Molecular characterisation of four cases of intrachromosomal triplication of chromosome 15q11-q14

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Abstract

Context—Chromosomal abnormalities that involve the proximal region of chromosome 15q occur relatively frequently in the human population. However, interstitial triplications involving one 15 homolog are very rare with three cases reported to date.

Objective—To provide a detailed molecular characterisation of four additional patients with interstitial triplications of chromosome 15q11-q14.

Design—Molecular analyses were performed using DNA markers and probes specific for the 15q11-q14 region.

Setting—Molecular cytogenetics laboratory at the University of Chicago.

Subjects—Four patients with mild to severe mental retardation and features of Prader-Willi syndrome (PWS) or Angelman syndrome (AS) were referred for molecular cytogenetic analysis following identification of a suspected duplication/triplication of chromosome 15q11-q14 by routine cytogenetic analysis.

Main outcome measures—Fluorescence in situ hybridisation (FISH) was performed to determine the type of chromosomal abnormality present, the extent of the abnormal region, and the orientation of the extra chromosomal segments. Molecular polymorphism analysis was performed to determine the parental origin of the abnormality. Methylation and northern blot analyses of the SNRPN gene were performed to determine the effect of extra copies of the SNRPN gene on its methylation pattern and expression.

Results—Fluorescence in situ hybridisation (FISH) using probes within and flanking the Prader-Willi/Angelman syndrome critical region indicated that all patients carried an intrachromosomal triplication of proximal 15q11-q14 in one of the two chromosome 15 homologues (trip(15)). In all patients the orientation of the triplicated segments was normal-inverted-normal, suggesting that a common mechanism of rearrangement may have been involved. Microsatellite analysis showed the parental origin of the trip(15) to be maternal in three cases and paternal in one case. The paternal triplication patient had features similar to PWS, one maternal triplication patient had features similar to AS, and the other two maternal triplication patients had non-specific findings including hypotonia and mental retardation. Methylation analysis at exon 1 of the SNRPN locus showed increased dosage of either the paternal or maternal bands in the paternal or maternal triplication patients, respectively, suggesting that the methylation pattern shows a dose dependent increase that correlates with the parental origin of the triplication. In addition, the expression of SNRPN was analysed by northern blotting and expression levels were consistent with dosage and parental origin of the triplication.

Conclusions—These four additional cases of trip(15) will provide additional information towards understanding the phenotypic effects of this abnormality and aid in understanding the mechanism of formation of other chromosome 15 rearrangements.

Keywords: chromosome 15 triplication; Prader-Willi syndrome; Angelman syndrome; autism

The proximal long arm of human chromosome 15 is frequently involved in molecular rearrangements including deletions, duplications, triplications, translocations, and inversions, as well as in the formation of supernumerary marker chromosomes. Approximately 70% of patients with Prader-Willi syndrome (PWS) or Angelman syndrome (AS) can be characterised by the presence of a de novo interstitial deletion of 15q11-13 spanning approximately 4 Mb. This recurring deletion is one of the most common observed in humans and identifies at least three hotspots for chromosome breakage in the 15q11-13 region, referred to as BP1, BP2, and BP3. Whether this deletion results in PWS or AS depends upon the origin of the affected homologue (paternal or maternal, respectively). This parent of origin effect is the result of the presence of oppositely imprinted gene(s) within the 15q11-13 region.

Interstitial duplications of 15q11-q13 of maternal origin have been identified in patients with developmental delay/learning difficulties and patients with autistic behaviour. Subjects with paternally derived duplications have a normal phenotype instead. The duplications frequently cover the entire PWS/AS critical region.

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Intrachromosomal triplication of 15q11-q14

Table 1: Summary of patients with intrachromosomal 15q11-q14 triplication

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex and age of patient</th>
<th>Phenotype</th>
<th>Extent of triplication (probe order in fig 1)</th>
<th>Middle repeat inverted</th>
<th>Parental origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>F, 12 y</td>
<td>Mild mental retardation, cleft palate, obesity</td>
<td>D15S18-D15S1019</td>
<td>Yes</td>
<td>Paternal</td>
</tr>
<tr>
<td>Patient 2</td>
<td>F, 6 y</td>
<td>Ataxia, mild mental retardation, speech delay</td>
<td>D15S11-D15S11019</td>
<td>Yes</td>
<td>Maternal</td>
</tr>
<tr>
<td>Patient 3</td>
<td>M, 7 mth</td>
<td>Severe mental retardation, generalised hypotonia, strabismus</td>
<td>D15S18-D15S1019</td>
<td>Yes</td>
<td>Maternal</td>
</tr>
<tr>
<td>Patient 4</td>
<td>F, 2-4 y</td>
<td>Severe mental retardation, mild hypotonia, strabismus, brachycephaly</td>
<td>D15S18-D15S1019</td>
<td>Yes</td>
<td>Maternal</td>
</tr>
<tr>
<td>Ref 4, 11</td>
<td>F, 7 y</td>
<td>Mental retardation, hypotonia, seizure, autistic features</td>
<td>D15S18-D15S1043</td>
<td>Yes</td>
<td>Maternal</td>
</tr>
<tr>
<td>Ref 5</td>
<td>F, 4 y</td>
<td>Developmental delay, seizure, ataxia</td>
<td>D15S18-D15S24</td>
<td>Yes</td>
<td>Maternal</td>
</tr>
<tr>
<td>Ref 3, 11</td>
<td>F, 6 y</td>
<td>Developmental delay, hypotonia</td>
<td>—</td>
<td>—</td>
<td>Maternal</td>
</tr>
</tbody>
</table>

Case reports

The clinical findings associated with these patients are summarised in table 1 and are compared to other cases with molecularly characterised triplications.
with a short forehead and mild frontal bossing, extension of the temporal hairline to the lateral eyebrows, mild synophrys, bilateral inner epicanthic folds, slightly upward slanting palpebral fissures, myopia, strabismus, broad nasal bridge and upturned nares, slightly small jaw, normal position of the ears with midhelical hypoplasia, mild hypertrichosis of the back, mild swelling of the dorsa of both hands and feet, and slightly tapering fingers. Neurological examination showed only mild hypotonia.

Materials and methods

CYTOGENETICS AND FISH ANALYSIS

Cytogenetic analysis was performed on peripheral blood lymphocyte cultures from patients 3 and 4 and their parents using standard techniques. Preparations were GTG stained. For analysis of patients 1 and 2 see Pettigrew et al26 and Clayton-Smith et al.

Lymphoblastoid cell lines were established for each patient and chromosome preparations were made from these cells using standard methods. The probes used in these studies are listed in order from proximal to distal where P1=P1 clone, b=BAC, p=PAC, and c=cosmid: P1-5022 (D15S18), c512 (D15S543), p151G14 (ZNF127), D15S11, p158H23 (UBE3A), b150L13 (D15S931), b72P22 (D15S1019), b53C6 (D15S165), b184N23 (D15S144). All BACs, PACs, and P1 clones were acquired from Genome Systems Inc (St Louis, MO). p158H23 (UBE3A) was kindly provided by Dr James S Sutcliffe. D15S11 was acquired as a commercially available FISH probe (Oncor, now Vysis Inc, Downer’s Grove, IL). The relative positions of the probes are shown in fig 1.

MOLECULAR POLYMORPHISM ANALYSIS

Genomic DNA of the patients and their parents was prepared either directly from peripheral blood lymphocytes (parents) or from Epstein-Barr virus transformed lymphoblastoid cell lines (patients). Nine short tandem repeat polymorphic markers (STRs) inside the PWS/AS region were used to determine the parental origin of the triplicated chromosome, as previously described.31 The locations of the polymorphic markers used in this analysis are shown in fig 1. Polymerase chain reaction (PCR) assays were carried out using published methods.32 PCR products were separated on a 6% polyacrylamide/urea gel and visualized by autoradiography.

METHYLATION ANALYSIS

SNRPN exon 1 methylation analysis was performed using a methylation specific PCR assay (M-PCR), described previously,26 with the addition of 0.15 µl of α-[32P]dCTP/sample. Radiolabelled PCR products were separated on an 8% acrylamide gel and maternal (174 bp) and paternal (100 bp) bands were quantified using a phosphorimager with ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA, USA). The ratio of the area under the curve of the maternal band relative to that of the paternal band and vice versa was determined for a control subject with a normal karyotype and for the four patients. The values obtained for the patients were then compared to the normal control.

NORTHERN ANALYSIS

Total RNA was purified using TRizol Reagent from Life Technologies Inc (Rockville, MD). Twenty µg of total RNA were loaded in each lane for northern blot analysis. A cDNA probe for the SNRPN region exon –1 to exon 1 (RN175, RN140) was generated as previously described.13 The PCR product was cloned into the Bluescript II KS(−) vector (Stratagene, La Jolla, CA). Twenty µg of total RNA were loaded in each lane for northern blot analysis. A cDNA probe for the SNRPN region exon –1 to exon 1 (RN175, RN140) was generated as previously described.13 The PCR product was cloned into the Bluescript II KS(−) vector (Stratagene, La Jolla, CA).
using a pGEM-T Easy vector (Promega Madison, WI, USA) and was 32P-labelled by random priming. Hybridisation was performed using ExpressHyb Solution (Clontech, Palo Alto, CA, USA) at 65°C. The filters were washed in a solution of 0.2 x SSC, 0.1% SDS at 65°C. An oligonucleotide specific for 28S ribosomal RNA (5'-AACGATCAGAGTAGTGGTATTTCACC -3') was directly 32P-labelled and used to correct for RNA loading differences. Relative intensities of the hybridised bands were quantified using ImageQuaNT software.

Results
EXTENT OF THE TRIPlicated SEGMENTS
G banding analysis of patient 1 and patient 2 was previously reported and originally interpreted as indicating interstitial duplications. G banding analysis of patient 3 and patient 4 showed a 46,XX,15q+ karyotype that was also initially interpreted as probable duplication of the 15q11-q13 region (data not shown).

Dual colour FISH analysis using combinations of clones P1-5022, p158H23, b72P22, b53C6, or b184N23 was performed on inter-
phase nuclei and metaphase chromosomes. The locations of these clones within chromosome 15q11-q14 are presented in fig 1. Patient 1 (fig 2A-C) and patient 3 showed identical results, including three signals on one chromosome 15 homologue for probes P1-5022, p158H23, and b72P22, and a single signal for clones b53C6 and b184N23. This indicates that these patients have triplications extending from a site proximal of S18 to a point between S1019 and S165 (fig 1). Patient 4 (fig 2D-F) showed three signals for clones P1-5022, p158H23, and b72P22 as well as for clone b53C6, while only clone b184N23 was present in single copy. This larger triplication, therefore, includes both S18 and S165 (fig 1). These results indicate that in these three patients the triplicated region extends from the most proximal breakpoint, BP1, to a distal breakpoint that lies between D15S1019 and D15S165 in patients 1 and 3 (BP4), and S15S165 and D15S144 in patient 4 (BP5) (fig 1).

FISH analysis using the same clones on interphase nuclei and metaphase chromosomes of patient 2 also showed three signals using clones p158H23, b72P22, and b53C6, indicating that the distal extent of the triplication in patient 2 is the same as that in patient 4 (BP5) (fig 1). The proximal breakpoint in this patient, however, involves a previously unobserved breakpoint that was mapped using clones c512, p151G14, and D15S11. These results indicate that in patient 2 the proximal breakpoint lies between ZNF127 and D15S11 (fig 1).

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**Figure 3** Orientation of the triplicated segments. Hybridisation with probes c512 (red) and b150L13 (green) to metaphase chromosomes of patient 4 (A) and ideogram of the triplicated chromosome 15 showing the relative position of the probes used (B). For probe c512 (red) the telomeric signal was of double intensity as compared to the centromeric signal; for probe b150L13 (green) the centromeric signal was stronger than the telomeric signal, indicating that the repeats of the triplication are normal (N) in the proximal and distal regions and inverted (I) in the central region. The same results were obtained in patients 1, 2, and 3. Note that in patient 2, c512 was replaced by D15S11 as proximal probe.

**Figure 4** Microsatellite analysis at locus D15S1035 in family of patient (Pt) 1 (A) and at locus D15S542 in family of Pt 4 (B). The triplication is of paternal origin in patient 1 as determined by the presence of three paternal alleles and one maternal allele. Note that the intensity of allele 4 is higher than the same allele in the father, indicating that in patient 1 allele 4 is duplicated. In patient 4, the triplication is of maternal origin as indicated by the presence of three maternal alleles and one paternal allele. The increase of intensity of allele 3 in patient 4 indicates that this is the duplicated allele.
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Figure 5  Methylation analysis of the CpG island at the 5' end of the SNRPN gene. In each lane the upper band (174 bp) is from the maternal chromosome and the lower band (100 bp) is from the paternal one. The Angelman syndrome patient AS with a deletion of the maternal 15q11-q13 chromosome shows only the paternal band, while the Prader-Willi patient PWS with a paternal deletion of the same region shows only the maternal band. Patient (Pt) 1 has a more intense paternal band that indicates paternal inheritance of the patient PWS with a paternal deletion of the same region shows only the maternal band. The Angelman syndrome patient AS with a deletion of 15q11-q13 maternal 15q11-q13 chromosome shows only the paternal band, while the Prader-Willi syndrome patient PWS with a paternal deletion of the same region shows only the maternal band.

Table 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Mat/Pat Ratio</th>
</tr>
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<tbody>
<tr>
<td>Normal control</td>
<td>1</td>
</tr>
<tr>
<td>Pt 1 (pat trip)</td>
<td>2.6</td>
</tr>
<tr>
<td>Pt 2 (mat trip)</td>
<td>2.8</td>
</tr>
<tr>
<td>Pt 3 (mat trip)</td>
<td>3.0</td>
</tr>
<tr>
<td>Pt 4 (mat trip)</td>
<td>3.0</td>
</tr>
<tr>
<td>Patient 1 PWS</td>
<td>3.3</td>
</tr>
<tr>
<td>Patient 2 PWS</td>
<td>2.6</td>
</tr>
<tr>
<td>Patient 3 PWS</td>
<td>2.8</td>
</tr>
<tr>
<td>Patient 4 PWS</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Discussion

This work reports the clinical and molecular findings for four subjects with de novo interstitial triplications of the 15q11-q14 region. The clinical manifestations of the four patients are summarised in table 1 and compared with the other three known cases of triplication of the same region.

Molecular analysis shows that in patients 2, 3, and 4 the triplication is maternal in origin. A maternal triplication in a case with mild features of AS was previously described by Schinzel et al. More recently, Long et al reported a patient with developmental delay who was found to have a small supernumerary inv dup(15) and an interstitial triplication of proximal 15q11-q13 maternal in origin. The absence of euchromatin from the PWS/AS critical region within the inv dup(15) suggests that the trip(15) is responsible for that patient’s abnormal phenotype. Moreover, all the de novo supernumerary marker 15 chromosomes that include the PWS/AS critical region are maternal in origin and are associated
with moderate to severe mental retardation. These observations indicate that maternal rearrangements of the 15q11-q13/q14 region can be associated with an abnormal phenotype.

A paternal triplication of the 15q11-q13 region was previously found by Cassidy et al. in a hypotonic, developmentally delayed child with non-specific findings. Patient 1, described here, has a triplication of the 15q11-q14 region on the paternal chromosome that is associated with mental retardation, obesity, and cleft palate. These results indicate that paternal triplication of the PWS/AS critical region may also have phenotypic effects, unlike duplications that show a normal phenotype.

By using the three markers D15S1019, D15S165, and D15S144, we have shown that the triplication involves two breakpoints located between D15S1019 and D15S165 (BP4) and between D15S165 and D15S144 (BP5) that are likely to represent the breakpoints for most large inv dup(15) chromosomes. Both BP4 and BP5 contain portions of the genomic duplication (duplcon) present in BP2 and BP3A/3B (unpublished data) that are likely to be hotspots for recombination.

Intrachromosomal triplications of chromosome 15 seem to result from a common mechanism as indicated by the inverted orientation of the middle repeat found in our patients and in previously reported cases. Wang et al. recently reviewed the published reports since 1993 and identified at least 11 cases of intrachromosomal triplication, affecting chromosomes 2q, 5p, 7p, 9p, and 15q, involving an inverted orientation of the middle repeat in all cases investigated. These authors proposed a mechanism of two U type exchanges involving three chromatids, as previously described in Drosophila by Slizynska. For chromosome 15, this would include a U type exchange in meiosis I between homologues at the distal breakpoint region and a second U type exchange at the proximal breakpoint region between sister chromatids of a single homologue (see fig 4 in Wang et al. ). This mechanism gives rise to a small inv dup(15), as was seen in the case of Long et al. , but may more often be lost, and a chromosome 15 which contains three sequentially arranged segments of which the first and third repeats are in direct orientation while the second one is inverted. Another possible mechanism of recombination was previously described by Schinzel et al. , where the authors proposed that the first step in the formation of the triplication involves an unstable dicentric inv dup(15)(pter→q13::q13→qter) chromosome of maternal origin that contains both a direct and an inverted repeat. Triplication would be the result of recombination between this broken chromosome and the remaining normal chromosome 15 followed by the loss of the supernumerary marker.

The phenotypic abnormalities associated with the chromosomal rearrangements of the 15q11-q14 region can be determined by alterations in the expression of the imprinted genes mapping within the PWS/AS critical region. At least five imprinted, paternally expressed genes have been localised to the centromeric end of the PWS/AS critical region, including ZNF127, NDN, MAGEL2, SNURF/SNRPN, and IPW. PAR-1 and PAR-5 are paternally expressed sequences which remain poorly characterised. UBEDA is preferentially expressed from the maternal chromosome in brain and is localised distally to the paternally expressed cluster of genes. The region also includes a cluster of three GABA receptors with unclear imprinting status.

In each patient we studied the methylation status of the CpG island located upstream of the SNRPN gene. Our results showed that the presence of the 15q11-q14 triplicated region does not interfere with SNRPN methylation status, maintaining hypomethylation on the paternal chromosome and hypermethylation on the maternal chromosome. Accordingly, SNRPN RNA levels increased only in the patient with paternal triplication (patient 1), while in the three maternal triplication patients, SNRPN expression was normal. These results suggest that an alteration in the levels of SNRPN RNA may play a role in the

<table>
<thead>
<tr>
<th>Patient</th>
<th>Relative SNRPN expression</th>
</tr>
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<tbody>
<tr>
<td>AS</td>
<td>1.2</td>
</tr>
<tr>
<td>PWS</td>
<td>0.2</td>
</tr>
<tr>
<td>Pt 1</td>
<td>2.2</td>
</tr>
<tr>
<td>Pt 2</td>
<td>1.2</td>
</tr>
<tr>
<td>Pt 3</td>
<td>0.9</td>
</tr>
<tr>
<td>Pt 4</td>
<td>0.9</td>
</tr>
<tr>
<td>Normal controls</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Figure 6. Expression of the SNRPN gene. (A) Autoradiograph of a gel loaded with 20 µg of total RNA extracted from lymphoblastoid cell lines from each patient and hybridised sequentially with a cDNA probe for SNRPN and with an oligonucleotide specific for 28S rRNA to correct for RNA loading differences. (B) Relative SNRPN expression for each sample. Only in patient 1 (Pt 1), who has a paternal triplication, is the SNRPN expression more than two times increased compared to the normal controls. Patients 2, 3, and 4 (Pt 2, Pt 3, Pt 4) with a triplication of maternal origin have SNRPN RNA levels similar to the normal controls, SNRPN RNA levels were not different from the normal controls in a patient with Angelman syndrome (AS) with a deletion of the maternal 15q11-q13 chromosome, and almost absent in a patient with Prader-Willi syndrome (PWS) with a deletion of the same region on the paternal chromosome.
pathogenesis of the phenotype of patient 1. However, the expression of other imprinted and non-imprinted genes mapping in the 15q11-q14 region was investigated in this study and probably also contributes to the abnormal phenotype observed in these patients.

Results presented in this paper suggest that a single, common mechanism gives rise to the proximal 15q triplications observed in our patients and additionally identify breakpoints in common with other chromosome 15 rearrangements, as well as the derivation and potential familial recurrence of AS, PWS, and pervasive developmental delay/autism. Taken together, this information may aid in providing appropriate genetic counseling, especially in the context of prenatal diagnosis.

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