A study on the patterns of expression of the *DAZ* and *HSP* genes in the testicular tissue of men with azoospermia

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Spermatogenesis is known to be regulated by a number of genes and several factors such as hormones, growth factors, cytokines and others. This study was done to evaluate the relationship between HSPs and DAZ genes in human spermatogenesis; we observed the expression pattern of HSP gene in azoospermia men with DAZ gene that regulated the gene expression related with human spermatogenesis. RT-PCR method was used to detect DAZ, HSP70A, and HSP70B transcripts in all RNA samples. Total RNA was extracted from 21 testis tissues using TRIZOL reagent. cDNAs were synthesized with reverse transcriptase, AMV. All PCR reaction were performed on a PCR themocycler with DAZ, HSP70A, and HSP70B-specific primers. Semen analysis, karyotyping and testis histology were performed. DAZ gene, known as a candidate gene of azoospermia factor(AZF), was deleted in 2 of 21 patients. To evaluate the only effects of HSPs in this patients, 2 DAZ deleted cases were removed. We observed the mRNA of HSP70B in 5 whereas none could be seen with regard to HSP70A. Furthermore, the sperm of these 5 men were discovered to be immature. In conclusion, HSP70B as well as DAZ gene seem to be involved causing spermatogenic failure. We suggest that HSP70B plays an important role in spermatogenesis and it is one of factors induced sperm maturation in human.

Keywords: azoospermia, DAZ, expression, HSP70, testis

INTRODUCTION

Due to a serious defect in the process of spermatogenesis 2% of all male adults are proven to be infertile, and most of them did not have other health problems. Of the infertile patients, those who show azoospermia have defects in spermatogenesis and unable to produce sperm. According to recent reports, the cause of this is the deficiency in the gene associated with spermatogenesis. (Tiepolo and Zuffardi, 1976; Sandberg, 1985; Chandley and Cooke, 1994; Reijo *et al.*, 1995; Lee *et al.*, 1997)

The main subject in the genetic study of spermatogenesis is the Y chromosome and its role was first advocated by Tiepolo and Zuffardi. They reported that microdeletions in the long arm of the Y chromosome were discovered in patients with azoospermia. Based on these results Tiepolo and Zuffardi have suggested that there is a gene on the long arm of the Y chromosome which plays an important role in spermatogenesis, thus confirming the presence of the *azoospermia factor (AZF)* and also reported of another structural abnormality besides the deletion on the Y chromosome as a result of chromosomal studies in patients with azoospermia. The abnormal cytogenetic findings of Yq in men with azoospermia or oligospermia anticipated that the genetic element controlling spermatogenesis is in Yq11 (Tiepolo and Zuffardi, 1976; Sandberg, 1985); they named the genetic factor AZF (i.e. azoospermia factor; Tiepolo and Zuffardi, 1976). According to studies on men observed to have translocations between the Y chromosome and autosomal chromosomes or showing large deletions at Yq, all euchromatic areas of the Y chromosome were reported to be necessary for normal development of gametes (Vergnaud *et al.*, 1986), and there were observations that men with deletions detectable through chromosomal studies were azoospermic. These observations made certain the assumption that gene(s) associated with azoospermia were at deletion interval 6 on the long arm of

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the Y chromosome. (Burgoyne, 1987; Bhasin *et al.*, 1994; Chandley and Cooke, 1994)

According to latest reported among the many genes thought to be associated with the control of spermatogenesis in men, genes on the long arm of the Y chromosome, especially the gene in deletion interval 6 is considered to be the most definite one. (Burgoyne, 1987; Bhasin *et al.*, 1994; Chandley and Cooke, 1994). A certain amount of proof has been reported that this region, that is, the gene at Yq11.23, plays an important role in spermatogenesis (Bhasin *et al.*, 1994), and the location of AZF at Yq11 has been confirmed at levels of cytogenetics (Yunis *et al.*, 1977; Chandley *et al.*, 1989) and molecular biology (Ma *et al.*, 1992) in many other laboratories. But the structure of AZF and its function has not been clearly verified yet.

According to Ma *et al.* (1992) and Vogt *et al.* (1992), the locus of AZF is said to be present between the proximal and distal parts of Yq11 and since the microdeletions of these proximal and distal parts do not overlap, the AZF locus seemed to be very large genes, several or a set of genes located at intervals 5 and 6 of Yq11.23 which were associated with spermatogenesis. After the suggestion of the presence of AZF associated with spermatogenesis by Tiepolo and Zuffardi (1976), Ma. *et al.* (1993) reported microdeletions of the Y chromosome, especially those at interval 6 through experiments using DNA probes, therefore obtaining definite knowledge of the presence of AZF and during the progress of many studies Ma *et al.* found a gene at interval 6. This gene, *YRRM* (RBM), has an RNA recognition motif and is assumed to function in the processing of RNA or the control of translation. But the functions of *YRRM1* and *YRRM2* have not been clearly defined yet and are located at various sites on the Y chromosome, thus while raising questions as AZF, Reijo *et al.* (1995), according to the results of experiments on 89 subjects with azoospermia, microdeletions could be observed in 12, and these were identified nothing to do with *YRRM1* and *YRRM2* reported by Ma *et al.* and therefore reported as *DAZ* (deleted in azoospermia), novel genes associated with spermatogenesis. This *DAZ* gene, like *YRRM1* and *YRRM2*, is a protein that has an RNA binding motif and was shown to be expressed only in the testes. Recently, a mouse gene that exhibits similarity to the *DAZ* gene has been cloned and termed as Dazla (Cooke *et al.*, 1996; Reijo *et al.*, 1996). These genes which were identified in fruit flies unlike the human *DAZ*, located not in the sex chromosomes but in the autosomal chromosomes and discussions are being made on the role of the RNA binding protein in the autosomal chromosome, regardless of the Y chromosome. Thus while on autosomal chromosomes genes associated with spermatogenesis are being discovered, it has been shown that *DAZH* (Saxena *et al.*, 1996) is located on chromosome 3 in humans and reported that human spermatogenesis is controlled by many complex factors besides the Y chromosome.

Owing to these studies in molecular biology and molecular genetics, studies in the field of male infertility are in active progress and there are reports that about 13% of the patients with non-obstructive azoospermia are caused by deletions of AZF on the Y chromosome (Reijo *et al.*, 1995)

On the other hand, HSP which is expressed in the testes is present in the spermatogenic cell and is known to be associated with spermatogenesis. According to Wolgemuth *et al.* (1987), HSP is reported to be specifically expressed in male haploid spermatogonia and associated with the differentiation of germ cells and the metabolic action during meiotic division in the early development of spermatogonia. (Raab *et al.*, 1995; Allen *et al.*, 1996; Wolgemuth, 1996). HSP, a protein expressed by external heat or other stimuli, is reported to play a role in protecting the testes and the HSP gene, which is functionally responsive to heat in the normal testes, is also found in the middle piece or tail of the sperm in humans and is known to play an important part in spermatogenesis (Sarge *et al.*, 1994; Allen *et al.*, 1996). Therefore whether this protein is expressed in the tissue of patients with azoospermia is considered to greatly affect the process of spermatogenesis.

In this study, we, by using PCR directed microdeletions at the long arm of the Y chromosome, investigated the degree of microdeletion based on male subjects with infertility, and observed the aspects of expression of both DAZ proposed by AZF and HSP, therefore analyzing its correlation with the process of spermatogenesis.

MATERIALS AND METHODS

The subjects

Of the infertile men who visited Samsung Cheil Hospital from November 1996 to January 1997, the sera and testicular tissues of 21 patients proven to be azoospermic through semen analysis were chosen as subjects with women designated as the negative controls, and fathered males as positive.

Semen analysis and chromosome analysis

In order to confirm the sperm in the extracted testicular tissue fine dissections were made and morphological analysis was executed via observation under high power fields and staining. Furthermore blood samples taken from the patients were treated with heparin and cultured in 10 ml of RPMI (Gibco BRL, USA) under the conditions of 37°C and 5% for 3 days. For the acquisition of chromosomes in metaphase 0.1 ml of colcemid (10 μg/ml) was added an hour before the completion of the culture, and then after centrifuging the cultured cells

at 100 rpm for 10 minutes and collecting them, they were treated with 5 ml of a hypotonic solution (0.075 M KCl). 5 ml of a mixture of methanol and acetic acid at the ratio of 3:1 was added to fix the cells collected after centrifugation. The fixed cells were then applied onto slide glasses and dried. After emerging the slides, which were previously dried overnight in a slide warmer set at 55-60°C, into a 0.05% solution of trypsin for 1 to 2 minutes they were stained with a 4% Giemsa solution for another 5 minutes and observed microscopically for chromosome analysis.

Isolation of Genomic DNA and the RNA in testicular tissue

Isolation of Genomic DNA

Sampling of the peripheral blood of the patients in vacuum tubes treated with anticoagulants was done, then centrifuged at 3,000 rpm followed by separation of the buffy coat and kept at -70°C. Genomic DNA was extracted by using a QIAamp Blood Kit (QIAGEN, Inc, Chatsworth, CA) and preserved again at 4°C.

Isolation of the RNA

Tissue samples taken from the patients were immediately put into a refrigerator set at -70°C and Trisol solution (Gibco BRL, USA) was applied to isolate the RNA. In order to obtain pure RNA only the genomic DNA was removed by using DNase I at 37°C for an hour, then after extracting phenol and isolating the water-soluble layer the RNA was precipitated with 100% ethanol 2.5 times that volume followed by washing the precipitate with 70% ethanol. The washed RNA was dried and used after dissolving in sterile water treated with DEPC.

Polymerase Chain Reaction (PCR) and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The reverse transcription reaction for the synthesis of cDNA was done by mixing 50 mM of Tris-HCl (pH 8.5), 8mM of, 30 mM of KCl, 1 mM of DTT, I mM of dNTP each, 0.5 units of RNase inhibitor, 2.5 µM of oligodT_{15}, 0.25 units of reverse transcriptase, AMV (Boehringer Mannheim) in a DNA thermal cycler (Perkin-Elmer) at 65°C for 10 min; 42°C for 60 min; and 99°C for 5 min. The synthesized cDNA was kept at -20°C before carrying out the PCR.

The primers used in PCR were, as listed in Table 1, DAZ, HSP70A, and HSP70B (Mivechi and Rossi, 1990) and Sry served as a control. The reaction consisted of mixing into the final 20 µl 10mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM 0.2 mM dNTPs, 20 pmol of coupled primers, 0.5 units of DNA polymerase (Boehringer Mannheim, Germany), and 50-100 ng of genomic DNA of the patient in the DNA thermal cycler (Perkin-Elmer, Stratagene), first at 94°C for 2 min; then for 35 cycles consisting of the following sequence: at 94°C for 40 sec; 58°C, 60°C or 62°C for 1 min respectively; and 72°C for 1 min, lastly, at 72°C for 5 min. The amplified product was analyzed through a 2% agarose gel electrophoresis and for those who gave negative results the final data were confirmed through at least three independent tests, in order to rule out all errors of PCR.

RESULTS

Sperm Analysis and Chromosomal Abnormalities

According to the results obtained by the analysis of the sperm in the testicular tissue extracted from the 21 patients, 4 did not show any sperm and 17 had sperm that could be extracted. When the extracted sperm was morphologically analyzed, maturation arrest was displayed in 7, whereas normal sperm could be seen in the rest. Also as seen in the chromosomal analysis from blood, all patients had normal karyotypes of 46, XY and there were no chromosomal abnormalities.

Microdeletions of DAZ, HSP70A, and HSP70B

By using PCR with primers for DAZ, HSP70A, and HSP70B, respectively, amplified products were demonstrated in the positive controls, whereas in the case of DAZ, none could be observed in the negative controls. In an analysis of an experiment requiring 21 men with azoospermia, 2 (9%) showed microdeletions of DAZ, whereas none of the patients displayed microdeletions in HSP70A and HSP70B. (Table 2)

The aspects of expression of DAZ

In order to observe the aspects of expression of the *DAZ*

Table 1. Primer sequences (5' to 3') of *DAZ* and *HSP* genes

Primers	Forward	Reverse
SRY	gAATATTCCCgCTCTCCggA	gCTggTgCTCCATTCTTgAg
DAZ	gACCACACAgCTAgAgCACC	gACTgTATCCTTCggATTCC
HSP70A	CTAgCCTgAggAgCTgCTgCgACAg	gTTCCCTgCTCTCTgTCggCTCggCT
HSP70B	ggTCggggAggTgCAAAAggAT	ggCTgAAgCTTCTTgTCggATgC

Table 2. The deletion and expression of DAZ and HSP70A, HSP70B gene in non-obstructive azoospermia

Gene	Deletion	Expression
DAZ	2/21 (9.5 %)	2/21 (9.5%)
HSP70A	0/21 (0.0 %)	0/21 (0.0 %)
HSP70B	0/21 (0.0 %)	5/21 (23.8%)

gene in patients with microdeletions and to see whether the *DAZ* gene could be normally expressed in patients without microdeletions, extraction of RNA from the testicular tissue in patients and reverse transcription and PCR resulted as follows: those who had a detectable microdeletion of the *DAZ* gene in the genomic DNA could not, as anticipated, synthesize the amplified products of DAZ mRNA and those who did not have a microdeletion could. (Table 2)

The aspects of expression of HSP70A and HSP70B

Microdeletions of HSP70A and HSP70B were not observed in the genomic DNA but according to observation of the aspects of expression of RNA in HSP70A and HSP70B, HSP70A could be seen in none of the patients, and while expression of the mRNA of HSP70B could be detected in 5 of the 21 patients, only immature sperm was to be seen in the testicular extracts of these 5 men. (Fig. 1, Table 2, Table 3)

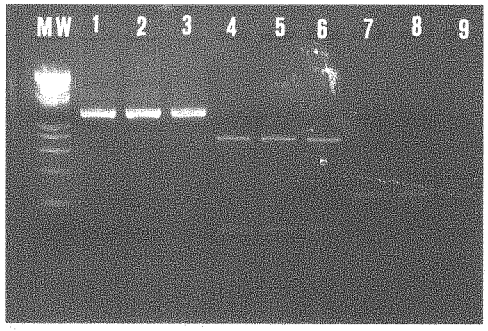

Fig.1. Expression of *DAZ* and *HSP70B*, MW; Molecular Marker XIV (B.M.); lane1-3; β-actin, lane4-6; *DAZ* lane7-9; *HSP70B*

DISCUSSION

Recently the number of patients being treated for male infertility has steadily increased among the causes of infertility. In addition to men with oligospermia or incompetent sperm, patients with total azoospermia also occupy a large number of the infertile. These patients have a defect in either spermatogenesis or an arrest in the process of sperm differentiation arising from other causes. Therefore the factors regulating spermatogenesis have been reported to be in the Y chromosome by many researchers, and the relationship between male infertility and the Y chromosome was first established by the suggestion of the existence of AZF on the long arm of the Y chromosome which is associated with spermatogenesis after discovering microdeletions there in men with azoospermia (Tiepolo and Zuffardi, 1976). While many studies with the aim to define AZF were taking place thereafter Ma *et al.* (1993) found a gene at interval 6, which led to a more active progress in terms of molecular genetic research. The genes, namely, *YRRM1* and *YRRM2*, contained RNA recognition motifs and were suspected to regulate the processing and translation of RNA in early spermatogenesis. But, while several reports of evidence which raises questions about YRRM as the AZF do exist, Reijo *et al.* (1995) reported a new gene, *DAZ*, and proved that it had nothing to do with the spermatogenesis regulated by YRRM1 and YRRM2 formerly informed by Ma *et al.*, (1993) thus strongly asserting that DAZ is the AZF that controls spermatogenesis. This *DAZ* gene is a protein which has an RNA binding motif and found to be specifically expressed only in the testes. These genes, unlike human DAZ, were located not in the sex chromosomes but in the autosomal chromosomes, suggesting the possibility of the function of the autosomal chromosomes towards RNA binding proteins in spermatogenesis. Recently, while autosomal chromosome genes associated with spermatogenesis are being discovered in mice and fruit flies, it has also been discovered that DAZH (Saxena *et al.*, 1996) is on the third

Table 3. Clinical characteristics of 5 infertile men with expression of HSP70B gene

Patient number	Indication	Age	Testes size (cc)	Histology	Karyotype	LH	FSH	T
1	Azoospermia	36	15/15	SCO	46 XY	6.7	9.5	1.2
2	Azoospermia	30	20/20	maturation arrest	46 XY	2.4	3.8	3.1
3	Azoospermia	48	16/16	severe hypoplasia	46 XY	3.4	7.3	3.9
4	Azoospermia	39	14/14	SCO	46 XY	4.3	23	4.7
5	Azoospermia	34	20/20	severe hypoplasia	46 XY	1.7	6.4	4.2

SCO, sertoli cell only; LH (mIU/ml), leuteinizing hormone; FSH (mIU/ml), follicular stimulating hormone; T (ng/ml), testosterone.

autosomal chromosome in humans. It has been shown that DAZH is located on chromosome 3 and reported that human spermatogenesis is controlled by many complex factors besides the chromosome.

As noted above there have been reports on the microdeletions in the DAZ region among patients with azoospermia or oligospermia and also places elsewhere, making it possible to know that the genes associated with spermatogenesis exist at many regions in the Y chromosome, based on the results of these studies.

According to the results of this experiment no microdeletions could be detected in the positive controls, but were seen in 2 men among the azoospermic patients, thus confirming that this microdeletion in the Y chromosome was one of the causes of the spermatogenic defect noticed in patients with microdeletions. As seen in the studies done by Reijo *et al.* (1995), it has been reported that microdeletions in the autosomal chromosomes, besides those in the Y chromosome, also cause abnormalities in spermatogenesis, which means that spermatogenesis is controlled by the complex actions of other factors rather than solely by the specific gene *DAZ*. Therefore, this study proved the fact that many genes besides *DAZ* are associated with spermatogenesis and further studies should be made.

Observing the mode of expression of the *DAZ* gene in both the patients who did or didn't display microdeletions, although not having any microdeletions it might be affected due to other different factors RNA extracts were taken from the testicular tissues of the patients, and the results of reverse transcription-PCR showed that no amplified products of DAZ mRNA could be detected as expected in the patients with microdeletions of the *DAZ* gene. Also, there have been reports of infertility due to chromosomal abnormalities (Ataya *et al.*, 1983; Chandley *et al.*, 1986; Micic *et al.*, 1990; Anderson *et al.*, 1988) but in this experiment the results of the karyotype analysis done on the 2 men with microdeletions were confirmed to be all normal with the karyotype 46, XY, reflecting the fact that it is impossible to find microdeletions of the Y chromosome through general methods of karyotype analysis and that this could not be directly associated with the diagnosis of spermatogenic defects.

On the other hand, we noticed that of the 21 patients 4 had no sperm detected in their testicular tissue while the rest did, and even in the cases of SCO patients whose sperm could not be found, based on the biopsy tissues obtained at various sites during the operation, sperm could be detected.

The *HSP70* gene acts on the process of differentiation seen in the germ lines of mammals (Zakeri and Wolgemuth, 1987), is expressed during spermatogenesis and acts on the differentiation of the spermatogenic cell. The mechanism of control is reported to be done by the transcription factors, that is, heat shock factors (HSF) 1 and 2 (Sarge *et al.*, 1994). Murgshov *et al.* (1996) found that, based on the results of examining the *HSP70* genes of the many tissues in mice, although expressed in other reproductive organs, it was expressed the most in the testes.

Results from the examination of the *HSP* gene in this study confirmed the forms of expression of the mRNA from the *HSP* gene in 5 patients with no deletions in the genomic DNA by using reverse transcription-PCR. Those with HSP70B showed SCO and maturation arrests, and the impression of the sperm discovered was one of the immature and these results mean that HSP70B is associated in the maturation process of sperm during spermatogenesis and by being expression temporarily at a certain time considered to play a functional part in the differentiation and maturation of sperm. Raab *et al.* (1995) discovered HSP70 in the spermatocytes and round spermatids of murine testes but not in the ejaculated sperm, thus reporting of its association with the differentiation and maturation process of sperm. In this study, however, HSP is considered to act as a maturation suppression factor, seeing that it is expressed in immature sperm and not in the mature. Hence, the mammalian *HSP* gene is thought to have various functions and play an important role in differentiation and development by acting specifically in each species (Raab *et al.*, 1995).

Due to the results of this study the DAZ microdeletion of the Y chromosome which is associated with the process of spermatogenesis in men with azoospermia has been confirmed in low ratios but it cannot be said that only this element controls spermatogenesis. According to current studies it is reported that spermatogenesis is controlled by many genes and that spermatogenesis is accomplished by the complex actions of genes besides *DAZ*. And since no microdeletions could be found in HSP70A and HSP70B they are thought not to be associated with differentiation and formation but function by being expressed at a fixed time in spermatogenesis, as observed that as an aspect of expression HSP70B exists only in the immature sperm of the testicular tissue, therefore acting as an important factor in the maturation process of sperm. In conclusion, DAZ is regarded to be associated with spermatogenesis and HSP70B to be of functional action in sperm maturation, making further studies on spermatogenesis including the *DAZ* and *HSP* genes necessary.

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