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ARTICLE

Polymorphisms of nucleotide-excision repair genes may contribute to sperm DNA fragmentation and male infertility

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Abstract The nucleotide-excision repair (NER) system is crucial for the removal of bulky DNA adducts during spermatogenesis. Dysfunction of its repair capacity is likely related to the increased susceptibility to DNA damage. In this study, four polymorphisms in NER pathway (XPA(−4) G/A, ERCC1 C8092A, XPD Lys751Gln and XPF Ser835Ser) were selected to evaluate their potential impact on sperm DNA damage and male infertility. Genotypes were determined by PCR-restriction fragment length polymorphism. Sperm DNA damage was evaluated by TdT-mediated dUDP nick-end labelling assay. A case-only study of 620 infertile men found a significant association between XPA(−4) G/A polymorphism and sperm DNA damage. Individuals with the XPA(−4) A allele showed more sperm DNA damage and lower sperm concentration than G allele carriers. Further analysis, including 620 patients and 385 controls, revealed a 1.52-fold risk (95% CI 1.08–2.02) of developing male infertility in the XPA(−4) AA carriers compared with noncarriers. Luciferase assay verified that the promoter with the XPA(−4) A allele had a lower transcriptional activity than that with the G allele. These data provide the first evidence that −4 G/A polymorphism in XPA promoter alters its transcriptional activity and, thus, might contribute to sperm DNA damage and male infertility.

KEYWORDS: male infertility, nucleotide-excision repair (NER), polymorphisms, sperm DNA damage, xeroderma pigmentosum group A gene (XPA)

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Introduction

About 25% of couples do not achieve pregnancy within 1 year, 15% of couples seek medical treatment for infertility and, ultimately, less than 5% remain childless. The male factor is at least partly responsible in about 50% of infertile couples. Despite a significant improvement in the diagnostic work-up of infertile men, the cause of abnormal spermatogenesis in about 50% of cases remains unknown (Dohle et al., 2005; Forti and Krausz, 1998; Guzick et al., 2001). It has been demonstrated that DNA damage in human spermatozoa is correlated with poor semen quality, low fertilization rates, impaired preimplantation development, increased abortion and an elevated incidence of diseases in the offspring, including childhood cancer (Borini et al., 2006; Cohen-Bacrie et al., 2009; Ji et al., 1997; Lewis and Aitken, 2005), while the origin of DNA fragmentation is still controversial. It is known that spermatogenic cells are constantly assaulted by endogenous and exogenous agents during spermatogenesis, which produce a variety of DNA lesions (Fisher and Aitken, 1997; Horak et al., 2003). To cope with the DNA damage, spermatogenic cells are equipped with defensive mechanisms, including several DNA repair pathways, among which the nucleotide-excision repair (NER) system is the most versatile in correcting localized small lesions and protecting against bulky endogenous DNA adducts (Xu et al., 2005).

Hitherto, a number of mice models with deletions of genes involved in the NER pathway have been generated and have provided invaluable information indicating that deficits in DNA repair pathways could influence germ cell survival and production of spermatozoa (Paul et al., 2008). For example, Hsia et al. (2003) found that male *Ercc1*-deficient mice were infertile with an increased level of DNA strand breaks and oxidative DNA damage in testis. Similarly, xeroderma pigmentosum group A gene (XPA)-deficient male mice become subfertile in an age-dependent manner, first observed at 24 months, with impaired spermatogenesis and diminished testis (Nakane et al., 2008; Tsai et al., 2005).

Recently, polymorphism analysis in candidate genes has helped us to understand the aetiology of and susceptability to male infertility (Kang et al., 2009; Tuttelmann et al., 2007). For example, polymorphisms in genes encoding complementing defective X-ray repair, RNA polymerase II subunit B4, androgen receptor, relaxin, acrosin and osteopontin are reported to be associated with sperm concentration, motility, semen volume per ejaculate or abnormal sperm rate (Gu et al., 2007; Lin et al., 2006). Several studies verified some polymorphisms in NER genes decreased the DNA repair efficiency in somatic cells (Shen et al., 2006; Spitz et al., 2001; Vodicka et al., 2004) and germ cells (Liang et al., 2009). It is highly likely that polymorphisms in the NER genes involved in spermatogenesis might be responsible for some idiopathic forms of spermatogenic disturbances. Four polymorphisms, XPA(−4) G/A (rs1800975), ERCC1 C8092A (rs3212986), XPD Lys751Gln (rs1052559) and XPF Ser835Ser (rs1799801) that localize in 9q22.3, 19q13.3, 19q13.3 and 16p13.12, respectively, were indicated to be associated with the reduced DNA repair capacity and the increased susceptibility to genomic DNA damage and cancers (Hu et al., 2004; Monzo et al., 2007; Wu et al., 2003). Nevertheless, few studies have focused on the contribution of the NER gene polymorphisms to sperm DNA damage. The present study selected these four polymorphisms to evaluate their associations with the sperm DNA integrity, semen quality and the risk of developing male infertility.

Materials and methods

Subjects and sample collection

A total of 984 infertile patients were candidates seeking treatment in the Centre of Clinical Reproductive Medicine between April 2005 and March 2009 (NJMU Infertile Study). All patients underwent at least two semen analyses, a series of physical examinations and serum determination, which helped us to exclude 364 individuals: 228 azoospermia (eight obstructive azoospermia), 65 severe oligozoospermia (sperm count $<5\times10^6$/ml), 18 with abnormal karyotype, nine with cryptorchidism and 44 secondary sterility cases. In total, 620 subjects from 25 to 38 years old with sperm concentration $>5\times10^6$/ml were chosen for this study. The 385 fertile men with normal semen parameters who had fathered at least one healthy child were chosen as controls. Each participant completed an informed consent as well as a questionnaire including detailed information, such as age, cigarette smoking, alcohol, tea and vitamin consumption and abstinence time, and donated 5 ml of blood for genomic DNA extraction. In addition, the semen samples of 620 patients were collected after 3—7 days of sexual abstinence. The liquefied ejaculates were divided into two parts, one for the routine semen analysis and the other for the assessment of sperm DNA fragmentation. The study was approved by the Ethics Review Board of Nanjing Medical University.

Semen quality analysis

Semen samples were obtained and analysed according to World Health Organization guidelines (World Health Organization, 2004) by using Micro-cell slide and the computer-aided semen analysis (CASA, WLJY 9000; Weili New Century Science and Tech Dev). Semen parameters such as sperm concentration and motility were recorded. Each sample was assessed twice with strict quality control, successively.

Tdt-mediated dUTP nick-end labelling assay

Tdt (terminal deoxynucleotidyl transferase)-mediated dUTP nick end labelling (TUNEL) assay to evaluate the sperm DNA fragmentation according to the manufacturer's instructions (APO-DIRECT kit; BD Biosciences Pharmingen). Briefly, spermatozoa were washed and resuspended in 2% paraformaldehyde for 30 min at room temperature. After rinsing with phosphate-buffered saline, each sample was resuspended in the permeabilizing solution (0.2% Triton X-100, 0.1% sodium citrate) for 10 min on ice and then 50 μl of TUNEL reagent was added. For each batch, a negative control without the addition of TdT enzyme and a positive control with DNase I treatment were set up. After incubating at 37°C for 1 h, samples were analysed immediately by flow cytometry (FACSCalibur; BD Biosciences Pharmingen).

Genotyping

Genotypes of XPA(−4) G/A, ERCC1 C8092A and XPD Lys751Gln were determined by polymerase chain reaction restriction fragment length polymorphism assay (PCR-RFLP) using the condition as described previously (De Ruyck et al., 2007; Ji et al., 2008). XPF Ser835Ser polymorphism was detected using the following primers: 5′-CTGAAACAAAGCAAGCCACA-3′ (forward) and 5′- GACAGGGCTGCTAATTCTGC-3′ (reverse). The 194 bp PCR products were then digested overnight by Cail enzyme (MBI Fermentas) and the A/A (194 bp), A/G (194, 130, 64 bp) and G/G (130, 64 bp) genotypes were obtained. For quality control, 10% of the samples were randomly genotyped again and the reproducibility was 100%. All the polymorphisms were in Hardy-Weinberg equilibrium.

Construction of reporter plasmids

To evaluate the potential effects of the XPA(−4) polymorphism on transcriptional activity, two luciferases were constructed. The fragment encompassing the XPA promoter region from positions −214 to 58 was subcloned into a promoterless pGL3 luciferase basic vector (Promega, Madison, WI, USA). In detail, genomic DNA of a homozygote with either −4 G or −4 A allele was amplified by PCR using the following primers: 5′- GACGCTAGCGCAGGTAGTTAGGCGGG TAC-3′ (forward) and 5′- CGCG AAGCTTAGCTCCGCGGGTTGC TCTAA-3′ (reverse), consisting of the *Nhe*I and *Hin*dIII restriction sites (The protective nucleotides are marked in bold and restriction sites are underlined). Amplicons were directionally cloned into the pGL3-basic luciferase vector using restriction sites added to the primers. The pGL3-basic vector lacked eukaryotic promoter and enhancer sequences and expression of luciferase activity in transfected cells depended solely on the putative regulatory XPA sequences inserted upstream. The integrity of the inserts was confirmed by sequence analysis.

Transient transfection and luciferase assay

The two luciferase expression constructs described above were used for transient transfection in MCF-7, HeLa and COS-7 cells. The cells were seeded at 3×105 cells per well and transfected with 2.25 μg plasmid containing either the −4 G or −4 A allele using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) until 70−80% confluence. At the same time, pRL-SV40 vector containing the renilla luciferase gene was co-transfected as an internal standard. The pGL3-basic vector was used as a negative control. Cells were harvested 48 h later and lysed with lysis buffer (Promega, Southampton, UK). Firefly luciferase activity was assessed by the dual-luciferase reporter assay system (Promega, Madison, WI, USA) and normalized to renilla values. Independent triplicate experiments were performed and averaged.

Statistical analysis

The effects of selected individual characteristics on sperm DNA fragmentation and semen quality were analysed by the t-test. Since the sperm DNA fragmentation and sperm concentration were abnormally distributed, logarithmic transformation was used to stabilize the variance of the DNA fragmentation rate (Bland, 2000) and convert them to normal distribution for all statistical procedures. Multiple linear regression was used to examine the differences of sperm DNA fragmentation and semen quality among XPA, ERCC1, XPD and XPF genotypes. Hardy-Weinberg equilibrium of the controls' genotype distributions was tested by a goodness-of-fit chi-squared test. Multivariate logistic regression analysis was performed to obtain adjusted odds ratios (OR) for risk of male infertility and their 95% confidence intervals (CI). A P-value <0.05 was used as the criterion of statistical significance. The statistical tests were two-sided tests and all analyses were conducted using Statistical Analysis System (version 9.13; SAS Institute, Cary, NC).

Results

Sperm DNA damage and subject characteristics

Sperm DNA damage was evaluated by the TUNEL assay using flow cytometry. The distribution of TUNEL values among 620 patients is illustrated in Figure 1 and these underwent a logarithmic transformation for further association study. The associations between the individual characteristics and sperm parameters, including log transformation value of sperm DNA damage, sperm concentration and sperm motility, are presented in Table 1. The tea-consuming population had decreased DNA damage compared with non-consumers (2.43 versus 2.65, $P = 0.014$) (Table 1). However, other characteristics, such as smoking or alcohol intake, appeared to have no obvious effects on sperm DNA integrity.

Effects of NER gene polymorphisms on sperm DNA damage

The effects of NER gene polymorphisms on sperm DNA damage were evaluated as well as the motility and concentration (Table 2). Compared with the G allele, subjects who carried the XPA(−4) AA genotype displayed a markedly higher level of sperm DNA fragmentation (2.80 ± 0.97 versus 2.38 ± 1.07; $P = 0.002$). Figure 2 shows the distributions of TUNEL values, sperm concentration and motility among 620 subjects with the different genotypes. Similarly, the XPA(−4) A allele was also observed to be associated with the lower sperm concentration compared with the G allele ($P = 0.038$). A gradual decline of sperm motility was found among the three XPA(−4) subgroups (means 57.84%, 55.51% and 51.84% for GG, GA and AA, respectively), but the differences were not statistically significant. As to the other three genetic polymorphisms (XPD Lys751Gln, ERCC1 C8092A, XPF Ser835Ser), significant differences were not observed.

XPA(−4) polymorphism and risk of male infertility

Due to the relationship between the sperm DNA fragmentation and male infertility, the contribution of XPA−4 G/A polymorphism to the risk of male infertility was further explored based on a case−control study including 620 patients and 385 controls. As shown in Table 3, the XPA(−4) AA genotype appeared more frequently in the cases (23.06%) than in the controls (18.70%). The result also suggests that subjects with the AA genotype have a 1.52-fold

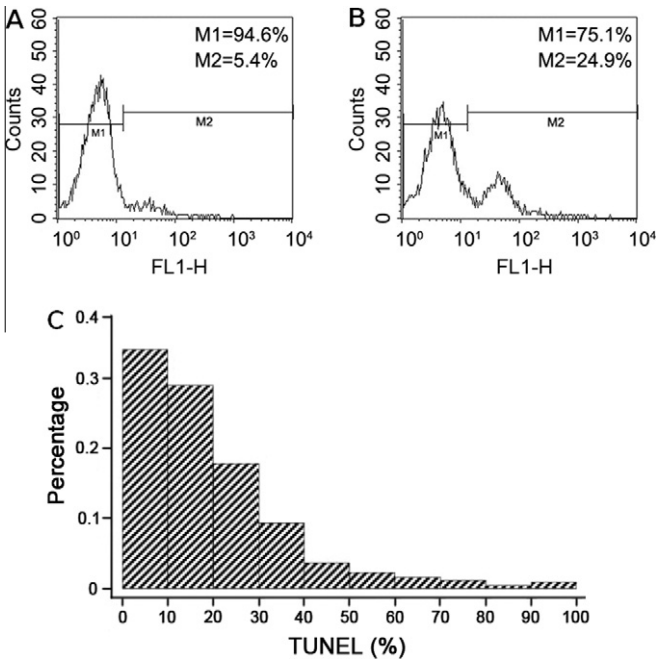

Figure 1 Detection of sperm DNA damage using Tdt (terminal deoxynucleotidyl transferase)-mediated dUTP nick end labelling assay. Histogram data from flow cytometry shows (**A**) a negative control, (**B**) one sample with 24.9% spermatozoa (M2) labelled with strong fluorescein isothiocyanate fluorescence and (**C**) distribution of sperm DNA damage in 620 ejaculates. The x axis in A and B: the percentage of fluorescence intensity measured in FL1-H channel.

Table 1 Association between the selected individual characteristics and sperm DNA damage and sperm parameters.

Variable (n)	*Sperm DNA damage*[a]	*Motility*	*Concentration*[a]
Age			
<31 years (307)	2.61 ± 0.07	59.34 ± 24.57	3.83 ± 0.07
≥31 years (313)	2.48 ± 0.07	56.58 ± 27.27	3.93 ± 0.06
Smoking status			
Yes (335)	2.51 ± 0.06	58.86 ± 25.14	3.89 ± 0.06
No (285)	2.59 ± 0.07	56.73 ± 27.06	3.88 ± 0.07
Alcohol drinking			
Yes (88)	2.53 ± 0.12	56.59 ± 24.37	3.80 ± 0.13
No (532)	2.55 ± 0.05	58.18 ± 26.06	3.89 ± 0.05
Tea consumption[b]			
Yes (171)	2.43 ± 0.85	59.66 ± 25.86	4.01 ± 0.08
No (449)	2.65 ± 0.06	57.18 ± 26.09	3.83 ± 0.06
Vitamin using			
Yes (83)	2.71 ± 0.14	55.45 ± 25.90	3.63 ± 0.11
No (537)	2.51 ± 0.05	58.19 ± 25.84	3.92 ± 0.05
Abstinence time			
3—5 days (333)	2.59 ± 1.01	56.55 ± 24.11	3.91 ± 0.90
6—7 days (287)	2.55 ± 1.01	58.11 ± 26.35	3.88 ± 0.91

Values are mean ± SD.
[a]Ln-transformed data.
[b]Sperm DNA damage was significantly lower in the tea drinkers (two-sided chi-squared test, $P = 0.014$). There were no other statistically significant differences.

Table 2 Effects of polymorphisms of nucleotide-excision repair gene on sperm DNA fragmentation and semen parameters.

Genotype (n)	*Sperm DNA damage*[a]		*Motility (%)*		*Concentration*[a]	
	Mean ± SD	*P-value*[b]	*Mean ± SD*	*P-value*[b]	*Mean ± SD*	*P-value*[b]
XPA(−4) G/A						
GG 156	2.38 ± 1.07	Reference	57.84 ± 28.63	Reference	4.08 ± 0.79	Reference
GA 321	2.64 ± 0.98	NS	55.51 ± 23.84	NS	3.84 ± 0.93	NS
AA 143	2.80 ± 0.97	0.002	51.84 ± 28.88	NS	3.72 ± 0.87	0.038
XPD Lys751Gln						
GG 528	2.56 ± 0.99	Reference	58.93 ± 25.98	Reference	3.91 ± 0.89	Reference
GA 90	2.54 ± 1.12	NS	54.18 ± 26.24	NS	3.87 ± 0.93	NS
AA 2	2.61 ± 1.35	NS	34.32 ± 30.25	NS	3.75 ± 0.84	NS
ERCC1 C8092A						
GG 273	2.59 ± 0.86	Reference	60.63 ± 28.92	Reference	3.89 ± 0.66	Reference
GA 285	2.62 ± 1.01	NS	58.80 ± 24.60	NS	3.85 ± 0.63	NS
AA 62	2.48 ± 1.06	NS	52.74 ± 33.14	NS	3.76 ± 0.75	NS
XPF Ser835Ser						
CC 326	2.50 ± 1.02	Reference	56.83 ± 26.36	Reference	3.89 ± 0.92	Reference
CT 251	2.59 ± 1.02	NS	59.19 ± 24.75	NS	3.87 ± 0.85	NS
TT 43	2.80 ± 0.84	NS	56.53 ± 31.13	NS	3.77 ± 1.06	NS

NS = not statistically significant.
[a]Ln-transformed data.
[b]Two-sided chi-squared test, adjusted for age, smoking, alcohol drinking, tea consumption, vitamin consumption and abstinence time.

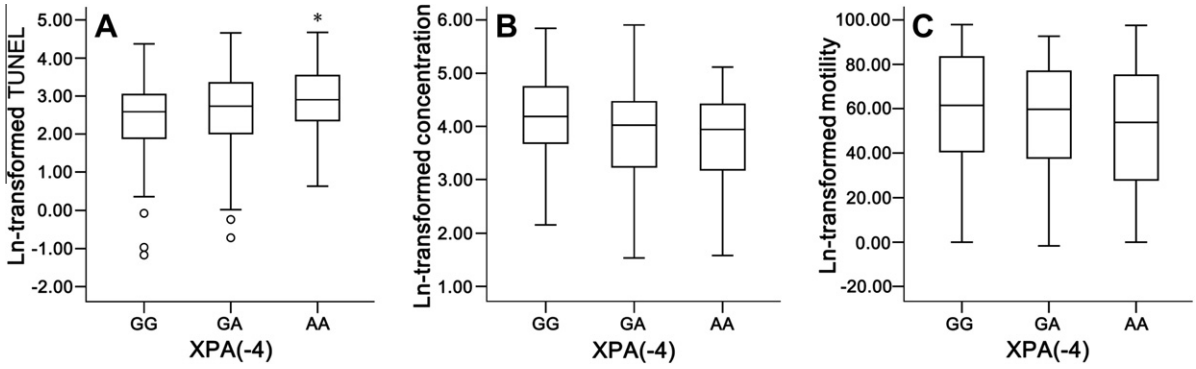

Figure 2 Association between XPA(−4) genotypes and the values of sperm DNA fragmentation, sperm concentration and sperm motility. The box-and-whisker plots depict the distribution of the transformed (**A**) TUNEL value, (**B**) sperm concentration and (**C**) motility among 620 subjects with the different genotypes. The boxes represent the 25th and 75th percentiles; whiskers cover the extent of the data on 1.5 interquartile range. The median value is denoted as the line that bisects the boxes. Circles represent the outlier values. Significant difference measured by multiple linear regression. *$P < 0.05$ compared with the GG groups.

increased risk of developing male infertility compared with the GG genotype ($P = 0.031$; 95% CI 1.08−2.02).

Effect of the *XPA*(−4) polymorphism on transcriptional activity

To directly determine the allele-specific effect of XPA−4 G/A polymorphism on native promoter activity, two luciferase reporter gene constructs were generated, which contained 272 bp of the XPA promoter region with a G or A at the −4 polymorphic site (Figure 3A, B). As shown in Figure 3C, the XPA promoter with the −4 G allele had an obviously higher activity than the one with the −4 A allele in all these cell lines transiently transfected with the constructs (increase from 135% to 214%; $P < 0.05$ for all). These results suggested that the −4 G allele in the promoter region might enhance transcriptional activity of the XPA gene.

Discussion

The alarming reports on a time-related impairment of male reproductive function, together with an increased incidence of testicular cancer and a possible decline in sperm counts, have drawn attention to the effects of DNA damage in male germ cells. Sperm DNA integrity is essential for the accurate transmission of genetic information and any form of sperm

Table 3 Association between XPA(−4) polymorphism and the risk of male infertility.

XPA(−4) genotypes	*Controls* (n = 385)		*Cases* (n = 620)		*P-value*[a,b]	*OR (95% CI)*[b]
	n	%	n	%		
GG	118	30.65	156	25.16	Reference	Reference
GA	195	50.65	321	51.77	NS	1.26 (0.94−1.71)
AA	72	18.70	143	23.06	0.031	1.52 (1.08−2.20)
GA/AA	267	69.35	464	74.84	NS	1.33 (1.02−1.78)

NS = not statistically significant.
[a]Adjusted for age, smoking, alcohol, tea and vitamin consumption and abstinence time.
[b]Two-sided chi-squared test.

DNA damage may result in male infertility regardless of the number, motility and morphology of spermatozoa (Agarwal and Said, 2003; Guzick et al., 2001). The clinical significance of sperm DNA damage lies in its association not only with natural conception rates but also with assisted reproduction success rates (Horak et al., 2007; Zini and Libman, 2006). Moreover, it is proposed to have a serious impact on the offspring (Ji et al., 1997; Marchetti and Wyrobek, 2008). As a major mechanism for the removal of a wide diversity of DNA lesions, NER is believed to play an important role in maintaining normal spermatogenesis and genetic stability. There is however limited knowledge of NER in spermatogonial stem cells. It was reported that polymorphic variants of the NER pathway could reduce DNA repair capacity and thus be associated with modest elevations in cancer risk (Berndt et al., 2006). However, to date, few studies have focused on the roles of the NER polymorphisms in male infertility. The current data provide the first evidence that XPA(−4) G/A polymorphism is associated with sperm DNA fragmentation based on a population study consisting of 620 infertile men. This case−control study of moderate sample size corroborate that this polymorphism is likely to contribute to an increased risk of developing male infertility. XPA(−4) G/A polymorphism is identified in the 5′ noncoding region of the XPA gene, located four nucleotides upstream of the ATG start codon (Butkiewicz et al., 2000; Richards et al., 1997). Until now, little is known about the exact function of this polymorphism. It is supposed that this polymorphism regulates gene expression through transcriptional or post-transcriptional mechanisms. Probably, the polymorphism in promoter affects the transcriptional efficiency (Hao et al., 2006). On the other hand, it is also possible that

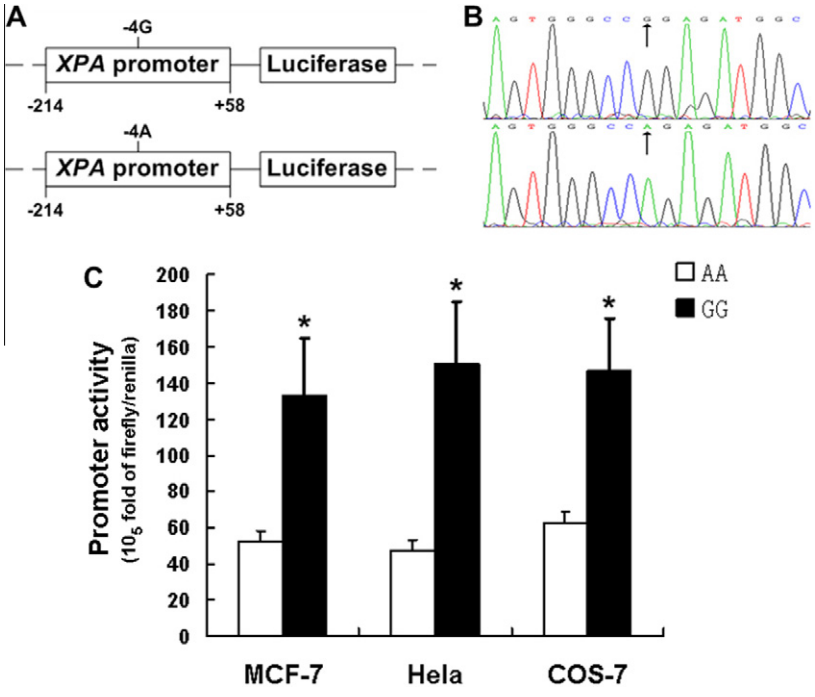

Figure 3 Effect of the XPA(−4) A/G polymorphism on the transcriptional activity. (**A**) Schematic representation of reporter plasmids containing the −4 G or −4 A allele, which was inserted upstream of the luciferase reporter gene in pGL3 basic plasmid. (**B**) DNA sequencing results of reporter plasmids containing the −4 G or −4 A allele; arrows indicate the positions of nucleotide changes. (**C**) The two constructs were transiently transfected into the MCF-7, HeLa and COS-7 cells, respectively. The luciferase activity of each construct was normalized against the internal control of renilla luciferase. The data indicate the mean values with the standard deviation from three independent experiments. *$P < 0.05$ compared with the construct counterpart.

the nucleotide substitution of A to G at position −4 preceding the AUG initiation codon (CCAGAGAUGG) might affect 40S ribosomal binding and thus alter the efficiency of XPA protein synthesis (Kozak, 1986; Morle et al., 1986). An alternative explanation could be the protective XPA allele is in linkage disequilibrium with a true susceptible allele at a quantitative trait locus controlling XPA concentrations.

To investigate whether the transition from A to G affected the transcription or translation efficiency, the luciferase reporter assay was performed. The in-vitro result offered strong evidence that the A allele containing construct displayed markedly lower promoter activity compared with the G allele, suggesting that it may reduce the XPA expression and lead to lower DNA repair capacity. The data obtained: (i) supported the population study data which showed the elevated sperm DNA fragmentation in A allele carriers compared with the G allele; (ii) confirmed the lower transcription activity of the XPA(−4) A allele; and (iii) more importantly, not only provided the biological basis for the hypothesis that −4 G allele might increase XPA expression (and increased DNA repair capacity) in healthy subjects (Wu et al., 2003), but also offer a new explanation for the protective effect of G allele on lung cancer found in a Korean and a European Caucasian study respectively (Butkiewicz et al., 2004; Park et al., 2002).

Considering the central role of XPA in the NER pathway and its importance during normal spermatogenesis, reduced expression of the protein would be expected to impair NER ability and, hence, increase the risk of developing sperm DNA fragmentation or male infertility. This work extends the previous understanding of the NER polymorphisms and their effects on the risk of idiopathic azoospermia by further evaluating the contribution of these polymorphisms in relation to sperm DNA fragmentation, although no relationship was found between the other three polymorphisms and sperm DNA integrity or semen quality.

Male infertility is a disease of low-penetrance alleles like cancer. Individual susceptibility alleles are unlikely to contribute much to diagnosis or prevention in the population, even though it might have an implication for the individual (Pharoah et al., 2008). The current data provide evidence that XPA(−4) G alleles confer relatively low penetrance for predisposition to the development of infertility, which imply that individuals carrying the XPA(−4) A allele will be at higher risk of infertility compared with G allele carriers.

Another interesting finding was that tea consumption was associated with reduced sperm DNA fragmentation. To date, few studies have investigated tea consumption on sperm DNA integrity. It is more likely that polyphenols in tea play an antioxidant role in the prevention of sperm DNA damage (Greco et al., 2005; Xie et al., 1993). Despite much research over the last few decades, there still remains considerable uncertainty as to the relationship between smoking or alcohol intake and sperm DNA fragmentation (Horak et al., 2003; Sergerie et al., 2000). This study did not find an obvious impact of cigarette smoking or alcohol consumption on sperm DNA fragmentation.

In conclusion, as far as is known, this study is the first mutation analysis of the XPA gene in sperm DNA damage and male infertility. It has identified a possible contribution of XPA(−4) A/G polymorphism to sperm DNA integrity and male infertility due to effects on the transcriptional mechanism. The results of this study might be helpful in improving the understanding of the genetic susceptibility on sperm DNA integrity and in providing diagnostic implications for assisted reproduction success rates. Further studies with larger cohorts or different ethnic populations are required to elucidate the exact effects of these genetic polymorphisms on human spermatogenesis and the risk of male infertility.

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