Elucidation of Distinctive Genomic DNA Structures in Patients with 46,XX Testicular Disorders of Sex Development Using Genome Wide Analyses

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Purpose: Although several genes, including the SRY gene, are involved in testicular differentiation, the entire mechanism of this differentiation remains unclear. We performed genome wide analysis in patients with 46,XX testicular disorders of sex development to comprehensively elucidate the mechanisms of testicular differentiation.

Materials and Methods: Whole genomic DNA was extracted from the peripheral blood of 4 patients with 46,XX testicular disorders of sex development who were SRY negative. Genomic DNA was hybridized to a GeneChip® human mapping 250K array set. Compared to normal female data, we detected common loss of heterozygosity and copy number variation regions in 4 patients using Genotyping Console™ software.

Results: Loss of heterozygosity was detected in 19 regions of 11 chromosomes. A total of 27 genes or nearby genomic areas were included in the applicable regions. Copy number loss was recognized in 13 regions of 10 chromosomes, and these regions included 55 genes. Copy number gain was detected in 6 regions of 4 chromosomes, which included the upstream region of the SOX3 gene.

Conclusions: The regions with loss of heterozygosity did not contain genes associated with testicular differentiation. However, the upstream area of the SOX3 gene, which is located in Xq27.1, was included in the region of copy number gain. These results suggest that high expression of the SOX3 gene led to testicular differentiation despite SRY gene loss. As this applicable area is not within a coding region, genome wide analyses were valuable for detecting the novel regions associated with testicular differentiation.

Key Words: comparative genomic hybridization, disorders of sex development, sex differentiation, testis

Abbreviations and Acronyms

CGH = comparative genomic hybridization
CN = copy number
CNV = copy number variation
DSD = disorders of sex development
LOH = loss of heterozygosity
PCR = polymerase chain reaction
SNP = single nucleotide polymorphism
TGCT = testicular germ cell tumor

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In humans bipotential gonads can differentiate into either testes or ovaries, and activation of the SRY gene initiates testicular development.[1] SRY is a transcription factor with a DNA binding high mobility group box domain.[2] Recent molecular biological studies have shown that a protein complex of SRY and Ad4Bp/SF1 binds to a *SOX9* enhancer element known as testis specific enhancer of *SOX9* core element.[3] When SOX9 further interacts with FGF9, PGD2, FOXL2 or WNT4, male

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sex determination and testis morphogenesis subsequently occur.[1] Although analysis of each gene function (using transgenic animals) and pedigree analyses in patients with disorders of sex development have revealed molecular biological mechanisms involved in sex differentiation, the entire mechanism remains unclear.

Although the incidence of disorders of sex development is quite low, detailed investigations would provide useful information. Previously we investigated the expression of representative gonad marker genes in SRY negative XX male testes and found that *SOX9* was up-regulated, and *Ad4Bp/SF1* and *DAX1* were down-regulated.[4] More recently we explored differentially expressed genes between XY^{SRY+} and XX^{SRY-} testes using PCR based subtractive hybridization and sequencing, and identified 13 up-regulated and 7 down-regulated genes in XX^{SRY-} testes. Furthermore, we performed an in vitro inhibitory assay and demonstrated that the product of *ROCK1*, which is up-regulated, phosphorylates and activates *SOX9* in Sertoli cells.[5]

However, this methodology has technical limitations that do not allow clarification of the comprehensive mechanisms of sex differentiation that involve some heterochronic genes and large gene interactions. Although functional analyses of candidate genes have been performed, genome wide analyses have recently been used as alternative methods for identifying regions involved in testicular differentiation. In particular recent advances in microarray technology, including CGH analysis and SNP genotyping, have allowed identification of genomic rearrangements.[6] In fact, studies involving CGH analyses in patients with DSD or genital malformation are increasing.[7–11] Genome wide analyses have shown the cryptic deletions or duplications of chromosomes and provided new insights into the mechanisms of gonadal development and sex differentiation. We hypothesize that several pathways are complementarily involved in testicular development besides the signaling cascade from SRY. In this study we performed genome wide analysis in patients with 46,XX testicular DSD to comprehensively elucidate the mechanisms of testicular development. To our knowledge only a few studies have analyzed patients with 46,XX testicular DSD.[12–14]

MATERIALS AND METHODS

Patient and Sample Preparation

Of patients with 46,XX testicular DSD who were followed at our hospital whole genomic DNA was extracted from the peripheral blood in 4 using a Wizard® genomic DNA purification kit, according to manufacturer instructions.[4,5] All patients had hypospadias with müllerian structures. In 3 patients the gonads were in the scrotal position on the first visit, and in 1 patient the left gonad was impalpable. Bilateral gonadal biopsy had histologically revealed immature testicular tissues. The absence of SRY was confirmed in all 4 patients using fluorescence in situ hybridization and PCR analyses.

CGH Array

Patient DNA was analyzed using the commercially available GeneChip human mapping 500K array set, which consists of 2 arrays, each capable of genotyping 250,000 SNPs on average with median probe spacing of 2.5 kb. SNP, a variation at a single nucleotide in DNA, is the most frequent type of variation in the genome. The development of SNP array has allowed LOH and CNV analyses among the whole genome. LOH due to loss of 1 parental copy in a region and CNV is rearrangement of the genome, such as deletions and duplications that result in an increase or decrease in the effective copy DNA number.[8] The labeling and hybridization of patient DNA was performed according to the manufacturer protocol. Genotyping calls were determined from the fluorescent intensities using the dynamic modeling algorithm with a 0.33 p value setting,[15] as well as the Bayesian robust linear model with the Mahalanobis distance algorithm.[16] In a comparison with genomic data of normal female (46,XX) we detected common regions in 4 patients with 46,XX LOH and CNV testicular disorders of sex development using Genotyping Console software. First, we detected the genomic region in each patient that was significantly different compared to that of a normal female. Next, individual data were integrated using Microsoft® Access® 2010 database software. Finally, we detected the common regions among all patients.

Validation of CNV

To validate the CN in the chromosomal region of Xq27.1 of all patients, quantitative TaqMan® CNV assays were performed. All reactions were performed in triplicate using a FAM™ dye label based assay (Hs05650308_cn, Hs05600961_cn and Hs05693436_cn) for the target of interest and a VIC® dye label based assay for the internal controls, according to the manufacturer protocol. Relative quantitation analysis was performed to estimate the CN for each sample using CopyCaller™ software, version 1.0.

Immunohistochemical Analysis

We performed immunohistochemical analysis on mice testes to evaluate the expression of SOX3, one of the candidate genes. Fetal (embryonic day 17) and juvenile (postcoital day 12) testes were removed and fixed in 4% paraformaldehyde or Bouin solution and then embedded in paraffin to enable histological examination. Signals were detected with a 1:500 dilution of S7443 anti-SOX3 antibody (Sigma-Aldrich®), as described previously.[5]

Ethics Statement

Studies using human genomic material were performed only after obtaining written informed consent from the families of the patients and Nagoya City University Hospital review board approval (No. 83). All animal experimental procedures were performed in accordance with protocols approved by the animal care committee of