ORIGINAL

A rapid and simple system of detecting deletions on the Y chromosome related with male infertility using multiplex PCR

Mayumi Umeno[1,2,6], Toshikatsu Shinka[1,6], Youichi Sato[1,6], Xin-Jun Yang[1,6], Yoshinobu Baba[4,5], Teruaki Iwamoto[3,6], and Yutaka Nakahori[1,6]

[1]*Department of Human Genetics and Public Health, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan;* [2]*Major in Laboratory Science, School of Health Sciences, Faculty of Medicine, The University of Tokushima, Tokushima, Japan;* [3]*Department of Urology, St. Marianna Medical University School of Medicine, Kanagawa, Japan;* [4]*Department of Medicinal Chemistry, Faculty of Pharmaceutical Sciences, The University of Tokushima, Tokushima, Japan;* [5]*Department of Applied Chemistry, Graduate School of Engineering, The University of Nagoya, Aichi, Japan; and* [6]*Core Research for Evolutional Science and Technology Corporation, Saitama, Japan*

Abstract : Around 10% of males with idiopathic azoospermia or oligozoospermia, which are important causes of male infertility, have partial deletions on the long arm of the Y chromosome. To develop a rapid and accurate detection system for screening major Y deletions found in infertile men, we developed a multiplex PCR system that can simultaneously amplify five loci on the Y chromosome, SRY, AMELY, DBY, RBMY, DAZ and one locus on the X chromosome, AMELX. The size of the PCR products was designed to increase gradually from the distal Yp to the distal Yq. Our system could detect deletions of three major candidate regions for the azoospermic factor, AZFa, AZFb and AZFc on the Y chromosome together with sex. The gradual increase in the size of the PCR products was convenient for imaging the location of deletions on the Y chromosome. Moreover, the multiplex PCR system was combined with microchip-based electrophoresis, and the PCR products derived from each locus were separated within 4min. Our system is useful for screening Y chromosomes bearing the structural anomalies including three major AZF deletions found among azoospermic or oligozoospermic males. J. Med. Invest. 53:147-152, February, 2006

Keywords **:** *Y chromosome, AZF, multiplex PCR, microchip electrophoresis, azoospermia*

INTRODUCTION

Around 10% of the males with idiopathic azoospermia and oligozoospermia, important causes of male infertility, have interstitial deletions on the Y chromosome (1-3). There are three major candidate regions for the azoospermic factor, AZFa, AZFb and AZFc, on the long arm of the Y chromosome (3). Each region itself has several candidates for the factor (4). AZFa contains USP 9 Y and DBY (DDX 3 Y) encoding an ubiqutin-specific protease and a RNA-helicase with a DEAD box, respectively (5-7). AZFb possesses numerous candidate genes, among which, RBMY encoding a RNA-binding protein presumed to function in splicing, has been analyzed with regard to genomic structure, partners and evolutional conservation (8, 9). AZFc has

Received for publication November 30, 2005; accepted January 18, 2006.

Address correspondence and reprint requests to Yutaka Nakahori, M. D., Department of Human Genetics and Public Health, Institute of Health Biosciences, The University of Tokushima Graduate School, Kuramoto-cho, Tokushima 770-8503 Japan and Fax:+81-88-633-7453.

the DAZ family, the CDY family and VCY2 (10, 11). Among the AZFs, AZFc is most frequently deleted in the males with azoospermia or oligozoospermia (12). Recent progress in analyzing the human genomic structure has unveiled that the long arm of the Y chromosome has many DNA sequences consisting of palindromes or inverted repeats (4, 8, 11). This characteristic structure of the human Y chromosome is presumed to cause the deletions of AZF regions found in males with azoospermia or oligozoospermia (4, 8, 11).

In the clinical setting, rapid and accurate gene tests are required for an appropriate diagnosis and genetic counseling. Recently, microchip devices for electrophoresis has been developed and applied to gene tests (13-15). Such devices have the advantages of requiring only small amounts of DNA and little time for analysis.

Here, we describe a newly developed multiplex PCR system convenient for the analysis of Y chromosomes bearing structural anomalies including AZF deletions together with identification of sex. Moreover, we show that the new system combined with microchip-based electrophoresis can provide rapid and accurate gene tests for male infertility.

MATERIALS AND METHODS

Subjects

A total of 50 fertile and 48 infertile men including 42 azoospermic and 6 oligozoospermic patients were studied. All the subjects have been described elsewhere (16-19). The infertile men had already been analyzed for Y-deletion according to routine testing performed in our laboratory (16, 19). In brief, microdeletions in AZF regions on the Y chromosome were analyzed by PCR with 9 sequence-tagged-sites (STSs) including SRY, AMELY, DYZ3, USP9Y, DBY, DYS211, RBMY, DAZ and DYZ 1 according to previous reports (1, 19). It had been shown that among the 48 infertile men, two were missing DBY or RBMY, five lacked DAZ, and two were losing both RBMY and DAZ.

This study was approved by the Ethics committee of the University of Tokushima and the Ethical Committee/ Institutional review board of St. Marianna University School Hospital. Every participant provided an informed consent.

Multiplex PCR

The primers for five loci on the Y chromosome and AMELX on the X chromosome were generated as listed in Table 1. SRY and AMELY are located on the short arm of the Y chromosome (20, 21). The former is known as the male testis-determining factor on the Y chromosome, and the later as the counterpart of amelogenin X (AMELX) responsible for X- linked amelogenesis imperfecta. DBY, RBMY and DAZ, which are assigned to the long arm of the Y chromsome, are located in AZFa, AZFb and AZFc, the candidate regions for the azoospermic factor suggested to be on the long arm of the Y chromosome (4)(Fig. 1).

Initially, two types of the Taq polymerase ready for hot start, Ampli-Taq Gold (Applied Biosystems, Branchburg, NJ, USA) and TaKaRa Ex Taq HS (TAKARA BIO INC. Shiga, Japan) were compared in terms of their specificity for PCR. The former was found to be appropriate for our multiplex PCR. The reaction was performed under different annealing temperatures, and it became clear that 56°C is most appropriate for multiplex PCR. Based on these preliminary experiments, we carried out the PCR reaction with Ampli-Taq Gold at an annealing temperature of 56°C.

Multiplex PCR was carried out in a total volume of 20μl containing 66ng of genomic DNA with, 0.5μM (except for AMELX) or 1μM (AMELX) of each primer listed in Table 1, 10 mM Tris-HCl buffer (pH 8.3), 50mM KCl, 2.5mM $MgCl_2$, 0.001% gelatin, 0.25mM of each dNTP, and 0.5units of Ampli-Taq Gold. The conditions for the PCR were as follows : initial denaturation at 94°C for 10min and 30 cycles consisting of denaturation at 94°C for 30 sec, annealing at 56°C for 1 min and extension at 72°C for 1 min. The final extension step was at 72°C for 10 min.

To confirm the multiplex PCR products, they were separated by electrophoresis with 2.5% agarose gel, and were visualized with ethydium bromide.

Electrophoresis with a microchip device

A Hitachi SV1210 microchip CE system (Hitachi Electronics Engineering Co., Ltd., Tokyo, Japan) was used for electrophoresis. We used a polymethyl methacrylate micro i-chip12 with i-SDNA12kit (Hitachi Chemical Co., Tokyo, Japan) that contains a gel for microchip electrophoresis and a fluorescent dye according to the manufacture's protocol. The multiplex PCR product 1μl was mixed with water 9μl and applied to the wells in the microchip device. This system can simultaneously separate and detect 12 samples.

RESULTS

Generation of a multiplex PCR system for detecting Y deletions

First, to establish a multiplex PCR system for the detection of Y deletions, we carried out PCR with a multiplex primer set for SRY, AMELY, DBY, RBMY, and DAZ on the Y chromosome together with one for AMELX on the X chromosome (see Fig. 1 and Table 1). Using agarose gel-based electrophoresis, six bands consistent in size with one of the PCR products generated with the primer sets for each locus were clearly observed (Fig. 2).

Next, we used the system to test for Y deletions. PCR products obtained with genomic DNA derived from azoospermic or oligozoospermic males who had been confirmed to have no Y deletion, DBY deletion, RBMY deletion, or DAZ deletion, were separated with agarose gel -based electrophoresis. Moreover, XX males who had been shown to have SRY were also analyzed. The PCR products derived from AMELX were observed in both males and females, suggesting that PCR was successful. The results were consistent with those obtained in our routine tests for Y deletions (Fig. 3)(16, 19). The gradual increase in the size of the PCR products according to direction from the distal Yp to the distal Yq was convenient for imaging the location of deletions on the Y chromosome.(see Fig. 1 and Table 1).

Finally, a total of 48 infertile males including 39 without Y deletions, two without DBY or RBMY and

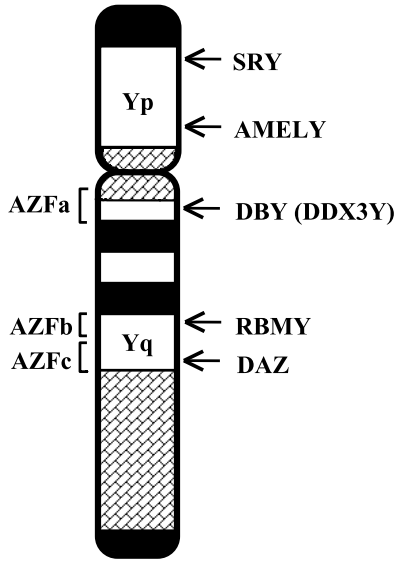

Fig. 1. Schematic representation of the Y chromosome and loci analyzed in this studty.
AZFa, AZFb and AZFc all have several candidate genes for an azoospermic factor, respectively.

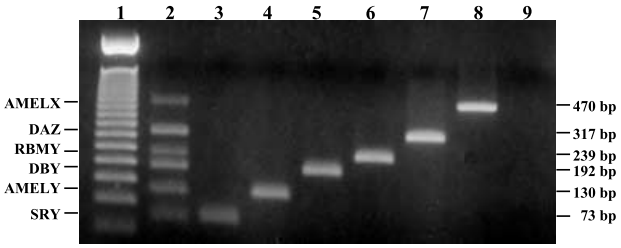

Fig. 2. Multiplex PCR for five Y loci and one X locus.
1, DNA marker of 50 bp ladder ; 2, multiplex PCR product for a male ; 3, SRY ; 4, AMELY ; 5, DBY ; 6, RBMY ; 7, DAZ ; 8, AMELX ; 9, water (multiplex PCR).

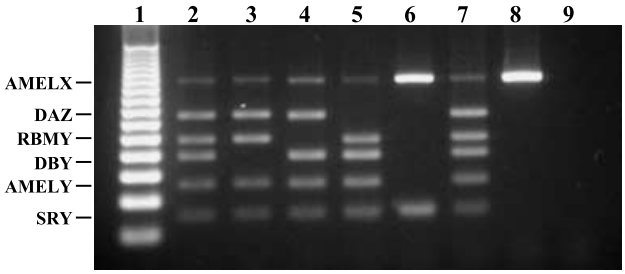

Fig. 3. Analysis of Y chromosome deletions with agarose gel-based electrophoresis.
lane 1, 50 bp ladder DNA marker ; lane 2, azoospermic male without Y deletion ; lane 3, azoospermic male without DBY ; lane 4, azoospermic male without RBMY ; lane 5, azoospermic male without DAZ ; lane 6, XX male ; lane 7, normal male ; lane 8, female ; lane 9, water.

Table 1. The primer sets used in this study

Locus	Primers	Sequence	%GC (%)	PCR product size
SRY	Forward : SRY-F	5'-AGCTGTGCAAGAGAATATTC	40	73 bp
	Reverse : SRY-R	5'-CTTAGAGTTACAGCTTTCAG	40	
AMELY	Forward : AMELY-CF	5'-TTCTGAACACCAGAGTGGTG	50	130 bp
	Reverse : AMELY-CR	5'-GAGGATCTTATGTCATTGCG	45	
DBY	Forward : DBYn-F	5'-CTTACACTTGCAGACTTTGC	45	192 bp
	Reverse : DBYn-R	5'-TTTAGTGGTGGACAATCATC	40	
RBMY	Forward : RBMY 1-F	5'-CGCATATAGTAATACACGAG	40	239 bp
	Reverse : RBMY 1-R	5'-TGCTTTAATATCTGCTCGAG	40	
DAZ	Forward : DAZ 1-F	5'-CTGGTGACTGAATTACAATG	40	317 bp
	Reverse : DAZ 1-R	5'-GTTCATGATGTATGTTAAGG	35	
AMELX	Forward : AMELX-CF	5'-GCTACTGCAAGAACCTTAAG	45	470 bp
	Reverse : AMELX-CR	5'-AGGCAAAGAGTTTCATCACG	45	

five without DAZ, two without both RBMY and DAZ were analyzed using our developed multiplex PCR system. All results were consistent with those of routine testing for Y deletions. Moreover, 50 fertile males previously shown to have some children were also analyzed using our developed multiplex PCR system. No deletions were observed in all fertile men analyzed in this study.

Combination of multiplex PCR with microchip-based electrophoresis for detecting Y deletions

To develop a rapid detection system for Y deletions, we applied our multiplex PCR system to microchip-based electrophoresis. The multiplex PCR products generated in the above experiments were applied to the microchip. Each product was separated within 4min with a clear chromatogram (Fig. 4). The peaks derived from each locus on the Y chromosome were clearly discriminated from the background. The interstitial deletions of the Y chromosome were easily identified. This microchip-based electrophoresis showed good reproducibility.

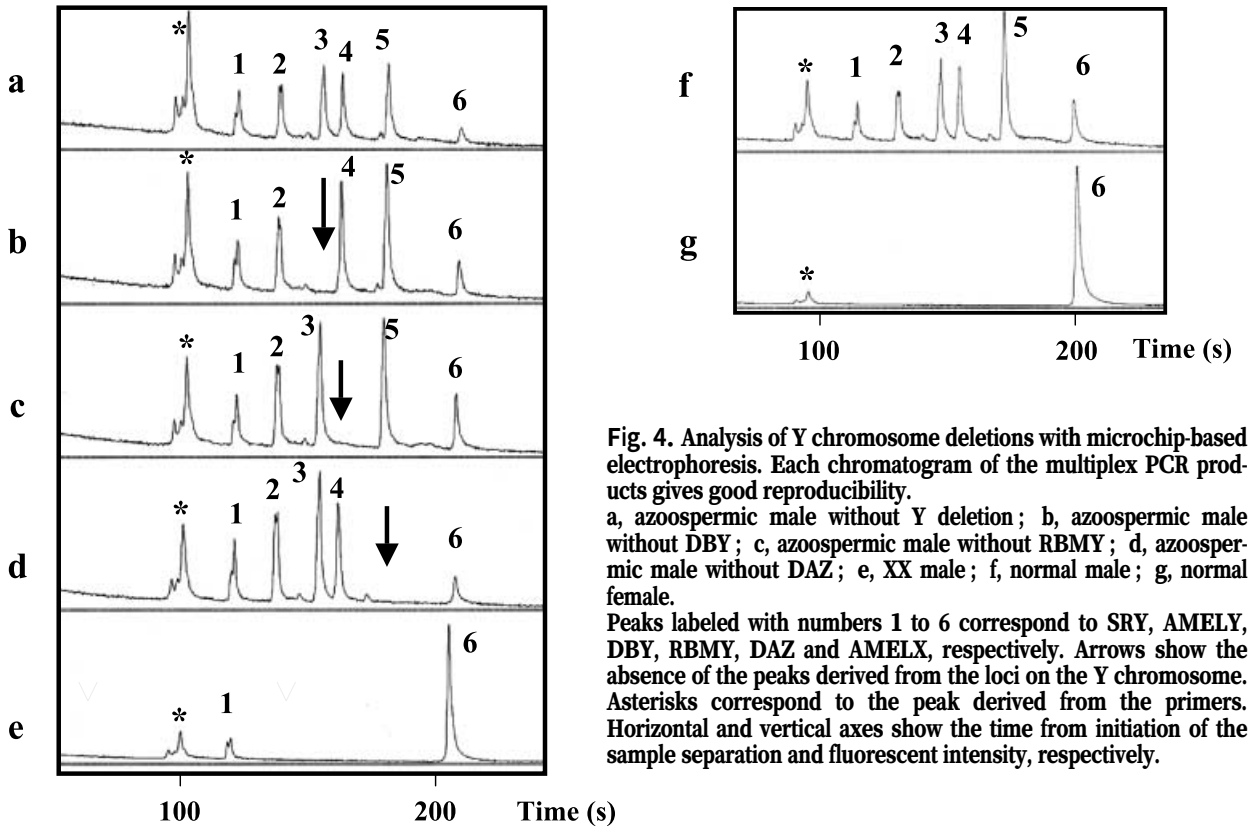

Fig. 4. Analysis of Y chromosome deletions with microchip-based electrophoresis. Each chromatogram of the multiplex PCR products gives good reproducibility.
a, azoospermic male without Y deletion; b, azoospermic male without DBY; c, azoospermic male without RBMY; d, azoospermic male without DAZ; e, XX male; f, normal male; g, normal female.
Peaks labeled with numbers 1 to 6 correspond to SRY, AMELY, DBY, RBMY, DAZ and AMELX, respectively. Arrows show the absence of the peaks derived from the loci on the Y chromosome. Asterisks correspond to the peak derived from the primers. Horizontal and vertical axes show the time from initiation of the sample separation and fluorescent intensity, respectively.

DISCUSSION

Here, we described a newly developed multiplex PCR system for detecting Y chromosomes with structural anomalies. Our system can detect three major deletions on the Y chromosome related to idiopathic spermatogenic failure. There are some reports that both USP9Y and DBY or only DBY was deleted in azoospermic males (7, 22). To our knowledge, the deletion of USP9Y is very rare in azoosepermic males without AZFa, while the deletion of DBY or of both DBY and USP9Y occurs frequently in such males (7, 22). RBMY is located in AZFb, and males without AZFb often lack RBMY(8, 9). The DAZ gene is located in AZFc, the most frequently deleted of the three AZFs in idiopathic azooseprmic or oligozoospermic males. It is shown that deletions of AZFa and AZFb are much less frequent than deletions of AZFc (12). Therefore, in the initial screening for Y deletions in infertile males, it is appropriate to choose DBY, RBMY, and DAZ as representative of the genes located in AZFa, AZFb and AZFc, respectively.

Since AMELX should be detected in both males and females, and AMELY is male-specific, AMELX/AMELY is widely used to identifying sex (23). In our system, we also used AMELX as an internal control for PCR. Although the original approach employs the same primer set for both AMELX and AMELY, our system does not. By the AMELX/AMELY sex

identification system, our method can simultaneously identify sex and Y deletions involving major AZF regions.

While microchip technology is applied to devices for biological analysis such as electrophoresis and gene expression analysis, nanochip technology is currently being developed for use in various fields (13-15, 24). In future, the combination of our newly developed multiplex PCR system and a nanochip device-based electrophoresis will provide a speedier gene test for Y deletions in infertile males (24).

Assisted reproductive technology (ART) including intracytoplasmic sperm injection (ICSI) is rapidly developed (25). The newly developed multiplex PCR system for Y deletion analysis together with karyotype analysis may provide useful information for prognosis of male infertility and indications of ART (26).

In conclusion, we showed that our multiplex PCR system is convenient for the analysis of Y chromosomes bearing structural anomalies including AZF deletions together with the identification of sex, and can provide a speedier gene testing in combination with electrophoresis based on a microchip device.

ACKNOWLEDGEMENTS

This study was supported in part by grants from the Core Research for Evolutional Science and Technology (CREST), and Japan Society for the Promotion of Science.

REFERENCES

1. Nagafuchi S, Namiki M, Nakahori Y, Kondoh N, Okuyama A, Nakagome Y : A minute deletion of the Y chromosome in men with azoospermia. J Urol 150 : 1155-7, 1993
2. Kobayashi K, Mizuno K, Hida A, Komaki R, Tomita K, Matsushita I, Namiki M, Iwamoto T, Tamura S, Minowada S, Nakahori Y, Nakagome Y: PCR analysis of the Y chromosome long arm in azoospermic patients: evidence for a second locus required for spermatogenesis. Hum Mol Genet 11 : 1965-7, 1994
3. Vogt PH, Edelmann A, Kirsch S, Henegariu O, Hirschmann P, Kiesewetter F, Kohn FM, Schill WB, Farah S, Ramos C, Hartmann M, Hartschuh W, Meschede D, Behre HM, Castel A, Nieschlag E, Weidner W, Grone HJ, Jung A, Engel W, Haidl G: Human Y chromosome azoospermia factors (AZF) mapped to different subregions in Yq 11. Hum Mol Genet 5 : 933-43, 1996
4. Skaletsky H, Kuroda-Kawaguchi T, Minx PJ, Cordum HS, Hillier L, Brown LG, Repping S, Pyntikova T, Ali J, Bieri T, Chinwalla A, Delehaunty A, Delehaunty K, Du H, Fewell G, Fulton L, Fulton R, Graves T, Hou SF, Latrielle P, Leonard S, Mardis E, Maupin R, McPherson J, Miner T, Nash W, Nguyen C, Ozersky P, Pepin K, Rock S, Rohlfing T, Scott K, Schultz B, Strong C, Tin-Wollam A, Yang SP, Waterston RH, Wilson RK, Rozen S, Page DC : The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. Nature 423 : 825-37, 2003
5. Lahn BT, Page DC: Functional coherence of the human Y chromosome. Science 278: 675-80, 1997
6. Sun C, Skaletsky H, Birren B, Devon K, Tang Z, Silber S, Oates R, Page DC: An azoospermic man with a de novo point mutation in the Y-chromosomal gene USP9Y. Nat Genet 23: 429-32, 1999
7. Foresta C, Ferlin A, Moro E : Deletion and expression analysis of AZFa genes on the human Y chromosome revealed a major role for DBY in male infertility. Hum Mol Genet 9: 1161-9, 2000
8. Repping S, Skaletsky H, Lange J, Silber S, Van Der Veen F, Oates RD, Page DC, Rozen S: Recombination between palindromes P 5 and P1 on the human Y chromosome causes massive deletions and spermatogenic failure. Am J Hum Genet 71 : 906-22, 2002
9. Ma K, Inglis JD, Sharkey A, Bickmore WA, Hill RE, Prosser EJ, Speed RM, Thomson EJ, Jobling MM, Taylor K, Wolfe J, Cooke HJ, Hargreave TB, Chandley AC : A Y chromosome gene family with RNA-binding protein homology: candidates for the azoospermia factor AZF controlling human spermatogenesis. Cell 75 : 1287-95, 1993
10. Reijo R, Lee TY, Salo P, Alagappan R, Brown LG, Rosenberg M, Rozen S, Jaffe T, Straus D, Hovatta O, de la Chapell A, Silber S, Page DC : Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. Nat Genet 10 : 383-93, 1995
11. Kuroda-Kawaguchi T, Skaletsky H, Brown LG, Minx PJ, Cordum HS, Waterston RH, Wilson RK, Silber S, Oates R, Rozen S, Page DC: The AZFc region of the Y chromosome features massive palindromes and uniform recurrent deletions in infertile men. Nat Genet 29 : 279-86, 2001
12. Foresta C, Moro E, Ferlin A : Y chromosome

microdeletions and alterations of spermatogenesis. Endocr Rev 22 : 226-39, 2001

13 Endo Y, Zhang L, Katashima R, Itakura M, Doherty EA, Barron AE, Baba Y: Effect of polymer matrix and glycerol on rapid single-strand conformation polymorphism analysis by capillary and microchip electrophoresis for detection of mutations in K-ras gene. Electrophoresis 26 : 3380-6, 2005

14. Jabasini M, Ewis AA, Xu F, Mohamadi MR, Ping G, Shinka T, Nakahori Y, BabaY: Multiplex PCR with multichannel microchip electrophoresis: an ultrafast analysis for genetic diseases. J Chromatogr Sci 43 : 221-5, 2005

15. Qin J, Liu Z, Wu D, Zhu N, Zhou X, Fung Y, Lin B : Genotyping the -6 A/G functional polymorphism in the core promoter region of angiotensinogen gene by microchip electrophoresis. Electrophoresis 26 : 219-24, 2005

16. Yang XJ, Shinka T, Nozawa S, Yan HT, Yoshiike M, Umeno M, Sato Y, Chen G, Iwamoto T, Nakahori Y: Survey of the two polymorphisms in DAZL, an autosomal candidate for the azoospermic factor, in Japanese infertile men and implications for male infertility. Mol Hum Reprod 11 : 513-5, 2005

17. Yan HT, Shinka T, Kinoshita K, Sato Y, Umeno M, Chen G, Tsuji K, Unemi Y,Yang XJ, Iwamoto T, Nakahori Y : Molecular analysis of TBL 1 Y, a Y-linked homologue of TBL 1 X related withX-linked late-onset sensorineural deafness. J Hum Genet 50 : 175-81, 2005

18. Shinka T, Sato Y, Chen G, Naroda T, Kinoshita K, Unemi Y, Tsuji K, Toida K, Iwamoto T, Nakahori Y: Molecular characterization of heat shock-like factor encoded on the human Y chromosome, and implications for male infertility. Biol Reprod 71: 297-306, 2004

19. Nakahori Y, Kuroki Y, Komaki R, Kondoh N, Namiki M, Iwamoto T, Toda T, Kobayashi K : The Y chromosome region essential for spermatogenesis. Horm Res 46 (Suppl 1):20-3, 1996

20. Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, Foster JW, Frischauf AM, Lovell-Badge R, Goodfellow PN : A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature 346 : 240-4, 1990

21. Nakahori Y, Takenaka O, Nakagome Y: A human X-Y homologous region encodes“ amelogenin ”. Genomics 9 : 264-9, 1991

22. Van Landuyt L, Lissens W, Stouffs K, Tournaye H, Van Steirteghem A, Liebaers I, Blagosklonova O, Bresson JL : The role of USP 9 Y and DBY in infertile patients with severely impaired spermatogenesis. Mol Hum Reprod 7 : 691-3, 2001

23. Nakahori Y, Hamano K, Iwaya M, Nakagome Y: Sex identification by polymerase chain reaction using X-Y homologous primer. Am J Med Genet 39 : 472-3, 1991

24. Kricka LJ : Microchips, microarrays, biochips and nanochips : personal laboratories for the 21st century. Clin Chim Acta 307: 219-23, 2001

25. Pinborg A, Loft A, Ziebe S, Nyboe Andersen A: What is the most relevant standard of success in assisted reproduction? Is there a single‘ parameter of excellence’’? Hum Reprod 19:1052-4, 2004

26. Foresta C, Moro E, Ferlin A: Prognostic value of Y deletion analysis. The role of current methods. Hum Reprod 16 : 1543-7, 2001