CHAPTER III

RESEARCH DESIGN, MATERIALS AND METHODS

1. Research design

1.1 Patient enrollment and specimen collection

Infertile males who attended the Infertile Clinic at Maharaj Nakorn Chiang Mai Hospital were screened for the causes of infertility. Semen analysis was performed according to the World Health Organization criteria [9]. Two semen samples per patient were analyzed at 4 weeks apart, each following a 3-day sexual abstinence. Patients were enrolled in this study if they had oligospermia (sperm count less than 10 million per ml) or azoospermia (no sperm in the ejaculated semen) in 2 consecutive semen analyses. Inform consent was obtained from all participants. After a complete medical history and a detailed physical examination, patients were asked for 5 ml of blood for laboratory tests.

1.2 Determination of reproductive hormone profiles and chromosome analysis

Serum prolactin, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone levels were measured by electrochemiluminescent technique at the Department of Obstetrics and Gynecology, Faculty of Medicine, Chiang Mai University. Chromosomal analysis was performed on culture lymphocytes, using the G- and Q-banding technique at the Department of Anatomy, Faculty of Medicine, Chiang Mai University.

1.3 Optimization of multiplex PCR technique for Y chromosome microdeletions detection

A multiplex PCR technique was set up to investigate Y chromosome microdeletions. Peripheral blood mononuclear cells (PBMCs) were isolated from

EDTA blood by Ficoll-Hypaque density gradient centrifugation method. Genomic DNA was extracted from the PBMCs and used as a template for PCR. Twelve oligonucleotide primer pairs specific for *sY14, DFFRY, DBY, SMCY, EIF1AY, RBM1, PRY, TTY2, sY277, sY283, CDY1* and *BPY2* genes were used in this study. The optimization process of multiplex PCR technique composed of the following steps.

1.3.1 Primer design and grouping

Published primer sequences specific for designated genes were verified. Some primer sequences were modified or redesigned in order to fit into multiplex PCR system. This was done using the complete sequences of the genes in the GenBank and the help of Primer Premier Software version 5.0 (PREMIER Biosoft International, CA, USA). The modified and redesigned primers were analyzed for their specificities, using the web-based BLAST program (http:// www.ncbi.nlm.nih.gov /BLAST/). Each primer pair was tested in a singleplex PCR system to ensure that the expected product size was obtained. The 11 primer pairs were then tested for primer-dimer formations and combined into 4 multiplexes, based on their product sizes and *Tm*. One primer pair specific for a control gene on the Y chromosome was finally added to each multiplex PCR set.

1.3.2 Optimization of multiplex PCR conditions

Hot start PCR with HotStarTaq (QIAGEN, Germany) polymerase was used for optimization of the four multiplex primer sets. The goal of optimization was to obtain the highest and comparable yields of individual primer-pair reaction. Conditions to be optimized included the annealing temperature, annealing time, extension time, dNTP concentration, $MgCl_2$ concentration, genomic DNA concentration, primer concentration and the number of cycles.

1.4 Data analysis

1.4.1 PCR result interpretation

A sample was considered “no-deletion” for the given gene-base markers when the PCR products of the expected sizes were present. It was considered ‘with-deletion’ if a product of the expected size was not found after three successful PCR reactions.

All samples were analyzed in a blinded fashion, without knowledge of the patient's details. The *SRY* gene was chosen as an internal control and amplification product should be shown in every sample. Normal male DNA with an absence of Y chromosome microdeletions was used as 'no deletion' control. Female DNA and double distilled water were used as negative controls.

1.4.2 All parameters analysis

Data from all tests including medical history, physical examination, hormone profiles and karyotyping were analyzed. Prevalence of Y chromosome microdeletions in Thai males with or without oligospermia or azoospermia was calculated. The steps of this study were summarized in Figure 3.1.

2. Materials and Methods

2.1 Subjects

Thai males, who attended the Infertile Clinic at Maharaj Nakorn Chiang Mai Hospital, were screened for the causes of infertility. A complete semen analysis was performed according to the World Health Organization guidelines [(9)]. Semen samples were obtained two times at 4-week interval, each following a 3-day period of sexual abstinence. A hundred and twenty infertile males were enrolled in this study. They were divided into two groups: 1) azoospermic group when no sperm was found in the ejaculate even after centrifugation (n = 40) and 2) oligospermic group when sperm count was found to be less than 10×10^6 spermatozoa/ml (n = 80). The attending physicians obtained medical history and performed physical examination on the infertile males before taking blood samples.

Fifty healthy men with normal semen analysis were studied along with the infertile men. Sample from a normal fertile male without Y chromosome microdeletions was used as positive control, whereas, a sample from a healthy woman was used as negative Y chromosome control in each multiplex PCR assay.

This study was approved by the Ethics Committee, Faculty of Medicine, Chiang Mai University and informed consent was obtained from each subject.

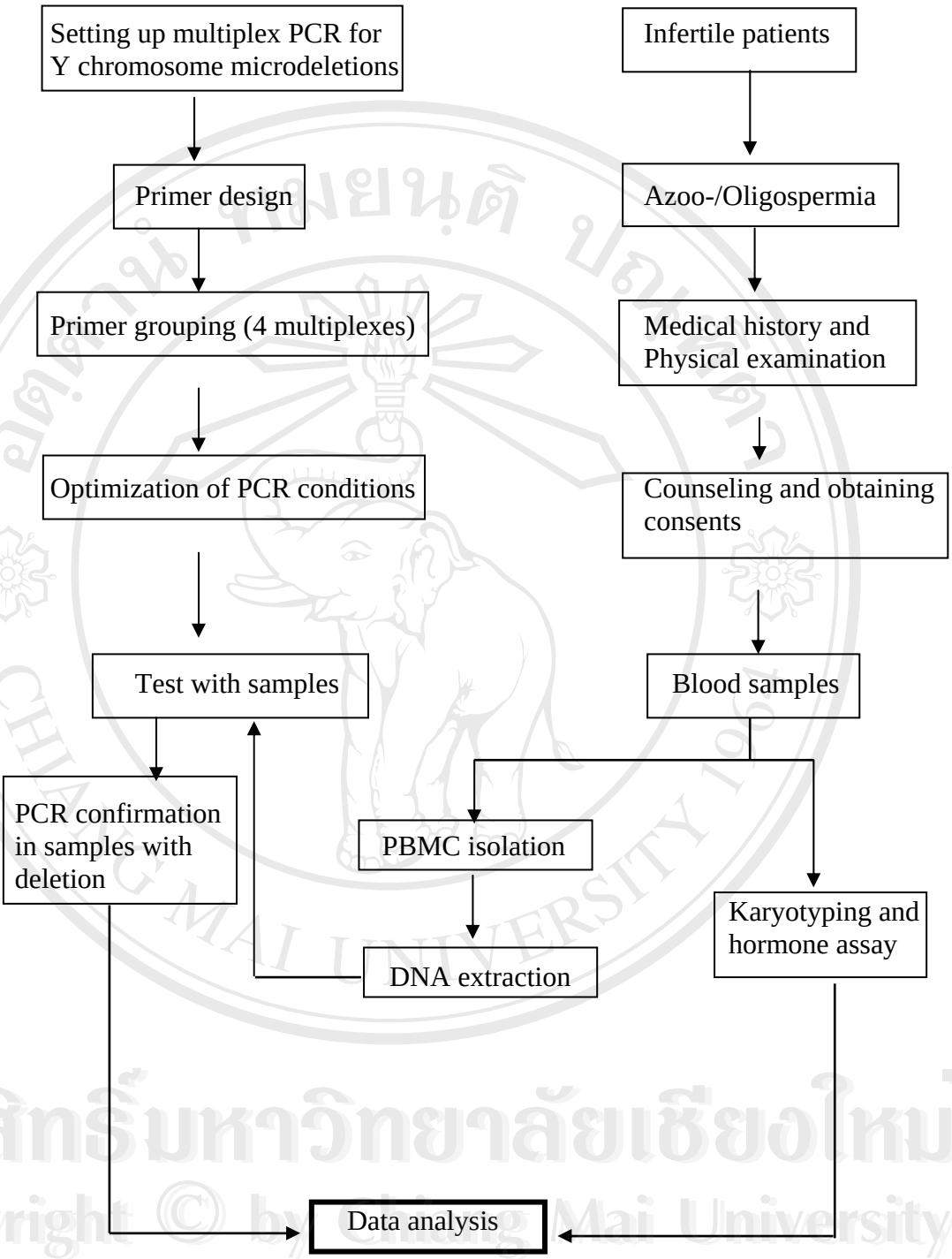

Figure 3.1 The schematic diagram of the research design in this study

2.2 Oligonucleotide primers

2.2.1 Primer design

Twelve primer-pairs were used in this study. They were specific for *sY14(SRY), DFFRY, DBY, SMCY, EIF1AY, RBM1, PRY, TTY2, sY277(DAZ), sY288(DAZ), CDY1 and BPY2* genes on the Y chromosome. Primers specific for *sY14(SRY), DBY, SMCY, RBM1 EIFIAY, sY277 and BPY2* genes were selected from previously published studies [35, 89, 99]. Primers specific for *PRY, CDY1 and TTY2* genes were modified from the reported sequences by Lin *et al*. [99]. Primer specific for *sY283* and *DFFRY* genes were newly designed by using Primer Premier software version 5.0 (PREMIER Biosoft International, CA, USA), based on the complete sequences of the genes in the GenBank. The primers were analyzed for their specificities using the web-based BLAST program (http:// www.ncbi.nlm.nih.gov/ BLAST/). All of them were specific to the intended targets. PCR oligonucleotide primers were ordered from a qualified company (QIAGEN, Hilden, Germany**).** A list of genes and nucleotide sequences of all primers used in this study were shown in Table 3.1.

2.2.2 Primer evaluation

The correct design of each primer-pair was checked by singleplex PCR amplification of 200 ng of fertile male DNA. A reaction volume of 50 µl was used with standard PCR conditions as follow: 1X PCR buffer with adjuvant, 200 µM dNTPs, 1.5 mM $MgCl_2$, 2.0 unit HotStarTaq (Qiagen, Hilden, Germany) and 0.2 µM of each primer. The PCR reaction consisted of denaturation at 95 °C for 15 min, followed by 35 cycles at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. Single PCR products were detected by electrophoresis on 1 % agarose gel. One-Kb ladders (Gibco BRL, USA) were used to verify the expected sizes of the amplicons.

2.3. Specimen collection and preparation

2.3.1 EDTA-anticoagulated blood sample

Five milliliters of peripheral blood were drawn by venepuncture and put into a tube containing ethylene diamine tetra-acetic acid (EDTA). The blood and EDTA were mixed well. The tube was kept refrigerated until further processing, within 24

hours. After centrifugation at 1500 x g for 15 minutes, the buffy coat was collected from the white blood cell layer and put into another tube.

Table 3.1 The sequences and product sizes of all 12 primers used in this study

Gene	Acc. No.	Left primer	Right primer	Product size (bp)
sY14[€]	G38356	GAA TAT TCC CGC TCT CCG GA	GCT GGT GCT CCA TTC TTG AGT	470
SMCY*	AF134849	CCT CCA GAC CTG GAC AGA AT	TGT GGT CTG TGG AAG GTG TCA	362
RBM1*	NM_005058	ATG CAC TTC AGA GAT ACG GGA	CTC TCT CCA CAA AAC CAA CAT G	800
EIF1AY[†]	AF00987	CTC TGT AGC CAG CCT CTT CTG	GAC TCC TTT CTG GCG GTT AC	84
DBY *	NM_004660	ATC GAC AAA GTA GTG GTT CCA G	AGA TTC AGT TGC CCC ACC AG	689
DAZ (sY283) (New)	G42071	ACT TGA TGC CTC TTG ACA CTG AT	GTT ATT TGA AAA GCT ACA CGG G	314
PRY[£]	NM_004676	GAG CAC ACC ACA CCA GAA ACA	CTC AGA CTG ACC TCG GAC TGT	80
DFFRY (New)	G38348	ATG TGG ACT ATA ATT TCT TCC CTT	CTG TCG TTC CCT CCT ACT GG	130
CDY1[£]	NM_004680	TGG GCG AAA GCT GAC AGC A	TTG GGT GAA AGT TCC AGT CAA	79
DAZ (sY277) *	G42070	GGG TTT TGC CTG CAT ACG TAA TTA	CCT AAA AGC AAT TCT AAA CCT CCA G	312
TTY2[£]	AF_000991	GAC AAC TCT GAC AGC CAG GG	TCA GAA CTC CCA AAC AGG C	87
BPY2*	NM_004678	GGG ATT ATC ACA TAT TGC GG	ATG ATA GTC GCG TCA GCT GG	370

[€] = Simoni M. 1999 [(89)], * = Lin Y.M. 2002 [(99)], [£] = modified from *

[†] = Kostiner DR. 1998 [(35)], New = new design in this study

2.3.2 Isolation of peripheral blood mononuclear cells (PBMCs) from buffy coat

PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation, using Isoprep reagent (Robbins Scientific, CA, USA) according to the manufacturer's instruction. In brief, the buffy coat was diluted with PBS (pH 7.2) to make a total volume of 3 ml. The diluted buffy coat was carefully layered on a 3-ml layer of Isoprep reagent in a 15-ml centrifuge tube. Mixing of buffy coat with the solution was avoided. The tube was then centrifuged at 1500 x g for 20 minutes at room temperature. After centrifugation, mononuclear cells (lymphocytes and monocytes) formed a distinct layer at the sample/Isoprep interface. The cells were collected from the interface using a sterile pipette, without removing the upper layer. Five milliliters of TE buffer (pH 8.0) were added to lyse contaminated red blood cell. The tube was centrifuged at 500 x g for 10 minutes. The harvested mononuclear cells were washed 3 times in PBS (pH 7.2) by centrifugation at 250 x g for 10 minutes. The final pellet was collected and stored at -20°C until DNA extraction process.

2.3.3 Extraction of genomic DNA from PBMCs

Genomic DNA was extracted and purified from the PBMCs by using the QIAmp® DNA Blood Mini kit (Qiagen, Hilden, Germany). The extraction was performed as recommended by the manufacturer. In brief, the PBMCs pellet was thawed and suspended in 200 µl of PBS (pH 7.2). Two hundred microliters of AL buffer and 20 µl of protenase K were added to the cell suspension and incubated at 56°C for 10 minutes. Two hundred microliters of absolute ethanol was added and the mixture was applied on a column of silica gel filter tube. After a centrifugation at 6000 x g for 1 minute, the nucleic acid bound to silica gel was washed with 500 µl of AW1 buffer to get rid of any PCR inhibitory residuals. Then, the proteins and other cellular contents were washed out from the filter tube with 500 µl of AW2 buffer. Finally, 200 µl of AE buffer was added and centrifuged at 6,000 x g for 1 minute to elute the purified DNA from the column. The purified genomic DNA was stored at -70°C until further analysis.

2.3.4 Quantification of genomic DNA

The concentration of human genomic DNA was measured by DNA fluorescence assay, using the Hoefer DyNA Quant 200 Fluorometer. Bisbenzimide, commonly known as Hoechst 33258 (H 33258) dye, exhibits changes in fluorescence characteristics in the presence of DNA that allows accurate DNA quantification. In the absence of DNA, the excitation spectrum of H 33258 peaks at 356 nm and the emission spectrum peaks weakly at 492 nm.

In a cuvette well, the sample is exposed to filtered light (365±7 nm) from a mercury lamp. This light excites the DNA-dye complex, causing a light peak at 458 nm emission. An emission filter in front of the photodetector allows only 460±15 nm of fluorescence to register. Thus, the measured fluorescence is a direct indicator of the DNA concentration. H 33258 binds to the minor groove of DNA. When 365 nm light (long UV) excites this bound dye, its fluorescence at 458 nm can be measured.

Two microliters of the standard (100 ng/µl) and the sample DNA were diluted in 2 ml of assay solution that contained H 33258 dye. Then, the light (458 nm.) emitted from the DNA-H 33258 dye complex in the mixture was measured by the Hoefer DyNA Quant 200 Fluorometer. The quantity of DNA was calculated by the Hoefer DyNA Quant 200 Fluorometer using the following equation:

$$\text{Quantity of sample DNA (ng/µl)} = \frac{OD_{458} \text{ of sample DNA x standard concentration (100ng/µl)}}{OD_{458} \text{ of standard}}$$

The calculated value was reported by the Hoefer DyNA Quant 200 Fluorometer, which directly displayed DNA quantity in ng/µl.

2.4. Multiplex PCR

2.4.1 Primer grouping

Eleven oligonucleotide primer-pairs were grouped into 4 multiplexes, based on their product sizes, *Tm* and primer-dimer formation. A pair of primers, specific for the control gene (*SRY*), was added to each multiplex set. The details of the 4 multiplex primers were shown in Table 3.2.

Polymerase chain reaction was performed to amplify discrete genes in a multiplex fashion. Each multiplex reaction contained three or four pairs of primers, including primers specific to a control gene. The combination of primers used in each multiplex PCR were designated as followed:

multiplex PCR set # 1: *sY14 (SRY), SMCY, RBM1*, and *EIF1AY*

multiplex PCR set # 2: *sY14 (SRY), DBY, DAZ (sY283)* and *PRY*

multiplex PCR set # 3: *sY14 (SRY), DFFRY* and *CDY1*

multiplex PCR set # 4: *sY14 (SRY), DAZ (sY277), TTY2*, and *BPY2*

Table 3.2 The details of the four multiplexes in this study

Mixture no.	Genes	Product size (bp)	Tm(F)/Tm(R)	Primer length,F/R (bp)
control*	*sY14 (SRY)*	470	61.5/59.2	20/21
1	*SMCY*	362	56.0/59.0	20/21
	RBM1	800	57.2/57.3	21/22
	EIF1AY	84	57.0/56.4	21/20
2	*DBY*	689	56.2/59.6	22/20
	DAZ(sY283)	314	58.1/56.7	23/22
	PRY	80	58.9/56.2	21/21
3	*DFFRY*	130	55.9/56.9	24/20
	CDY1	79	63.3/57.9	19/21
4	*DAZ(sY277)*	312	62.8/60.7	24/25
	TTY2	87	57.5/55.4	20/19
	BPY2	370	55.0/56.4	21/20

* Primers specific to a control gene (*SRY*) were added into each multiplex PCR set

2.4.2 Optimization of Multiplex PCR conditions

The multiplex PCR in this study was optimized in order to obtain balanced yields of all desired loci with as few nonspecific amplicons as possible. Towards this

aim, the twelve primer pairs were tested separately to determine their specificities and sensitivities. They were then grouped into 4 multiplexes, each consisting of 3-4 primer pairs. Each multiplex PCR reaction set was further optimized to give strong and specific products, well separated from each other on agarose gel electrophoresis.

The 12 gene-based primers on the Y chromosome were shown in Figure 3.2. The details of AZF marker genes were shown in Table 3.3.

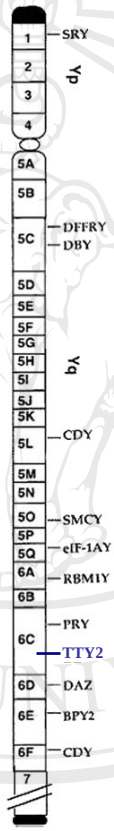

Figure 3.2. The location of all 12 AZF-marker genes on the human Y chromosome

2.4.2.1 Optimization of primer concentration

Lyophilized primers (Qiagen, Hilden, Germany) were dissolved in distilled water to make a final concentration of 400 µM and were kept frozen as stock primers. The stock primers were diluted into various concentrations before use in the multiplex mixtures.

Table 3.3 AZF marker genes in this study

Location	Gene Symbol	Gene name	Number of copies	Tissue expression
Yp (1A)	*SRY*	Sex determining regionY	1	predominantly testis
AZFa (5C)	*DFFRY*	Ubiquitin specific protease 9, Y chromosome	1	Ubiquitous
AZFa (5C)	*DBY*	Dead box Y	1	Ubiquitous
AZFb (5O)	*SMCY*	SMC (mouse) homolog, Y-linked	1	Ubiquitous
AZFb (5Q)	*EIF1AY*	Eukaryotic translation initiation factor 1A, Y chromosome	1	Ubiquitous
AZFb (6A)	*RBM1*	RNA binding motif protein 1	6	Testis
AZFb (6C)	*PRY*	Testis-specific PTP-BL-related protein on Y	2	Testis
AZFb (6C)	*TTY2*	Testis-specific testis transcript Y 2	1	Testis
AZFc (6D)	*DAZ*	Deleted in azoospermia	4	Testis
AZFc (6E)	*BPY2*	Basic protein on Y chromosome 2	3	Testis
AZFc (6F)	*CDY1*	chromodomain protein, Y-linked, 1	4	Testis

Initially, primer concentration of 0.2 µM was used in all multiplex PCR. In case of poor PCR efficiency, primer concentrations was gradually increased until satisfactory result was obtained. Once, primer competition was observed, the amount of the lower efficiency primers was increased while the amount of the higher efficiency primers was decreased. The final concentration of the primers was established empirically and varied considerably among the loci, from as low as 0.01 µM to as high as 0.5 µM.

2.4.2.2 Optimization of dNTP concentration

According to standard singleplex PCR condition, dNTP concentration of 200 µM is commonly used. Therefore, in order to find an optimal concentration for multiplex PCR, some higher dNTP concentrations were tested.

2.4.2.3 Optimization of DNA concentration

To evaluate the optimal concentration of DNA for multiplex PCR in this study, DNA concentrations of 100, 200, 300 and 400 ng (for 25 µl reaction volume) were tested .

2.4.2.4 Optimization of HotStarTaq DNA polymerase concentration

Theoretically, enzyme concentration in a reaction mixture can have tremendous impact on many aspects of multiplex PCR performance, including the yield and the locus-to-locus signal balance. In this study, titration of the HotStarTaq DNA polymerase was performed in concentrations of 1, 2, or 3 units/25-µl reaction volume.

2.4.2.5 Optimization of PCR conditions

2.4.2.5.1 Optimization of annealing temperature

As the multiplex PCR in this study contained some modified and some newly designed primers, the optimal annealing temperature could be different from that (60 ºC) described in previous reports. Five temperatures, 61 ºC - 65 ºC for *DBY* and 55 ºC – 60 ºC for *RBM1* primers, were tested. The yield and the specificity of PCR products were recorded and the efficiency of each annealing temperature was determined. The optimal annealing temperature was then selected for subsequent PCR in this study.

2.4.2.5.2 Optimization of the annealing time

To optimize the yields of PCR products, various annealing times of 1, 1.5 and 2 min were tested. The optimal annealing time was then selected for all subsequent multiplex PCR.

2.4.2.5.3 Optimization of the extension time

One-minute extension time is usually sufficient for the synthesis of PCR fragments up to 2 kb. To increase the amount of long PCR products, 1.5 and 2.0 min extension time were tested. The time, which gave visibly higher yields of PCR products, was then selected for further use.

2.4.2.5.4 Optimization of cycle number

Thirty cycles of PCR is commonly used in most standard PCR. However, some primers in the multiplex PCR system required more number of PCR cycle. Therefore, 35 and 40 cycles of PCR were tested and the cycle number that gave comparable bands among various PCR products on gel electrophoresis was selected for further use.

2.5. Detection of Multiplex PCR products

The multiplex PCR products were separated on 2.5 % agarose gels (SeaKem®, Rockland, ME, USA) by electrophoresis in TAE buffer at room temperature, using a voltage gradient of 10 volt cm^{-1}. PCR products from each reaction were mixed well with 6X loading buffer and loaded into gel slot. The DNA bands were visualized under ultraviolet light after staining with 10 mg/ml ethidium bromide for 15 minutes. Results were recorded by gel documentation system (Fotodyne, MI, USA).

2.6. Quantification of serum hormone levels

The serum concentrations of follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin and testosterone were measured by electrochemiluminescence (ECL) immunoassays, using Elecsys (Roche Diagnostics, Mannheim, Germany). This is a semi-automated analyzer operating on ECL technology (Elecsys1010). Normal reference ranges for men were: FSH 1.5-12.4 mIU/ml, LH 1.7-8.6 mIU/ml, prolactin 4.1-18.4.ng/ml and testosterone 2.8-8.0 ng/ml. All assays were done in the Endocrine Laboratory, Department of Obstetrics and

Gynecology, Faculty of Medicine, Chiang Mai University. Detection methods involved both the ECL-based competitive binding assay for testosterone and the ECL-based sandwich assays for prolactin, FSH and LH.

Competitive principle method. The competitive assay technique required two 9-minute incubations, for a total time of 18 minutes. A serum sample was incubated with a testosterone-specific biotinylated antibody for 9 minutes. In this manner, immune complexes were formed in an amount dependent upon the analyte concentration in the sample. Streptavidin-coated micro particles and testosterone derivative labeled with a ruthenium complex were added for another 9 minutes. Testosterone in the serum sample competed with the ruthenium-labeled testosterone derivative for the limited antibody binding sites. The interaction of biotin and streptavidin allowed the immune complexes to be bound to the solid magnetic particles, thus allowing capture of the immune complexes through interaction with a magnetic field. The reaction mixture was aspirated into a measuring cell with a buffer solution containing tripropylamine (TPA). The micro particles were magnetically captured onto the surface of an electrode and unbound substances were removed. A voltage was applied to the electrode, thus inducing a chemiluminescent emission which was then measured by a photomultiplier tube during a 0.4 second interval. Measurements were integrated to provide a single value and then compared to a calibration curve to calculate the sample result.

Sandwich principle method. In the first step, patient sample was combined with a reagent containing biotinylated FSH antibody and a ruthenium-labeled FSH-specific antibody in an assay cup. During the 9-minute incubation, antibodies captured the FSH present in the sample. In the second step, streptavidin-coated paramagnetic particles were added. During this incubation, the biotinylated antibody attached to the streptavidin-coated surface of micro particles. After 9 minutes, the reaction mixture containing the immune complexes was transported into the measuring cell. The immune complexes were magnetically entrapped on the working electrode, but unbound reagent and sample were washed away by the system buffer. In the ECL reaction, the conjugate was a ruthenium-based derivative and the chemiluminescent reaction was electrically stimulated to produce light. The amount of light produced was directly proportional to the amount of FSH present in the sample. Evaluation and

calculation of the antigen concentration were carried out by means of a calibration curve, that was established using standards of known antigen concentration.

2.7. Cytogenetic evaluation

Chromosome analyses were performed by using cultures of peripheral blood lymphocytes by the standard method of G- and Q-banding [132]. Briefly, lymphocytes were cultured in RPMI-1640 medium containing 20% fetal bovine serum and 10 µg/ml PHA (phytohemagglutinin). The cultures were incubated for 72 hours at 37ºC, then 0.1 µg/ml of colcemid was added and they were incubated for another 30 minutes. The chromosomes were harvested by conventional methods. In brief, the cells were exposed to 0.075 M KCl for hypotonic treatment and to a solution of methanol : acetic (3:1) for cell membrane fixation. Chromosome spreads were prepared by air drying technique. The chromosome spreads were kept overnight at room temperature. They were then stained with G- and Q-banding technique. The metaphase chromosomes were analyzed under a light microscope for G-banding and under a fluorescence microscope for Q-banding. At least 20 metaphases per patient were analyzed.

Cytogenetic evaluation was performed at the Department of Anatomy, Faculty of Medicine, Chiang Mai University.

2.8. Data analysis

Clinical characteristics of infertile men were expressed as mean ± standard deviation (SD) or median and ranges as appropriate. Unpaired t-tests were used for statistical comparisons of means when data were normally distributed. Otherwise Mann-Whitney tests were used for comparison of medians. Chi-square tests were used for categorical variables. *P* values of 0.05 were considered as statistically significant. All computations were carried out with the SPSS for Windows version 13 (SPSS Inc, Chicago, Illinois).