CHAPTER IV
RESEARCH METHODOLOGY

1) TARGET POPULATION

Patient presented with hypohidrotic ectodermal dysplasia and mental retardation who has been found to carry a constitutional chromosomal aberration, a 46,Y,inv(X)(p22.2q13) and his mother of 46,X,inv(X)(p22.2q13)

2) MATERIALS

Cell Lines

The lymphoblastoid cell line was established (as described by Koehler et al. [1995a]) by use of Epstein-Barr virus transformation of peripheral lymphocytes from the patient, his mother and his brother. The metaphase chromosomes, DNA and RNA were prepared from cultured lymphoblastoid cell line.

3) METHODS

Cytogenetics and FISH analysis

Patient presented with clinical signs of HED and mental retardation (MR) was investigated in this study. As HED is hardly associated with MR, other genetic disorders should be ruled out. We performed high resolution G-banding karyotype first to exclude chromosomal abnormalities. Initial cytogenetic analysis indicated that the patient had an inversion X chromosome and also his mother. One of the inversion breakpoints mapped to Xq13 region, the opposite breakpoint was at Xp22.2. Owing to the clinical signs of HED, we hypothesized that breakpoint on Xq13.1 involves *EDA* gene and the opposite breakpoint on Xp22.2 might disrupt a MRX gene. In order to narrow down the interval of both breakpoints and to exclude the involvement of known MRX

genes, we performed FISH analysis. We searched for appropriate BAC clones mapped to the regions of interest and contained MRX genes from genomic database of NCBI to use as FISH probes. BAC clone RP11-351K23 containing the human *ED1* gene was used to examine the breakpoint on Xq13.1 and other 3 BAC clones, RPCI11-42N20 containing *IL1* gene, RPCI1-218N20 containing the *ARX* gene and RPCI11-188G3 containing the *RSK2* gene (All of them were identified MRX genes on Xp21.3, Xp22.1 and Xp22.2 respectively), were used to investigate the opposite breakpoint on Xp. Each BAC clone was cultured separately. Plasmids were purified and Nick translated with fluorochrome conjugated dUTP. The probes were hybridized to metaphase chromosomes of the patient and his mother. If the FISH signal is found to split when metaphase chromosomes are hybridized with one of the probes, this means the breakpoint lies within that BAC sequence.

Fine mapping of the inversion breakpoints by polymerase chain reaction

For further analysis of the Xp22.2 and Xq13.1 breakpoints, we performed PCR to narrow down the breakpoints. The sequences of BAC RP11-804N7 and RP11-351K23 that crossed the breakpoints can be obtained from genomic database of NCBI and were used to design specific PCR primers for amplifying. Several primer pairs were designed to amplify the sequences within the vicinity of both BAC clones.

Expression analysis RT-PCR and quantitative real-time PCR

Cytoplasmic RNA was isolated from cultured lymphoblastoid cell line using the RNeasy kit (Qiagen). The RNA was treated with DNaseI (Roche) and RT-PCR was performed. First-strand cDNA was prepared with 2 μg total RNA, as determined by absorbance, random hexamers, and the Superscript II (Invitrogen). The relative expression of the *FAM51A1* gene in lymphoblastoid

cell line from the patient and 10 normal individuals was quantified using real-time PCR analysis. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control gene to normalize for the amount of RNA added to the real-time assays. Primers were designed for exon-to-exon amplification in an overlapping fashion. Each sample was run at least in triplicate. The final results are presented as *N*-fold differences in target-gene expression, relative to *GAPDH* according to the instruction manual of the *Taq*Man detection system.