomal anomalies and show heightened susceptibility to cancer. Here we show that cells from AT patients display considerable telomere attrition. Further, induced DNA damage and genomic instability were found to be more in DNA repair deficient $ATM^{-/-}$ cells than in normal cells. Results demonstrate that the ATM- deficient (heterozygous and homozygous) cells are sensitive to arsenite- and radiation-induced oxidative stress. Elevated numbers of chromosome alterations were seen in arsenic-treated and irradiated $ATM^{-/-}$ cells. The results might help in understanding the extent of susceptibility of AT patients to oxidative stress.

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Introduction

Ataxia Telangiectasia Mutated (ATM) protein is the most important and one of the first protein kinases to sense the DNA lesions like double stranded breaks (DSBs).[1,2] ATM also influences cell cycle check point proteins, apoptosis and recombination processes.[3-5] Deletion or abnormal coding sequence of *ATM* can cause a rare pleiotropic autosomal recessive disorder, Ataxia Telangiectasia (A-T).[6] The disease is identified by neuronal dysfunction (*ataxia*) or gradual cerebellar cortical atrophy, dilated blood vessels in the eye and facial area (*oculocutaneous telangiectasia*), chromosomal instability, immunodeficiency, susceptibility to cancer, increased radiation sensitivity, and cell cycle abnormalities.[7-13] Cells from AT patients have shown accelerated telomere shortening which is deleterious to the stability of the genome.[3]

Telomeres are terminal regions of linear chromosomes and contain repetitive sequences of DNA. They function in capping chromosomes to prevent end-to-end fusion, chromosomal degradation and maintain stability for progressive cell division and maintain genome integrity.[14] They are easily susceptible to damage such as chromosomal aberrations that can be effectively used as a biomarker for analysing those populations that have been exposed to mutagens, carcinogens, etc.[15] Previous studies have shown that functional inactivation of ATM leads to telomere shortening, chromosome instability, thereby suggesting an important role for the mammalian ATM gene in maintaining telomere integrity. It is observed that telomeric sequences are lost with cell division and from oxidative stress.[16] Telomere shortening is critical and may ultimately compromise the stability of chromosomes, with possibilities for aging and oncogenesis.[17,18]

A well-known and potent carcinogenic agent, arsenite is known to cause oxidative stress.[19] Dangerous levels of contamination can occur through long-term ingestion of high amounts of arsenic. It has also been shown that arsenite induces genotoxic effects in human fibroblasts, elevates NADH oxidase activity and induces DNA strand breaks by generating reactive oxygen species.[19-22] In addition, ionising radiation is known to cause DNA damage by direct effects and recent studies have shown how ATM kinase gets activated by ionizing radiation.[23]

Materials and Methods

Cells and Culture Conditions: AT or ATM cells were obtained from Coriell Cell Repositories (USA). The human patient fibroblast types

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were $ATM^{-/-}$ or homozygous knockout strain AG04405A, GM05823E and GM02052F and a heterozygous strain $ATM^{+/-}$ AG 03059A. Normal human lung fibroblasts, IMR-90 cells, also obtained from Coriell Cell Repositories were used as control in this study. All the cells were maintained consistently in complete Minimal Essential Medium with supplements as suggested by the supplier. These were kept in log phase in a humidified 5% CO_2 incubator at 37°C.

To induce oxidative stress, the stock solution of 1 mg/ml Sodium Arsenite was prepared using double distilled water and diluted with phosphate buffered saline or PBS. The cells were treated with arsenite for 24 hours after attachment. For all studies, appropriate volumes were added to achieve final concentration of 1.5 µg/ml (11.5 µmol) and 3.0 µg/ml (23 µmol). Every assay had a control without the drug. Cells were exposed to arsenite for 24 hours.[16,20] In addition, another set of the same cell lines, were irradiated with gamma rays (2Gy). They were allowed to undergo repair for 24hours and were then cultured and harvested for chromosomal studies.

Terminal Restriction Fragment (TRF) Length Analysis: TRF analysis was carried out using Telo-TAGGG Length Assay Kit (Roche Applied Science USA) and the chemiluminescence signals were examined and analysed by the Kodak Imaging software to calculate the measurements of the average TRF length of each of the sample.

Micronucleus Assay: Micronucleus analysis was done on cells (arsenite treated and untreated) as described earlier.[24]

Alkaline Single Cell Gel Electrophoresis (Comet Assay): Single cell gel electrophoresis or comet assay was performed to determine the DNA damage induced by oxidative stress in normal and AT cells.[20] About 50 comet samples were randomly analyzed using Comet Imager Software.

Peptide Nucleic Acid Fluorescence In Situ Hybridisation (PNA-FISH): Metaphase spreads prepared from the samples were subjected to two colour PNA-FISH using Cy3 labelled telomere probe and FITC-labelled centromere probes. The procedure for PNA-FISH was described earlier and for every sample 50 spreads were analysed.[3]

Comet Fluorescence In Situ Hybridisation (Comet FISH): The comet slides (minus SYBR staining) were prepared as per the regular comet assay. Over-night dehydration of slides in 100% ethanol at 4°C was carried out. Denaturation was carried out chemically by incubating the slides in 0.5M NaOH/1M NaCl (heat denaturation was not possible as agarose would melt). The slides were then subjected to neutralization and were immediately dehydrated in ice-cold ethanol series (70, 90, 100%, 5 min each). Clean cover slips with hybridization mix were affixed on to the air-dried slides and slides were placed in a humidified chamber at room temperature. Subsequent steps in hybridisation protocol followed as described earlier.[3,20] Once air dried, the slides were counterstained with anti-fade SYBR Green and placed in a light protected storage box.

Multicolour Fluorescence In Situ Hybridisation (mFISH): Multicolour FISH was performed on metaphase spreads derived from normal and AT cells to detect chromosome abnormalities if any in the samples. The detailed procedure was given in Hande et al.[25]

Results

Higher telomere attrition in $ATM^{-/-}$ cells

We began by determining the extent of telomere shortening in AT knock out cells by performing TRF to measure telomere length. $ATM^{-/-}$ showed acute telomere attrition when compared to $ATM^{+/-}$ and normal cells under untreated conditions.

Increased micronuclei in $ATM^{-/-}$ cells following oxidative stress

Micronucleus assay was performed on untreated and arsenite treated cells. It was observed that under untreated conditions, normal fibroblasts (IMR90 cells) showed minimal micronuclei induction when compared to $ATM^{+/-}$ and $ATM^{-/-}$ cells. It was also seen that genomic instability was higher in the DNA repair deficient $ATM^{-/-}$ when compared to $ATM^{+/-}$ cells. With arsenite treatment, a dose dependent increase in the number of micronuclei induced was observed.

Higher DNA damage in $ATM^{-/-}$ cells

DNA damage induction by arsenite treatment was measured by comet assay. A significant increase in tail moment was seen in $ATM^{-/-}$ cells when compared to control and heterozygous AT cells. Following arsenite treatment, a dose dependent increase in the tail moment of $ATM^{-/-}$ cells was evident. The normal untreated control cells did not show marked tail moment, however, $ATM^{-/-}$ cells treated with a high dose (23 µmol) displayed higher DNA damage.

Elevated telomeric DNA fragments in damaged nuclear regions

Further, to investigate for presence of telomeric fragments, if any, in $ATM^{-/-}$ cells, comet-FISH was carried out. In this qualitative assay, the DNA in the gel of the comet slides was processed with telomere specific Cy3-labelled probes in PNA-FISH assay. Fifty random cells from each sample were captured and analysed at the level of single comets. It was seen $ATM^{-/-}$ cells showed telomere signals in the comet tail while the control cells showed none.

Chromosome instability in $ATM^{-/-}$ cells

PNA-FISH revealed the presence of fusions and breaks in $ATM^{-/-}$ cells. The total number of aberrations in arsenite treated $AT^{-/-}$ cells was significantly higher than heterozygous AT knock-out cells and normal fibroblasts. The frequency of breaks seems to be nearly twice more than fusions. Upon treatment with arsenite (3.0 µg/ml), $ATM^{-/-}$ cells are more susceptible to damage than control and heterozygous AT cells.

Similarly, irradiation studies demonstrated that $ATM^{-/-}$ cells appeared to be highly sensitive when compared to the other samples. mFISH was carried out to identify the specific chromosomes involved in the translocations. There were no chromosomal abnormalities in the untreated cells. Karyotype of some of the irradiated $ATM^{-/-}$ cells

showed translocations like t(7;10), t(1;15), t(5;12), t(11;17) and t(11;22).

Discussion

It is imperative to have efficient DNA repair system and a stable genome for normal growth and development and to prevent cancer progression.[26] ATM can stimulate several damage-induced responses by increasing its protein kinase activity and thereby delay cell cycle progression and also propel the DNA-repair machinery into action.[27,28] It has been reported that AT carriers are at higher risk for cancer. Homozygous AT cells are very sensitive to ionising radiation and are repair-deficient after irradiation.[10]

The telomere length measurements revealed that the $ATM^{+/-}$ and $ATM^{-/-}$ cells have considerably shorter telomeres when compared to the normal IMR90 fibroblast cells. Hence, acute telomere attrition and chromosomal end to end fusions occur frequently in the $ATM^{-/-}$ cells as substantiated by our data. Previous investigators reported the role of ATM in DNA repair surveillance and recruiting the repair machinery to the site of DNA damage.[19,21,22,29-31] We report that the $ATM^{-/-}$ cells showed telomere signals in the damaged DNA in the comet tails as detected by comet-FISH. Earlier studies have shown this method to be effective in displaying telomeric and subtelomeric fragments in comet tail.[32]

Through this study, we were able to comprehend the critical role of ATM in telomere homeostasis and genome stability maintenance. The cells lacking ATM protein had chromosomal segregation deficiency during mitosis and physical separation of sister chromatids. This phenomenon is due to higher genomic instability and lack of functional telomeres in these cells.[16,21] The genomic instability is a result of shorter and dysfunctional telomeres at the tails of the ATM homo- and heterozygous knockout cells.

Structural chromosomal alterations were commonly seen in $ATM^{-/-}$ cells following treatment with DNA damaging agents. Additionally, higher number of fragments and breaks were also detected in these cells. Analysis by mFISH has revealed that $ATM^{-/-}$ cells produced complex chromosome aberrations following treatment with radiation or arsenite. Lack of sufficient telomere bases at chromosome ends in the ATM cells can cause the chromosomes to fuse and result in dicentrics and complex chromosomal translocations. Telomeres have been observed to function in meiotic chromosome pairing, in meiotic and mitotic chromosome segregation, and in nuclear organization. It is seen that positioning of telomeres within the nucleus is highly specific and dependent on the telomere interactions with the nuclear envelope directly or indirectly (through chromatin interacting proteins). Moreover, inactivation of ATM has been observed to enhance the frequency of chromosome end association and telomere loss.[33]

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