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Sequence Tagged Site (STS) Analysis of Y-Chromosome Micro Deletions in Phthalate Exposed Plastic Industry Workers in Tamil Nadu, India

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Abstract: It was found that Azoospermia factor locus (AZF) contains genes responsible for spermatogenesis. Any mutation in these regions would result in male infertility. This study was conducted to establish prevalence of micro-deletions in the Y chromosome in 60 occupationally exposed workers in plastic Industry, who are subjected to phthalates (plasticizers). Polymerase Chain Reaction (PCR) micro-deletions analysis was done in 60 test subjects and control as well. Genomic DNA was extracted from the blood. Seven set of primers were used encompassing AZFa, AZFb and AZFc regions. The result revealed that 14 micro deletions (46.6%) were observed in group: 3 (sample subject-25 to 35 age group). Only one micro deletion was (3.3%) observed in group: 2 (control subject-36 to 40 age group). When compared to control group: 2 (control subject-36 to 40 age group), high frequency of micro deletions were observed in group: 4 (sample subject-36 to 45 age group), 24 micro deletions (80.0%).

Key words: Phthalates % STS Primers % Azoospermia Factor % Micro-Deletion % Polymerase Chain Reaction (PCR)

INTRODUCTION

Infertility is found among affects 15% of couples worldwide and half of them are due to male factors [1]. India, being a second largest populated country in the world, consists of more men than women with respect to infertility. Of the childless Indian couples, who approach doctors for treatment, 23% were found to be was due to male factors. Genetic abnormalities would account for 15%–30% of male factor infertility. Genetics contributes to infertility by influencing a variety of physiological processes including hormonal homeostasis, spermatogenesis and sperm quality [2]. However, besides the genetic factors the other non-genetic factors such as exposure of chemicals for a long period or higher intensity of exposure for a short period contribute substantially to male infertility. Male infertility has been identified in 50% of all plastic industries.

Phthalates plays a vital role in hormone disruption. Studies show that common environmental exposures of plasticizers would lead to adverse effects, which would result in decreased sperm density of male across the world [3]. It is also explained that regional differences in male sperm density coincide with the use, manufacturing and exposure of endocrine disruptors [2]. Di ethyl hexyl Phthalate (DEHP) and other phthalates have been reported as androgen antagonists [4]. Sperm DNA damage was associated with Mono Ethyl Phthalate (MEP) and with Mono Ethyl Hexyl Phthalate (MEHP) [5].

Both qualitative and quantitative abnormalities in sperm production are responsible for infertility in 40% of the population. The origin of reduced testicular sperm function in about 60–70% of cases is unknown [6]. A study done reveals that, the long arm of the human Y chromosome is required for sperm production [7]. Deletion in three different regions can cause severe spermatogenic defects, ranging from non-obstructive

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azoospermia to oligozoospermia [8]. These regions are referred to as 'azoospermia factors' (AZFs). Three distinct non-overlapping regions, designated as AZFa, AZFb and AZFc, are located in interval 5–6 of the Yq chromosome and are associated with impaired spermatogenesis in humans [9]. Y chromosome micro deletions are most commonly detected in men with azoospermia (absence of sperm) or severe oligospermia (<1 million sperm/ml semen). Azoospermia factor (AZF) is a gene cluster found in the long arm of the chromosome Yq11. Y chromosome microdeletions test is widely used in the diagnostic evaluation of male infertility. Hence, incidence of Y chromosome microdeletions among the plastic industry workers using STS by PCR would be the most effective primary screening tool.

An understanding is essential to appropriately manage an infertile couple who suffers due to the occupation disclosure of endocrine disruptors. The male factor infertility is one of the most perplexing disorders in the reproductive domain. Its incidence keeps rising while its etiology remains elusive. Hence this paper investigates the relationship between phthalates and incidence of Y-Chromosome micro deletions in occupationally exposed workers in plastic industries.

MATERIALS AND METHODS

The study was carried out on 120 samples (60 test samples and 60 controls) obtained from two different age groups of volunteers in plastic Industry near Thiruchirapalli and Coimbatore. Upon enrolment, every individual signed an informed consent and filled out a comprehensive questionnaire concerning smoking habits, age, gender, lifestyle (i.e. drinking, diet, etc.) and medical history of disease and drugs. All the subjects were divided into in to four groups as follows according to their age factor. Each group contains 30 samples each.

Group1: Control subject in the age group of 25 to 35

Group 2: Control subject in the age group of 36 to 45

Group 3: Plastic Industry workers in the age group 25 to 35

Group 4: Plastic Industry workers in the age group 36 to 45

The experimental subjects were workers employed in plastic industry situated in Thiruchirapalli district and Coimbatore District. The Control subjects were normal men with a normal family history. All subjects were healthy individuals, who followed a well-balanced dietary practice and who had no health 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 from each subject by venous puncture. Main source of samples were drawn from the Plastic industry workers. No conflict of interest was recorded and all the information provided by the subjects was classified.

Yq Micro Deletion Analysis by STS-PCR: All the patients were analyzed for eight STS (Table 1) of AZF region located in the long arm of the Y chromosome including the internal control. All the STS markers studied, were genotyped by amplifying 100-200 ng of DNA in a 35 cycle three step PCR (Eppendorf Thermal Cycler, Germany). PCR reactions were performed in 25 µL of reaction volume containing Genomic DNA of 100-200 ng (5.0 µL), 2 X PCR Master Mix (12.5 µL), Primers (Forward and Reverse) 1.0 µL each and nuclease free

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	AZFa	209
	R-AATGGAAAATACAGCTCCCC		
sY 84	F-AGAAGGGTCTGAAAGCAGGT	AZFa	326
	R-GCGTAGCTGGAGGAGGCTTC		
sY 124	F-CAGGCAGGACAGCTTAAAAG	AZFb	109
	R- ACTGTGGCAAAGTTGCTTTC		
sY 254	F-GGGTGTTACCAGAAGGCAAA	AZFc	370
	R-GAACCGTATCTACCAAAGCAGC		
sY 255	F-GTTACAGGATTCGGCGTGAT	AZFc	126
	R-CTCGTCATGTGCAGCCAC		
sY 14	F-GAATATTCCCGCTCTCCGGA	Internal control SRY	472
	R-GCTGCTGCTCCATTCTTGAG		

water (5.5 µL). Amplification was carried out with the following thermal profile- Initial denaturation at 94° for 5 minutes followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 70°C for 30 s followed by final extension at 70°C for 5 min. The PCR products (amplicons) were subjected to electrophoresis in 1.5% agarose gels containing ethidium bromide 1% in TBE buffer 140 mA. A DNA ladder was also run to confirm the amplification of the desired gene. After electrophoresis the gels were examined under UV trans -illuminator (Bio-rad, USA).

Deletion of a particular STS was confirmed only when that STS failed to amplify after three PCR attempts in the presence of successful amplification of internal control. The presence of deletion results in the absence of synthesis of the desired product and therefore, the 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. An internal control (SRY) was 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 were performed using one-way analysis of variance (ANNOVA) followed by Duncan's multiple range test (DMRT) by SPSS (Version 17.0). 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 - 25 to 35 age group) no micro deletions were observed. When compared to group: 1, 14 micro deletions (46.6%) were observed in group: 3 (sample subject-25 to 35 age group). Only one micro deletion was (3.3%) observed in group 2 (control subject-36 to 40 age group). When compared to control group: 2 (control subject-36 to 40 age group), high frequency of micro deletions were observed in group: 4 (sample subject-36 to 45 age group), 24 micro deletions (80.0%). Table: 2 shows that the sY81 micro satellite marker is significantly increased in group: 2 (control subject-36 to 40 age) as compared to control subject (group: 1 control subject- 25 to 35 age group) at P<0.05. The sY84 satellite marker is substantially increased in group: 3 (sample subject-25 to 35 age) when compared to group: 4 (sample subject-36 to 45 age group) and also compared to other subjects at P<0.05. Micro satellite marker sY124 also showed amplified results in experimental group 3 and 4 (sample subject-25 to 35 and 36 to 45 age group) when compared to other subjects at P<0.05.

The sY128 satellite marker show considerable increase in group: 3 (sample subject-25 to 35 age group) when compared to group: 4 (sample subject-36 to 45 age) and also compared to other subjects at P<0.05. Micro satellite marker sY133 is increased group 3 and 4 (sample subject-25 to 35 age group and sample subject-36 to 45 age group) when compared to other subjects at P<0.05. The sY254 micro satellite marker is significantly increased in all experimental groups except control groups at P<0.05 it is significant when compared to sY255 micro satellite marker. The sY 255 micro satellite marker is significantly increased in all groups except control groups at P<0.05 and it is highly significant than the sY254 micro satellite marker. The sY14 micro satellite marker does not show any change in control subjects as well as the experimental subjects.

DISCUSSION

The main breakthrough of DNA-based molecular markers was driven by the invention of PCR [10]. For the first time, any genomic region could be amplified and analyzed in many individuals without the requirement for cloning or isolating large amounts of ultra-pure genomic DNA. Micro satellites as the first widespread markers to take full advantage of PCR technology were microsatellites [11-13]. High mutation rate of micro satellites allowed a more detailed analysis of their mutation pattern [14]. Micro satellites gain and lose repeat units by DNA-replication slippage, a mutation mechanism that is specific to tandemly repeated sequences.

Table 2: Showing the frequency of micro deletions 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-25 to 35 age	0a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
2: Control subject-36 to 40 age	0.03±0.004b	0 a	0a	0a	0a	0a	0a	0 a
3: Infertile subject-25 to 35 age	0.03±0.005 b	0.06±0.005 b	0.06±0.005b	0.06±0.004b	0.03±0.004b	0.1±0.02b	0.16±0.02b	0 a
4: Infertile subject-36 to 45 age	0.03±0.005 b	0.03±0.004c	0.06±0.005b	0.03±0.002c	0.03±0.004b	0.23±0.02c	0.36±0.04c	0 a

Values not sharing a common superscript are differ significantly at P<0.05

However, there is some limit to the application of these conventional methods in karyotyping, southern-blot and polymerase chain reaction (PCR) in infertility clinics, mainly due to its low specificity, inaccuracy and time-consuming procedure [15]. Even though most of the infertility clinics are currently using multiplex PCR rather than single PCR, more precise methodologies are necessary to efficiently pursue diagnostic studies for male infertility.

In the present study, 39 micro deletions were observed in 120 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 two main genes located in the AZFa region are USP9Yand DBY (also called DDX3Y). Deletions in the AZFa region that remove both of these genes cause Sertoli cell–only syndrome, a condition characterized by the presence of complete Sertoli cells in the testes but a lack of spermatozoa in the ejaculate [16, 17]. The present experiments show that, sY81 has 1 deletion in groups 3 and 4 in experimental subjects and no deletions were observed in control subjects. The sY81 micro satellite marker is significantly increased in all subject groups as compared to control subjects at $P<0.05$, but there is no significance observed between the experimental groups. Each 1 deletion was observed in both groups 3 and 4 of industry workers. The sY84 micro satellite marker did not show any deletions in groups: 1, 2 and 3 but 1 deletion was observed in group 4. Thus the sY84 micro satellite marker is significantly increased in group 4(infertile men: 36 to 45 age group) when compared to other groups at $P<0.05$. DBY, the major gene located in the AZFa region, has a probable role in infertility because it is localized in the testis and is involved in the development of premeiotic germ cells [18].

The AZFb deletions cause arrest of spermatogenesis at the primary spermatocyte stage, indicating that the region is essential for fertility [15]. The main gene in the AZFb region is RBMY and there are six copies of the gene located on the Y chromosome. RBMY1 codes for an RNA binding protein, which is a testis-specific splicing factor expressed in the nuclei of spermatogonia, spermatocytes and round spermatids [17]. In the present study three STS markers were used to detect the micro deletions in the AZFb region they are, sY124, sY128 and sY133.

Interestingly the micro satellite marker sY124 shows a micro deletion in group 2 (control subjects 36 - 45 age groups) and each 2 deletions were observed in groups 3 - 4 industry workers (25 - 35 and 36 – 45 age groups). Micro satellite marker sY124 is significantly increased in experimental group 2 (control subjects 36 - 45 age group), 3 and 4 (workers subject-25 to 35 and 36 to 45 age groups) when compared to other subjects at $P<0.05$. No significance was observed between these three groups. The sY128 does not show any micro deletion in control groups. A couple of micro deletions were observed in group 3 experimental subjects (Industry workers 25 - 35 age group) and 1 micro deletion were observed in group 4 experimental subjects (industry workers 36 - 45 age group). The sY128 satellite marker is significantly increased in group: 3 (workers subject-25 to 35 age group) when compared to group: 4 (workers subject-36 to 45 age group) and also compared to other subjects at $P<0.05$. Each one micro deletion was observed in both the group 3 and 4 experimental subjects (infertile subject-25 to 35 and 36 to 45 age). No micro deletions were observed in control subjects. Micro satellite marker sY133 is significantly increased group 3 and 4 (workers subject-25 to 35 age group and workers subject-36 to 45 age group) when compared to other subjects at $P<0.05$. But no significance was observed between groups 3 and 4.

Deletions in the AZFc region produce a wide range of phenotypes, many of which are associated with low sperm concentration due to reduced spermatogenesis [19]. AZFc deletions cause approximately 12% of nonobstructive azoospermia and 6% of severe oligozoospermia [20]. Studies demonstrate that only the AZFa and AZFb regions are needed to initiate spermatogenesis, but without the AZFc region, spermatogenesis will not be completely normal [21]. Studies of complete deletions of the AZFc region may occur in two different ways: either as a result of a previous deletion within the AZFc or spontaneously from a normal AZFc region. A study was found that there were more complete deletions of the AZFc region in groups with existing partial deletions in that area of the Y chromosome [22]. This result was replicated in a study of Italian men with a high frequency of partial deletions in the AZFc region. A deletion of the AZFc region may also predispose men to Y chromosome loss, leading to sexual reversal. A couple of STS markers were used to identify the AZFc deletions in the present study, which are sY 254 and sY 255. Maximum number of micro deletions was observed in the group 3 and 4 experimental subjects. 7 numbers of microdeletions were observed in group 3 experimental subjects (subject sample: 25 - 35 age group) and 10 number of micro deletions were observed in group 4 experimental subjects (subject sample: 36 - 45 age

group). The sY 254 micro satellite marker is significantly increased in all experimental groups except control groups at P<0.05. It is significant when compared to sY255 micro satellite marker. The sY 255 micro satellite marker is significantly increased in all groups except control groups at P<0.05 and it is highly increased than the sY254 micro satellite marker.

However, the lack of association between testicular phenotype and genotype in affected men forces clinicians to employ inefficient and costly methods, such as polymerase chain reaction (PCR), to determine diagnosis. The Y chromosome contains 300 sequence tagged sites (STS), which correspond to the AZF regions and could be exploited for easier characterization of micro deletions [23]. The utility of this strategy by developing a targeted multiplex PCR using STS specific to the Indian population was demonstrated [24]. This type of procedure could be used as an initial screen for Y chromosome micro deletions before employing more expensive and technically challenging testing methods. However, to be effective, specific STS would need to be defined for different ethnic populations.

In aggregate, these results suggest that the frequency of Y-chromosome micro deletions were remarkably high in experimental subjects, when compare to the 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 the exposure of phthalates (plasticizers) also affected the other sperm end points. Thus, at this time, a cautious approach is recommended for the plastic industry workers and proper safety measures should be taken by the employers in turn to avoid the potential genetic hazard capable of producing trisomy in future unexposed embryos, fetuses and children.

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