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Sequence Tagged Site (STS) Analysis of Y-Chromosome Micro Deletions in Environmental Tobacco Smokers [ETS] in Tamilnadu, India

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Abstract: Azoospermia factor locus (AZF) is assumed to contain the genes responsible for spermatogenesis. Deletions in these genes are thought to be pathologically involved in some cases of male infertility associated with azoospermia or oligozoospermia. An attempt was made to establish the prevalence of micro-deletions on the Y chromosome in 120 Environmental Tobacco Smokers [ETS] Tamil Nadu, India. Polymerase chain reaction (PCR) micro-deletion analysis was done in 180 EST males including control. For this, genomic DNA was extracted from the peripheral blood. Seven sets of primers were used encompassing AZFa, AZFb and AZFc regions.

Key words: Environmental Tobacco Smokers (EST) · STS primers · Azoospermia factor · Micro-deletion · Polymerase chain reaction (PCR)

INTRODUCTION

Reproductive health is a state of complete physical, mental and social well-being in all aspects relating to the reproductive system and to its function and processes [1]. Infertility is a problem that affects both men and women everywhere in the world. Medical statistics from the U.S. show that approximately 15% of all couples of reproductive age are unable to conceive naturally [2].

Approximately 10% of couples at child bearing ages suffered from some kind of infertility and about half of these cases are because of male factors [3], which recently became a hot issue in the relevant studies. [4] from India reported a decrease in sperm count and motility from 38.18 millions/ml and 61.16% in 1993-1994 to 26.61 millions/ml and 47.14% respectively by 2004-2005. Sperm with normal morphology was 40.51 % in 1993-1994 and was decreased to 19.75% by 2004-2005.

Tobacco smoke is a known carcinogen, which has been associated with cancer of several sites. Since many compounds in tobacco smoke are mutagens, it has been suggested that smoking may affect male reproduction and the offspring's health. However, the effects of smoking on sperm morphology are inconsistent. A review conducted a few years ago [5] suggests that smoking is associated with a modest reduction in sperm concentration, motility and morphology, but this effect seems to be limited to healthy men and to disappear when infertile men are included.

Smoking seems to have little effect on male fertility [6], although a recent study showed that infertile couples are more likely to smoke than fertile ones [7]. Although paternal smoking seems to be associated with congenital abnormalities and childhood cancer [8], sperm mutagenicity of smoking is still debated [9].

The Y chromosome micro-deletions are the most common genetic causes of male infertility due to spermatogenesis failure and have been reported in 2.7-55.5% of infertile men [10] and [11]. The frequency of Y chromosome micro deletions increases with the severity of spermatogenesis defect [12]. Micro-deletions in the Y chromosome long arm (Yq) are known to represent the pathogenic mechanisms for infertile males. Three distinct non-overlapping regions designated as AZFa, AZFb, AZFc are located in interval 5-6 of long arm of Y chromosome and are associated with impaired spermatogenesis in humans [13].

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The micro-deletions in these AZF loci are associated with azoospermia as well as varied testis histology ranging from sertoli cell only syndrome (SCO) to hypo spermatogenesis (HSG) and maturation arrest. Normal testicular histology reveals two types of cells, i.e. Leyding cell and sertoli cell, both of which are required for normal functioning. Presence of only sertoli cell in testicular histology is known as SCO syndrome and is associated with infertility. These AZF regions are putative RNA binding proteins and so may be involved in the regulation of gene expression. Infertility can be now treated with the help of recently developed techniques such as intracytoplasmic sperm injection (ICSI) and *in vitro* fertilization (IVF). However, deletions on the Y chromosome may pose problems as they might be spread to the male offspring, causing the assiduousness of infertility problem over the next generations. This study was carried out to identify the substantial prevalence of Yq micro-deletions in environmental tobacco smokers [ETS]. PCR technology was employed in South Indian ETS (males) to identify the micro-deletions linked with male infertility.

MATERIALS AND METHODS

Experimental Design: The study was carried out on 180 white-collar workers. Upon enrolment, every individual signed an informed consent and filled out a comprehensive questionnaire concerning smoking habits, number of cigarettes smoked per day, age, gender, lifestyle (i.e. drinking, diet, etc.) and medical history of disease, drugs. All the subjects were divided into in to six groups as follows according to their age factor.

Group1: Control subject-20 to 40 age
Group 2: Control subject-41 to 60age
Group 3: Active smoking subject-20 to40 age
Group 4: Passive smoking subject-20 to40 age
Group 5: Active smoking subject-41 to 60 age
Group 6: Passive smoking subject-41 to 60 age

Smokers smoked 10-30 cigarettes/day, passive smokers (ex-smokers) were exposed at about 6 h/day for at least 1 year and ex smokers had stopped at least 2 years for at least 1 year. All subjects were healthy individuals who followed a generally similar well-balanced diet and who had no problems in their medical history. Informed consent was obtained from all participating subjects. The work was carried out in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Sampling: Blood samples (5 ml) were taken at 8.00 AM in the morning from each subject by venous puncture. Germline DNA was isolated by the standard protocol developed by [14] for the genotypic analysis. No conflict of interest was recorded and all the information's of the subjects were classified.

Yq MICRO-DELETION ANALYSIS BY STS-PCR BASED STRATEGY: Each patient was tested for eight sequence tagged sites of AZF region located at long arm of Y chromosome including the internal control. The internal control used was SRY (sex determining region) gene. The primer sequences and PCR product size of different STSs primers [15] are represented in Table 1. PCR was carried out in 13 µl reaction volume containing 150 ng of DNA, 1.5 mM MgCl2, dNTPs mix (0.2 mM each), oligonucleotide primers [15] (10 pm each), Taq DNA polymerase (1 unit). Amplification was carried out in a Eppendorf-Thermo cycler with following thermal profile—initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55°C for 30 s, extension at 65 °C for 1 min followed by final extension at 65 °C for 5 min. PCR products were separated on a 2% agarose gel containing ethidium bromide (0.5 µg/ml), by electrophoresis in TAE buffer.

Deletion of a particular STS was confirmed only when that STS failed to amplify after three PCR attempts in presence of successful amplification of internal control.

Table 1: Primer sequences and PCR product size of Y STS

STS Marker	Primer sequence	AZF region	PCR product size (bp)
sY 81	F-AGGCACTGGTCAGAATGAAG, R-AATGGAAAATACAGCTCCCC	AZFa	209
sY 84	F-AGAAGGGTCTGAAAGCAGGT, R-GCGTAGCTGGAGGAGGCTTC	AZFa	326
sY 124	F-CAGGCAGGACAGCTTAAAAG, R-ACTGTGGCAAAGTTGCTTTC	AZFb	109
sY 128	F-GGATGAGACATTTTTGTGGG, R-GCCCAATGTAAAACTGGACA	AZFb	228
sY 133	F-ATTTCTCTGCCCTTCACCAG, R-TGATGATTGCCTAAAGGGAA	AZFb	177
sY 254	F-GGGTGTTACCAGAAGGCAAA, R-GAACCGTATCTACCAAAGCAGC	AZFc	370
sY 255	F-GTTACAGGATTCGGCGTGAT, R-CTCGTCATGTGCAGCCAC	AZFc	126
sY 14	F-GAATATTCCCGCTCTCCGGA, R-GCTGCTGCTCCATTCTTGAG	Internal control SRY	472

Table 2: Showing the frequency of STS deletion in the controls and experimental groups

Groups	sY81	sY 84	sY 124	sY 128	sY 133	sY 254	sY 255	sY 14
1: Control subject-20 to 40 age	0^a	0^a	0^a	0^a	0^a	0^a	0^a	0^a
2: Control subject-41 to 60age	0^a	0^a	0.034 ± 0.18^b	0^a	0^a	0^a	0^a	0^a
3: Active smoking subject-20 to40 age	0.034 ± 0.18^b	0^a	0.034 ± 0.18^b	0.034 ± 0.18^b	0.034 ± 0.18^b	0^a	0.034 ± 0.18^a	0^a
4: Passive smoking subject-20 to40 age	0.006 ± 0.25^c	0^a	0^a	0^a	0^a	0.034 ± 0.18^b	0.034 ± 0.18^a	0^a
5: Active smoking subject-41 to 60 age	0.006 ± 0.25^c	0.034 ± 0.18^b	0^a	0.034 ± 0.18^b	0.034 ± 0.18^b	0.034 ± 0.18^b	0.034 ± 0.18^a	0^a
6: Passive smoking subject-41 to 60 age	0^a	0.034 ± 0.18^b	0.034 ± 0.18^b	0.034 ± 0.18^b	0.034 ± 0.18^b	0^a	0.006 ± 0.25^c	0^a

Values are Mean ± S.E from 30 subjects of each group.

Values not sharing a common superscript are differ significantly at P<0.05 (DMRT).

The presence of deletion results in the absence of synthesis of the desired product, therefore, absence of band corresponding to any of the studied region is indicative of presence of the deletion of the particular region. Similarly the presence of band corresponding to the amplified region is indicative of absence of deletion. A positive control (fertile male DNA) and a negative control (female DNA) were also included with each set of reaction to ensure that all primers worked and that no contamination occurred during the course of the study.

Statistical Analysis: Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) by SPSS software package, version 17.0 (SPSS Inc, Chicago, IL, USA). Results were expressed as mean ± S.D. from 30 subjects in each group. The *P* values < 0.05 were considered to be significant.

RESULTS

In Group: 1 (control subject-20 to 40 age group) no micro deletions were observed. When compared to group: 1, 4 micro deletions (13.3%) were observed in Group: 3 (Active smoking subject-20 to 40 age group) and 3 micro deletions (10%) were observed in Group 4 (ex smokers 20 to 40 age groups). In group: 2 (control subjects 41 to 60 age group), 1 micro deletion (3.3%) was observed. When compared to control Group:2 (control subjects 41 to 60 age group), 8 micro deletions (26.6%) were observed in group: 5 (Active smoking subject-41 to 60 age group) and 6 micro deletions (20%) were observed in group:6 (ex smokers 41 to 60 age).

Table: 2 shows that the deletion in the sY81 micro satellite marker is significantly increased in all EST groups as compared to control subjects at P<0.05. Deletion in the sY84 micro satellite marker is significantly increased in 5 and 6 EST groups (Active smoking subject and ex smokers: 41 to 60 age group) when compared to other groups at P<0.05. The sY124 satellite marker is significantly increased in both control subjects (40-60 age) and all EST groups except group 3 (Passive smoking subject-20 to 40 age) and group 5 at P<0.05.

Elevated levels of micro deletions were observed in the sY 128 micro satellite marker it is significantly increased in all EST groups except group 3 (Passive smoking subject-20 to 40 age) as compared to control subjects at P<0.05.The sY 133 satellite marker is significantly increased in all EST groups except group 3 (Passive smoking subject-20 to40 age) as compared to control subjects at P<0.05.The sY 254 micro satellite marker is significantly increased in EST groups 3 (Passive smoking subject-20 to40 age) and 5 (Active smoking subject-20 to 40 age) as compared to control subjects at P<0.05. High frequency of micro deletion were recorded in the the sY 255 micro satellite marker it is significantly increased in all groups except control groups at P<0.05. The sY14 micro satellite marker does not show any change in control subjects as well as the experimental subjects.

DISCUSSION

In humans, about 15% of couples are infertile for various reasons, of which male factors account for 50% of the cases. In approximately 30%-50% of all cases of azoospermia or severe oligozoospermia, etiology is idiopathic [16]. In the present study, 22 micro deletions were observed in 180 subjects with the aid of 8 STS micro satellite markers. sY81 and sY84 are the two STS markers corresponding to the AZFa region of the Y-chromosome. The present experiments shows that, sY81 has 1 deletions in group:3 expiremental subjects and each 2 deletion in group:5 and 6 expiremental subjects. The sY81 micro satellite marker is significantly increased in all EST groups as compared to control subjects at P<0.05.

The sY84 micro satellite marker shows did not showed any deletions in group: 1, 2, 3 and 4 but each 1 deletion was observed in group: 5 and 6. Thus the sY84 micro satellite marker is significantly increased in 5 and 6 EST groups (Active smoking subject and ex smokers: 41 to 60 age group) when compared to other groups at P<0.05. Therefore the PCR based Y-chromosome screening is becoming necessary both for providing accurate diagnosis as well as for proper management of

the patient clinically and for counseling. Each AZF locus of Y chromosome is associated with different stages of spermatogenesis and deletion of each locus interrupts spermatogenesis at a particular stage [13]. Deletion of AZFa is associated with complete absence of germ cells and presence of sertoli cells in seminiferous tubules.

sY124, sY128 and sY133 micro satellite markers were mainly associated with the AZFb region of the Y-chromosome. Interestingly in the present study, group:2 control subjects shows a deletion in the STS sequence and it also showed each 1 deletion in the group:3 and 6. Hence the sY124 satellite marker is significantly increased in both control subjects (40-60 age) and all EST groups except group 3 (Passive smoking subject-20 to 40 age) and group 5 at $P<0.05$. The sY128 satellite marker is significantly increased in all EST groups except group 3 (Passive smoking subject-20 to 40 age) as compared to control subjects at $P<0.05$.

The sY 133 satellite marker is significantly increased in all EST groups except group 3 (Passive smoking subject-20 to40 age) as compared to control subjects at $P<0.05$. Deletion of AZFb is associated with germ cell development arrest at pachytene stage. These observations are consistent with the observations of that subjects with AZFb deletion range from azoospermia to mild oligozoospermia [17].

sY254 and sY255 are directly associated with the AZFc region of the Y-chromosome. sY254 shows each one deletion on the group: 4 and 5 besides this sY255 shows a high frequency of micro deletions, 4 deletion each 1 deletion in group:3,4,5 and 6. Thus the sY254 micro satellite marker is significantly increased in EST groups 3 (Passive smoking subject-20 to40 age) and 5 (Active smoking subject-20 to 40 age) as compared to control subjects at $P<0.05$. The sY255 micro satellite marker is significantly increased in all groups except control groups at $P<0.05$. AZFc deletion is associated with germ cell development arrest at spermatid stage and also with HSG and maturation arrest. sY254 and sY255 are mainly associated with the DAZ gene. The results falls in the above mentioned groups will have a deletion in the DAZ gene and DAZL1 gene. Deletion in the sY254 and sY254 may definitely show azoospermia condition or oligozoospermia condition with a clinical feature of maturation arrest, distorted sperm motility and sperm morphology and even show the dead sperm condition or highly restricted motility. The present experimental observations are supported by the results of several investigators [18] and [19].

The rapid growth of molecular biology has determined that micro deletions of the Y chromosome represent an important cause of male infertility and the most frequent genetic etiology of severe testiculopathy. Such findings are fundamental both for a careful diagnosis of male infertility and for its treatment and Y chromosome screening is now a reality in the major andrological and infertility centers. The detection of a deletion in an infertile man provides a proper diagnosis of the disease, allows the clinician to avoid empirical, unnecessary and often expensive treatments to improve fertility (*e.g.*, hormonal treatments) and has important ethical consequences if the patient is a candidate for assisted reproduction techniques. Furthermore, it is now clear that a molecular diagnostic test of Y chromosome micro deletions should be at least performed in all men with a sperm concentration of less that 53-106/ml, regardless of the presence of other apparent concomitant causes of testicular damage, such as varicocele or cryptorchidism.

The identification of the actual role played by the AZF candidate genes in spermatogenesis will provide significant advances to our understanding of the biology of spermatogenesis, as well as the analysis of novel Y-chromosomal genes with a potential role in male germ cell development will clarify other important features of this important chromosome.

To date, most studies assess either knowledge or perceived risk and questions primarily focus on heart and lung disease and not other smoking related risks (e.g., other cancers, infertility), or the potential impact of these illnesses (e.g., premature death or disability). This study examines whether various health risks of cigarette smoking are known and are perceived to be a personal health risk by cigarette smokers. Evaluating knowledge and perceived risk can provide insight into whether more patient education is needed and in what areas, or if clinicians should place more emphasis on personalizing these risks for an individual smoker. Determining whether knowledge and perceived risks of smoking differ by age, gender, socioeconomic status and general health could also be used to inform smoking cessation education and treatment programs.

In aggregate, these results suggest that the frequency of Y-chromosome micro deletions were remarkably high in active smokers, when compare to the ex smokers and controls. The ability to resolve differences between exposed and controlled subjects using blood is known to depend on the magnitude of the expected effect, the variance and distributional characteristics of the

specific parameter in the study population and the number of men available for study. A larger study population would be needed to determine whether a smoking lifestyle also affected the other sperm end points. Thus, at this time, a cautious approach is recommended and active cigarette smoking (particularly in teenage men who consume alcohol and pursue a smoker's lifestyle) should be considered a potential genetic hazard capable of producing trisomy in future unexposed embryos, fetuses and children.

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