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Molecular cytogenetic characterization of a familial pericentric inversion 3 associated with short stature

Usha R. Dutta [a,b,*], Ingo Hansmann [a], Dietmar Schlote [a]

[a] Institut fuer Humangenetik, Martin Luther University, Halle-Wittenberg, Halle (Saale) 06097, Germany
[b] Centre for DNA Fingerprinting and Diagnostics, Nampally, Hyderabad 500 001, India

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ABSTRACT

Short stature refers to the height of an individual which is below expected. The causes are heterogenous and influenced by several genetic and environmental factors. Chromosomal abnormalities are a major cause of diseases and cytogenetic mapping is one of the powerful tools for the identification of novel disease genes. Here we report a three generation family with a heterozygous pericentric inversion of 46, XX, inv(3) (p24.1q26.1) associated with Short stature. Positional cloning strategy was used to physically map the breakpoint regions by Fluorescence *in situ* hybridization (FISH). Fine mapping was performed with Bacterial Artificial Chromosome (BAC) clones spanning the breakpoint regions. In order to further characterize the breakpoint regions extensive molecular mapping was carried out with the breakpoint spanning BACs which narrowed down the breakpoint region to 2.9 kb and 5.3 kb regions on p and q arm respectively. Although these breakpoints did not disrupt any validated genes, we had identified a novel putative gene in the vicinity of 3q26.1 breakpoint region by *in silico* analysis. Trying to find the presence of any transcripts of this putative gene we analyzed human total RNA by RT-PCR and identified transcripts containing three new exons confirming the existence of a so far unknown gene close to the 3q breakpoint.

1. Introduction

Short stature (SS) refers to the height of an individual which is below expected and is one of the major features found in many syndromic cases. The causes of SS are many and influenced by several genetic and environmental factors hence the systematic classification of the various syndromes involving SS is not possible [Enders, 1992]. The genetic factors may include chromosomal anomalies in both autosomes as well as in sex chromosomes. The most common cytogenetic cause of SS in females is Turner syndrome, with different chromosomal variants affecting X chromosome. The Short stature HOmeobox-containing gene on the short arm of X and Y (SHOX) is an important determining factor for stature phenotype [Musebeck et al., 2001]. Whereas in autosomes; the SHOX2 gene is localized on chromosome 3q25-26 [Baere et al., 1998; Blaschke et al., 1998]. SHOX2 is closely related to SHOX on sex chromosome [Clement-Jones et al., 2000]. But till date there are only two reports on the chromosomal analyses with SS [Lam et al., 2002; Moreno-Garcia et al., 2005].

In general, the structural rearrangements alter the genome architecture and may result in human disease phenotypes. The patients with translocations and inversions often have breakpoints located within the disease gene, or very close to it [Chen et al., 2010]. In order to identify the disease gene, characterization of the breakpoints has often been a promising start point in the molecular elucidation of early-onset of Mendelian disorders [Kalscheuer et al., 2003; Moller et al., 2008]. The constitutional pericentric inversion on chromosome 3 occurs rarely [Gahrton et al., 2010], accounting for about 4% of the normal population [Soudek and Sroka, 1978]. Most of the inversions are not clearly visualized by GTG banding [Hasle et al., 1992] and therefore not frequently reported [Welborn, 2004]. Nevertheless a few cases of chromosome 3 abnormalities associated with SS were reported in literature [Barajas-Barajas et al., 2001], like a 3q deletion with SS reported by Nguyen [Nguyen et al., 2005] and a partial monosomy 3q with SS reported by Brueton et al., 1989. Most of other cases were associated with other chromosomal rearrangements [Kondo et al., 2006; Stine et al., 1982; Yip et al., 1996]. But to the best of our knowledge there is no case reported with a familial inversion

* Corresponding author. Diagnostics Division, Center for DNA Fingerprinting and Diagnostics, Tuljaguda complex, 4-1-714, Hyderabad 500 001, Andhra Pradesh, India. Tel.: +91 40 24749337; fax: +91 40 24749448.
E-mail addresses: ushadutta@hotmail.com, usha@cdfd.org.in (U.R. Dutta).

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3 associated with SS. In this study, we characterized a familial inversion inv(3) (p24.1q26.1) associated with SS and identified a novel putative gene on 3q26.1 breakpoint region.

2. Patient data

A 16-year old girl was referred to our center with SS. She was the first child born at term to a non-consanguineous couple after an uneventful pregnancy. Her milestones were normal. She was only 146 cm tall, significantly under the 3rd centile, whereas her 7 year old brother was 125 cm tall, consistent with the 50th centile. The detailed family history and written consent was taken from the patient's family.

3. Methods

3.1. Cytogenetic analyses

Chromosomal analyses were carried out on the peripheral blood lymphocytes in the patient, her parents, brother, maternal aunt and maternal grandmother by standard methods. Metaphases were analyzed by G-banding using Trypsin and Giemsa (GTG).

3.2. Fluorescence in situ hybridization

Initially 18 Yeast Artificial Chromosomes (YAC) clones were selected based on the chromosomal band position and then 20 Bacterial Artificial Chromosome (BAC) clones were randomly selected using GRCh37/hg19 assembly utilizing the Ensembl and UCSC Genome browser. The international standard nomenclature was used for clone names, which were obtained from ImaGenes, Berlin, Germany [Kent et al., 2002; Pruitt et al., 2007]. BAC DNA was isolated using NucleoBond Plasmid Midi kit (Macherey-Nagel, Dueren, Germany) according to manufacturer's instructions. The isolated BAC DNA was labeled with biotin-16-dUTP (Roche Diagnostics, Mannheim, Germany) by nick translation and FISH analysis was done on the patient metaphase slides as described by the standard protocols [Langer et al., 1981].

3.3. PCR amplification

To narrow down the breakpoints, primers specific for breakpoint spanning clones CTD-2007B5 and RP11-12N13 were designed and amplified by standard PCR. Thus two specific products of size 2.9 kb (5′-CTGATGATTAAAGGGATGAAGAC-3′; 5′-ACCTGGTTGTTGGAGCTTATC-3′) with CTD-2007H5 and 5.3 kb (5′-CTCGGTTCGTCTAAAGCTGC-3′; 5′-ATTAACCCAAGACCTTAGCG-3′) with RP11-12N13 as templates were amplified. For nested or semi-nested PCR, 2 μl of the first reaction mixture was used as the template for the second amplification. The amplified PCR products were electrophoresed on an agarose gel with a molecular weight DNA standard and stained with ethidium bromide.

Long range PCR was performed with Long PCR enzyme mix (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. Specific primers (5′-CTGTGTCTTAGGGTCTACTTTGGTCAGAAT-3′; 5′-AGTGTTGAAATAGTTATCACCATGAGGAC-3′) for the breakpoint spanning BAC clone RP11-12N13 amplified a product of 13.7 kb.

3.4. Reverse Transcription (RT-PCR)

Reverse Transcription was performed using the M-MLV Reverse Transcriptase, *RNase H* Minus (Promega, Mannheim, Germany) according to the specification provided by the supplier. As template; testes, brain, whole embryonic and fetal human total RNA (Biochain, Heidelberg, Germany) were used. Specific products were amplified with exon 5 specific primers p5 (5′GACATTGTCTGGGAGCAGC-3′), p5n (5′-AGTAGATCCTGAAGGCGTG-3′) and exon 11 specific primers p11 (5′-CTTGTCTAAGGTTGCAGACTCA-3′), p11n (5′-GTGAAGCCTCGTTTCATCC-3′) in a first round of standard PCR and re-amplified using the nested primers. For the splice variant SV2 an exon 9a specific primer p9a (5′-GGAAAGAAGCAGAGGTAGCC-3′) was used as forward primer.

3.5. Subcloning

The breakpoint spanning clone RP11-12N13 on 3q26.1 region was digested with *Sac I* restriction enzyme and the desired fragments were excised from the gel, eluted and subcloned into pBluescript SK Vector which were further labeled and used as probes for FISH mapping.

3.6. Restriction digestion

Several different restriction enzymes were selected to generate fragments of desired size for the breakpoint spanning clones. These fragments were gel eluted and directly labeled for further FISH experiments which helped to substantially narrow down the breakpoint region.

3.7. DNA sequencing

PCR amplified bands were excised from the gel and purified using a QIAGEN gel extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA sequencing was performed using a BigDye v.1.1 Terminator Cycle sequencing kit (Applied Biosystems, Darmstadt, Germany). The products were analyzed on an ABI Prism® 3100 Genetic Analyzer.

3.8. Database searches and sequence alignments

Homology searching was performed using the National Center for Biotechnology information BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against nt and the dbEST databases.

4. Results

Cytogenetic analysis of the GTG banded metaphase chromosomes of the patient revealed a karyotype of 46, XX, inv(3) (p23q25q26) (Fig. 1B) according to ISCN (Fig. 1C). The chromosomal analyses of the patient's father and brother showed a normal 46,XY male karyotype and her maternal aunt also showed a normal 46,XX female karyotype. But the chromosomal analyses of both her mother and maternal grandmother showed the same pericentric inversion 3 (Fig. 1A).

The human chromosome 3 is metacentric and the inversion event in the patient is also equidistant from the centromere making it difficult to identify the p and q arms. So to rule out the arm identity one 3p terminal clone RP11-91K04 was used as a reference for all initial FISH experiments. To narrow down the breakpoint regions it was co-hybridized with specific probes to the patients metaphase spreads as double hybridization FISH. Initial FISH with YAC clones helped in the identification of breakpoint spanning YACs which had further helped in the easy selection of the BAC clones (data not shown). Subsequently, breakpoint spanning BAC contigs were assembled by screening genomic libraries. The 3p breakpoint interval was covered by a contig of 4 BAC clones (Fig. 2E), of which RP11-666G20 (186 kb) and CTD-2007B5 (97.5 kb) (Fig. 2A) showed split signals. For further analysis CTD-2007B5 clone was taken as the breakpoint spanning clone.