CLINICAL & BASIC RESEARCH

Individual Radiosensitivity Assessment of the Families of *Ataxia-Telangiectasia* Patients by G2-Checkpoint Abrogation

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التقييم الفردي للحساسية الإشعاعية لعائلات مرض اختلاج الحركة الناشئ عن تمدد الشعيرات الدموية بواسطة إلغاء نقاط تفتيش ج2

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ABSTRACT: *Objectives: Ataxia-telangiectasia* (A-T) is an autosomal recessive multisystem disorder characterised by cerebellar degeneration, *telangiectasia*, radiation sensitivity, immunodeficiency, oxidative stress and cancer susceptibility. Epidemiological research has shown that carriers of the heterozygous *ataxia-telangiectasia mutated* (*ATM*) gene mutation are radiosensitive to ionising irradiation and have a higher risk of cancers, type 2 diabetes and atherosclerosis. However, there is currently no fast and reliable laboratory-based method to detect heterozygous *ATM* carriers for family screening and planning purposes. This study therefore aimed to evaluate the ability of a modified G2-assay to identify heterozygous *ATM* carriers in the families of A-T patients. *Methods:* This study took place at the Tehran University of Medical Sciences, Tehran, Iran, between February and December 2017 and included 16 A-T patients, their parents (obligate heterozygotes) and 30 healthy controls. All of the subjects underwent individual radiosensitivity (IRS) assessment using a modified caffeine-treated G2-assay with G2-checkpoint abrogation. *Results:* The mean IRS of the obligate *ATM* heterozygotes was significantly higher than the healthy controls (55.13% ± 5.84% versus 39.03% ± 6.95%; *P* <0.001), but significantly lower than the A-T patients (55.13% ± 5.84% versus 87.39% ± 8.29%; *P* = 0.001). A receiver operating characteristic (ROC) curve analysis of the G2-assay values indicated high sensitivity and specificity, with an area under the ROC curve of 0.97 (95% confidence interval: 0.95–1.00). *Conclusion:* The modified G2-assay demonstrated adequate precision and relatively high sensitivity and specificity in detecting heterozygous *ATM* carriers.

Keywords: Ataxia-Telangiectasia; Chromosome Breakage; Genetic Carrier Screening; Heterozygote; Radiation Sensitivity; Sensitivity and Specificity.

الملخص: الهدف: مرض اختلاج الحركة الناشئ عن تمدد الشعيرات الدموية هو اضطراب جسدي متعدد الوجوه متنحي يتميز بتدهور وظائف المخيخ، تمدد الشعيرات الدموية، حساسية الإشعاع، نقص المناعة، الإجهاد التأكسدي وقابلية حدوث السرطان. أظهرت الأبحاث أن حاملي الطفرات الجينية المتغايرة لهذا المرض لديهم حساسية للإشعاع المؤيّن وقابلية أعلى لحدوث السرطان، وداء السكري من النوعالثاني ومرض تصلب الشرايين. ومع ذلك، لا يوجد حاليا طريقة مختبرية سريعة وموثوق بها لأكتشاف حاملي الطفرات الجينية المتغايرة لهذا المرض لفحص أفراد الأسرة بغرض التخطيط الأسري. تهدف هذه الدراسة إلى تقييم قدرة اختبار نقاط تفتيش ج2 المعدلة على تحديد حاملي الطفرات الجينية المتغايرة لهذا المرض في عائلات مرضى اختلاج الحركة. الطريقة: أجريت هذه الدراسة في جامعة طهران للعلوم الطبية، طهران، إيران، بين فبراير وديسمبر 2017 وشملت 16 مريضًا من اختلاج الحركة الناشئ عن تمدد الشعيرات الدموية، وأولياء أمورهم (حاملي المرض الجبريين) و 30 شخصا صحيحا كمجموعة ضابطة. خضع جميع المشاركين لتقييم الحساسية للأفراد الفردية باستخدام اختبار مقياس الكافيين ج2 المعدل مع إلغاء نقاط التفتيش ج2. النتائج: كانت متوسطات تقييم الحساسية للأفراد المجازية لحاملي الطفرات الجينية المتغايرة لهذا المرض أعلى بكثير من المجموعة الضابطة (5.84% ± 55.13 مقابل 6.95% ± 39.03؛ 0.001> P)، ولكنها أقل بكثير من مرضى اختلاج الحركة (5.84% ± 55.13 مقابل 8.29% ± 87.39؛ P = 0.001). أشار تحليل منحنى التشغيل المتميز لقيم اختبار ج2 إلى حساسية عالية وخصوصية، مع وجود منطقة تحت المنحنى تبلغ 0.97 (95% فاصل الثقة: 1.00–0.95). الخلاصة: أظهر اختبار ج2 المعدل دقة كافية وحساسية عالية نسبيا في الكشف عن حاملي الطفرات الجينية المتغايرة لهذا المرض.

الكلمات المفتاحية: اختلاج الحركة الناشئ عن تمدد الشعيرات الدموية؛ تكسر الكروموسومات؛ فحص حاملي المرض؛ الطفرات الجينية المتغايرة:الحساسية للأشعاع؛ الحساسية و الخصوصية.

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Advances in Knowledge
- *This study demonstrates the relatively high sensitivity and specificity of a modified caffeine-treated G2-assay in the detection of heterozygous carriers of the ataxia-telangiectasia mutated (ATM) gene mutation.*

Application to Patient Care
- *A modified caffeine-treated G2-assay is proposed as an alternative method for identifying heterozygous ATM carriers within the families of patients with ataxia-telangiectasia for family screening and planning purposes.*

ATAXIA-TELANGIECTASIA (A-T) IS A RARE AUTOsomal recessive pleiotropic syndrome characterised by cerebellar *ataxia*, oculocutaneous *telangiectasia*, chromosomal instability, radiosensitivity, variable immunodeficiency, oxidative stress and a high risk of malignancy.[1,2] Worldwide, only one in 40,000–100,000 neonates is homozygous for the *A-T mutated (ATM)* gene mutation; however, 0.5–2% of the general population are heterozygous carriers.[1] Although heterozygous *ATM* carriers are commonly asymptomatic, they have a higher sensitivity to ionising radiation (IR) and a higher risk of type 2 diabetes mellitus, cardiovascular diseases and cancers.[2,3] However, the exact mechanism and the responsible mutations for this increased susceptibility to certain cancers are not yet understood.

The *ATM* gene codes for a ubiquitous serine/threonine protein kinase that has been implicated in the maintenance of genomic stability.[1] Over 480 different mutations—which occur across the full-length transcript without any hotspots—have been described in A-T patients.[3] In addition, the *ATM* gene has many polymorphisms/missense DNA changes that must be distinguished from deleterious mutations.[1] Early identification of the syndrome is useful for establishing prognosis and initiating genetic counselling for the family members of affected patients.[4] However, the diagnosis can be problematic and is usually based on clinical symptoms as well as high levels of serum α-fetoprotein (AFP), acquired translocations between chromosomes 7 and 14 in the blood karyotype and reduced cell sensitivity to IR or the absence of the *ATM* protein.[5]

Despite advancement in functional and sequencing-based assays, no reliable low-cost test is yet available for identifying A-T patients and heterozygous *ATM* carriers.[6] However, certain techniques developed to evaluate radiosensitivity in A-T patients may constitute methods of differentiating heterozygous *ATM* carriers, such as G2 chromosomal radiosensitivity assays, comet assays, fluorescence *in situ* hybridisation, messenger ribonucleic acid (RNA)/microRNA expression profiling of the *ATM* gene and gamma-H2AX and *PSMC1* gene assays.[6–16] However, these findings need to be confirmed among additional individuals carrying different *ATM* gene mutations.

The G2-checkpoint eases chromosome damage repair, probably by inhibiting the deleterious progression of cells through the cell cycle.[17] Caffeine has been reported to increase radiosensitivity and abrogate radiation-induced cell cycle inhibition/delays by inhibiting the G2/M checkpoint, either with or without inactivating the *ATM* and *A-T Rad3*-related genes.[18] Terzoudi *et al*. reported that caffeine treatment increased G2-assay sensitivity and reduced interlaboratory and intra-experimental variations.[19] Similarly, Pantelias *et al*. proposed a standardised caffeine-treated G2-assay for predicting individual radiosensitivity (IRS).[20] This study aimed to evaluate the ability of a modified caffeine-treated G2 breakage assay to identify heterozygous *ATM* carriers in the families of A-T patients.

Methods

This study was conducted at the Tehran University of Medical Sciences, Tehran, Iran, from February to December 2017. A total of 78 individuals were included in the study, comprising 16 A-T patients, their 32 parents (obligate heterozygotes) and 30 healthy controls. The A-T patients were recruited from previously diagnosed cases or from those who were being followed-up at the Children's Medical Center Hospital in Tehran. In all cases, the A-T diagnosis was based on evidence of disabling mutations on both alleles of the *ATM* gene and either increased radiation-induced chromosomal breakage in cultured cells or progressive cerebellar *ataxia*.[5] Additional supportive tests, including specific serum AFP and immunoglobulin measurements, were also carried out. The control subjects were recruited from members of staff at the Children's Medical Center Hospital and the Department of Medical Genetics, Tehran University of Medical Sciences. Only individuals who were unrelated to A-T patients and had no family history of malignancy, atherosclerosis or type 2 diabetes were included as controls. The controls were approximately matched for age and gender with the heterozygous *ATM* carriers.

A total of 2 mL of peripheral blood was collected from each subject. A modified G2 breakage assay was performed based on previously published protocols.[20] Briefly, 1 mL of whole blood was added to 10 mL of Gibco™ Roswell Park Memorial Institute 1640 medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and supplemented with 15% fetal bovine serum, 2% phytohaemagglutinin, 1% L-glutamine and 1% penic-
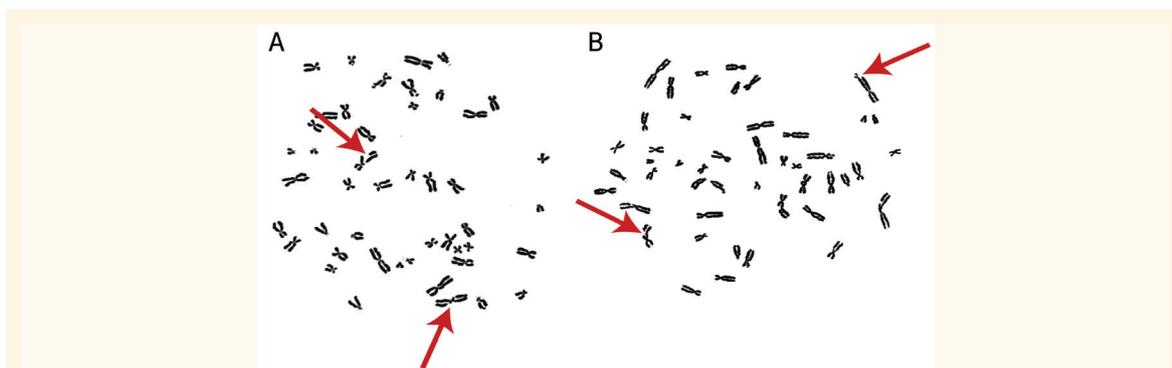
Figure 1: Diagrams showing chromatid breaks and gaps (arrows) in a peripheral blood lymphocyte at metaphase stage from a heterozygous carrier of the *ataxia-telangiectasia mutated* gene mutation after 0.9 Grays per minute of gamma irradiation using (**A**) conventional and (**B**) modified G2-assays.
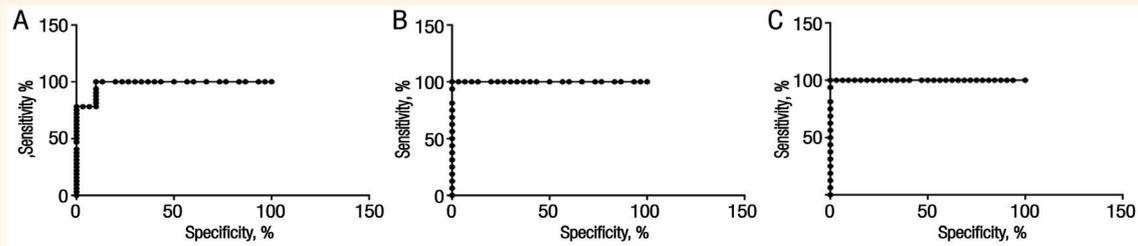
Table 1: Demographic and clinical data of *ataxia-telangiectasia* patients (N = 16)

Patient	Presence of consanguinity	Age in years/ gender	Age at *ataxia* onset in years	Age at *telangiectasia* onset in years	AFP level in mg/dL	IgG level in mg/dL	IgA level in mg/dL	IgM level in mg/dL	IgE level in IU/mL
1	Yes	14/M	5	6	103	960	0	195	40
2	Yes	9/M	2	3	161	670	7	225	7
3	Yes	7/M	1	1	380	809	173	111	10
4	Yes	4/F	2	3	90	982	16	100	1
5	Yes	16/F	1	2	75	<5	4	284	14
6	No	11/M	2	5	406	1,200	145	200	NA
7	No	11/F	2	8	104	5	1	208	<1
8	No	9/M	1	5	150	613	<5	60	<1
9	Yes	10/F	7	9	319	687	595	149	5
10	No	4/M	1	1	30	468	7	89	<1
11	No	10/F	2	4	520	NA	NA	NA	NA
12	Yes	22/M	NA	NA	666	1,185	249	112	NA
13	Yes	10/M	3	2	619	826	108	43	<1
14	Yes	10/F	1	1	155	<1	<1	430	<1
15	Yes	7/F	2	7	87	143	9	2,257	<1
16	Yes	8/F	1	8	157	643	1	71	<1

AFP = α-fetoprotein; Ig = immunoglobulin; M = male; F = female; NA = not available.
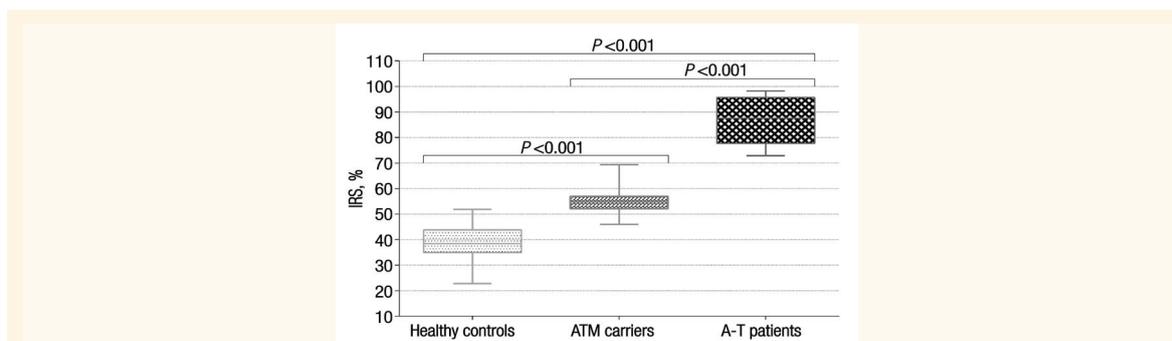
Figure 2: Box plot of the individual radiosensitivity percentages of healthy controls, heterozygous *ataxia-telangiectasia* (*A-T*) *mutated* gene carriers and A-T patients after 0.9 Grays per minute of gamma irradiation (N = 78).
IRS = individual radiosensitivity; ATM = ataxia-telangiectasia (A-T) mutated.

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Figure 3: Receiver operating characteristic curve analysis of G2-assay data for (**A**) healthy controls versus heterozygous *ataxia-telangiectasia mutated* (*ATM*) gene carriers, (**B**) healthy controls versus *ataxia-telangiectasia* (A-T) patients and (**C**) heterozygous *ATM* carriers versus A-T patients (N = 78).

illin/streptomycin. The mixture was then incubated for 72 hours at 37 °C in a humidified incubator with 5% carbon dioxide. Each sample was cultured in two flasks to assess spontaneous breakage and radiation-induced breakage. After 72 hours, gamma irradiation was performed using a Theratron® 780E teletherapy machine (MDS Nordion Inc., Ottawa, Ontario, Canada) at a dose rate of 0.9 Grays (Gy) per minute for 1.12 minutes at room temperature. After irradiation, each culture was divided into two flasks, of which one was treated with 4 mM of caffeine, before being incubated for 20 minutes at 37 °C. The cultures were then treated with demecolcine for 75 minutes. After incubation, the cells were harvested, prepared on slides and stained with Giemsa according to conventional cytogenetic techniques.[21]

Stained slides were coded and underwent blinded analysis by two separate researchers. Only well-spread metaphases with 44–47 chromosomes each were evaluated. For each sample, a total of 50 metaphases were examined for chromatid aberrations (i.e. breaks and gaps). Moreover, the level of spontaneous chromatid anomalies was evaluated in non-irradiated lymphocyte cultures and the resultant number was subtracted from the radiation-induced G2 yield of chromatid breaks. A chromatid gap was defined as an interruption in the staining of a chromatid greater than the width of the chromatid, without displacement of the segment distal to the lesion.[22] A lesion was considered to be a chromatid break if there was distinct dislocation and misalignment of the broken segment [Figure 1].

For each individual, two different yields of chromatid aberrations were calculated. The first yield was similar to that of a conventional G2-assay and the second was the maximum yield of chromatid breaks achieved when the G2-checkpoint was abolished by caffeine. The

$$IRS = \left(1 - \frac{G2_{caf} - G2}{G2_{caf}}\right) \times 100 \quad [Equation \ 1]$$

was calculated as below [Equation 1]:
where $G2_{caf}$ is the yield when the G2-checkpoint is abolished by caffeine and G2 is the conventional G2-assay yield. For samples which deviated by more or

less than 5% from formerly published cut-off values, an additional 50 metaphases were analysed.[20] The frequency of exchanges and rings was not included in the calculation of aberration yields. The inter-day precision of the G2-assay was determined by analysing six samples of peripheral blood mononuclear cells from one healthy donor on six different days. The precision of the test was assessed only at between-run levels.

Data were analysed using GraphPad Prism software, Version 6 (GraphPad Software, San Diego, California, USA). Statistical differences between groups were determined using Kruskal-Wallis and Dunn's multiple comparison post-*hoc* tests. Receiver operating characteristic (ROC) curves were calculated to assess the relevant sensitivity and specificity of the G2-assay values. A significance level of $P \leq 0.050$ was used throughout the experiments. The inter-day precision was presented as a coefficient of variation.

This study was approved by the Research Ethics Committee of the Tehran University of Medical Sciences (#26489-154-02-93). All subjects gave informed consent prior to their inclusion in the study.

Results

The male-to-female ratios of the A-T patients and controls were 0.9 and 1.4, respectively. The mean ages of the A-T patients, heterozygous *ATM* carriers and healthy controls were 10, 37 and 32 years old, respectively (ranges: 4–22, 26–55 and 19–46 years, respectively). The clinical and demographic data of the A-T patients are presented in Table 1.

There was a significant increase in IRS across all three groups ($P <0.001$). The mean frequency of induced chromatid aberrations in cells from heterozygous *ATM* carriers were significantly higher than in those from healthy controls (55.13% ± 5.84% versus 39.03% ± 6.95%; $P <0.001$) and significantly lower than in A-T patients (55.13% ± 5.84% versus 87.39% ± 8.29%; $P = 0.001$) [Figure 2]. An ROC curve analysis of G2-assay values demonstrated high sensitivity and specificity for the identification of heterozygous *ATM*

Table 2: Diagnostic potential of a modified G2-assay in the differentiation of healthy controls, heterozygous *ataxia-telangiectasia mutated* gene carriers and *ataxia-telangiectasia* patients (N = 78)

	Healthy controls versus heterozygous *ATM* gene carriers	Healthy controls versus A-T patients	Heterozygous *ATM* gene carriers versus A-T patients
AUC (95% CI)	0.97 (0.95–1.00)	1.00 (1.00–1.00)	1.00 (1.00–1.00)
Sensitivity, % (95% CI)	100 (89–100)	100 (80–100)	100 (79–100)
Specificity, % (95% CI)	90 (73–98)	100 (88–100)	100 (89–100)
Cut-off value, %	45	60	70

A-T = ataxia-telangiectasia; ATM = A-T mutated; AUC = area under the curve; CI = confidence interval.

carriers versus controls, with an area under the curve of 0.97 (95% confidence interval: 0.95–1.00) [Figure 3 and Table 2]. The overall inter-day coefficient of variation of the assay was 4.28% (mean: 45.84% ± 1.96%).

Overall, the G2-checkpoint efficiency was easily discriminated in A-T patients; however, there was an overlap of chromatid aberration frequencies observed between controls and heterozygous *ATM* carriers. Aberrations in non-irradiated lymphocytes were not noticeable in any of the three groups.

Discussion

Along with chromosomal instability, cells with *ATM* gene mutations display extreme radiosensitivity both *in vivo* and *in vitro*.[3] These phenotypes suggest that the *ATM* gene plays a critical role in the DNA repair system. Furthermore, as serine/threonine kinase acts as a sensor of oxidative stress in normal cells, the loss of this protein in individuals with *ATM* mutations disturbs the redox balance and increases levels of reactive oxygen species and oxidative stress, contributing to increased radiosensitivity.[23,24] However, the exact role of the *ATM* gene in these systems and the tissue specificity of such defects remains unknown. The *ATM* gene coordinates cell cycle checkpoint signalling via the phosphorylation of different substrates, including p53, checkpoint kinase 2, structural maintenance of chromosomes 1 (SMC1), activating transcription factor 2 and nibrin.[1] A lack of serine/threonine kinase leads to the loss of these checkpoints and, therefore, the potentially deleterious progression of cells through the cell cycle.

The screening and identification of heterozygous carriers of *ATM*-related haploinsufficiencies and allelic variants is an important public health goal due to their high frequency in the population and increased susceptibility to cancer, diabetes and atherosclerosis.[1,2] The current study evaluated the IRS of A-T patients, heterozygous *ATM* carriers and healthy controls by assessing the frequency of radiation-induced chromatid aberrations during the G2/M transition stage in stimulated blood lymphocytes using a modified caffeine-treated G2-assay with G2-checkpoint abrogation. A significantly higher frequency of chromatid breaks and gaps was observed in the cells of A-T patients compared to those of the heterozygous *ATM* carriers. Similarly, there was a significantly higher frequency of chromatid breaks and gaps among the cells of heterozygous *ATM* carriers compared to those of the healthy controls. Unfortunately, the range of these aberrations overlapped to some extent between the latter two groups, thus precluding the absolute detection of heterozygous *ATM* carriers. Nevertheless, this method—which required only 2 mL of peripheral blood from the subjects—showed acceptable inter-day variation and relatively high sensitivity and specificity for the identification of heterozygous *ATM* carriers.

Several previous studies have attempted to distinguish heterozygous *ATM* carriers from normal controls using G2-assays.[7,8,25] Tchirkov *et al.* detected 13 heterozygous *ATM* carriers without any overlap of results with those of normal donors.[10] However, these findings were not consistent with those of other studies.[22,26–28] This may be due to variations in the number of subjects, type of cells under consideration, nature of the *ATM* mutation, technical procedures, treatment dose and dose rate, number of analysed metaphases, time interval between irradiation and fixation and the type of analysed aberration. Leonard *et al.* distinguished obligate heterozygotes from controls utilising lymphoblast cell lines which had been treated with camptothecin; however, this method requires further study using peripheral blood samples from a larger cohort.[11] Likewise, Terzoudi *et al.* introduced a new approach to G2/M cell cycle checkpoint abrogation for assessing IRS using caffeine treatment; this method demonstrated better discrimination and minimal intra-individual and inter-laboratory variations compared to the conventional G2-assay.[19,20]

Claes *et al.* reported the use of a modified S-G2 micronucleus assay in which caffeine treatment formed a complementary assay for the identification of atypical A-T patients and heterozygous *ATM* carriers.[29] Prodosmo *et al.* proposed a new, fast and cost-effective test to determine *ATM* zygosity based on p53 centrosomal localisation visualised by immunofluorescence staining.[30] Porcedda *et al.* identified heterozygotes using a two-tier analysis of histone H2AX phosphorylation, H2AX phosphorylation levels and residual gamma-H2AX

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foci.[16] In addition, A-T patients and heterozygous *ATM* carriers have been identified by evaluating serine/threonine kinase-dependent phosphorylation of SMC1 following DNA damage; however, this assay could not discriminate between carriers and controls via enzyme-linked immunosorbent assay.[31] A more recent, quick, labour-intensive cell division assay using flow cytometry reported similar results to that of a colony-survival assay (CSA).[32] A CSA is the gold-standard method of evaluating radiosensitivity as it resolves the technical difficulties involved in standardisation and optimisation which interfere with predictive accuracy.[5]

This study was subject to certain limitations. Some of the healthy controls may have been DNA repair-deficient or radiosensitive.[20,22] On the other hand, some of the obligate heterozygotes may have had a range of *ATM* mutations; consequently, these individuals may, like A-T patients, have exhibited various degrees of radiosensitivity both *in vitro* and clinically.[7–11,19,25,28,29] Furthermore, technical issues in different laboratories can intensify such variations.[22] Finally, the assay was not specific for *ATM* mutations and may therefore have identified mutations in other genes which modify DNA repair system efficiency. For these reasons, it is recommended that this assay be used only for the screening of families of confirmed A-T patients, as hypersensitivity in such cases is more likely to truly reflect heterozygosity of the *ATM* gene.[33,34] Further investigation is recommended to confirm the practicality of using this assay. The results of experiments like those conducted in the current study are preliminary and need to be further confirmed in additional subjects carrying different *ATM* mutations.

Conclusion

The results of this study indicate that a modified caffeine treated G2-assay is a potential method of identifying heterozygous *ATM* carriers within the families of A-T patients.

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