Inherited DNA amplification of the proximal 15q region: cytogenetic and molecular studies

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Abstract

In a 15 year old girl, referred for growth retardation, conventional cytogenetic analysis detected an abnormal 15q+ chromosome with extra material in the proximal region, inherited from her father and grandfather. Using various molecular cytogenetic techniques, including comparative genomic hybridisation, we showed that the extra chromatin resulted from in situ amplification of DNA sequences located between the loci D15Z1 and D15S18. On the basis of the clinical features of our patient and the late replication of the large amplified region, we searched for functional modifications in the adjacent Prader-Willi syndrome region.

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The proximal 15q11-q12 region appears to be prone to various types of rearrangements including deletions, translocations, duplications, triplications, and inv dup(15) supernumerary chromosomes. Deletions, and less frequently translocations, have been reported in association with Prader-Willi syndrome (PWS) and Angelman syndrome (AS).¹ Inv dup(15) extra chromosomes are associated with both normal and abnormal phenotypes including dysmorphism, mental retardation, and severe epilepsy.²⁻⁶ Finally, the few reported cases of intrachromosomal duplications and triplications of the same region are associated with a normal phenotype, PWS or AS, or a phenotype very similar to that found in inv dup(15) patients.⁷⁻⁹

We report here a 15 year old girl referred for cytogenetic analysis because of short stature. Her karyotype showed an abnormal chromosome 15 with extra chromatin in the proximal long arm. Fluorescence in situ hybridisation (FISH) techniques, including comparative genomic hybridisation (CGH), further defined an amplification of DNA sequences located between the centromere of chromosome 15 and the D15S18 locus. This extra chromatin appeared as a large late replicated segment, adjacent to the PWS region. That the patient shared some clinical signs with PWS raised the possibility that the amplification modifies the expression of genes in the Prader-Willi syndrome region by a mechanism similar to position effect variegation (PEV) seen in *Drosophila*.^{10 11} Indications of functional modi-

fication in the PWS region were sought by two methods, DNA replication analysis using FISH and methylation study.

Replication timing and genetic activity have been correlated in various situations, and tissue specific genes generally replicate earlier when they are expressed than when they are not.¹²⁻¹⁴ Moreover, asynchronous replication has been reported for imprinted genes that display allele specific expression.^{15 16} We therefore searched for a modification in the replication timing of the imprinted SNRPN gene (small nuclear ribonucleoprotein polypeptide N) in our patient. SNRPN maps to the PWS region.

The PWS region has been characterised by specific DNA methylation imprinting at several loci.¹⁷⁻²⁰ We therefore analysed a possible modification of the methylation status at one of these loci, D15S63, in our patient. We conclude that the extra chromatin in our patient can be likened to a homogeneously staining region, without phenotypic effect.

Case report

The proband, a white female, was the product of an uneventful term pregnancy and a normal delivery. Birth weight was 3330 g and length 48 cm. The postnatal course was normal; however, slight hypotonia was noticed from birth to 3 months of age, without sucking difficulties. The proband was referred at the age of 15 years for short stature (height 139 cm, -3 SD). At the time of the consultation, her weight was 38 kg and she had a tendency to hyperphagia. She did not show any dysmorphic features, mental retardation, or hypotonia and had a normal puberty. She had had eczema and asthma from 2 years of age. Her family history was unremarkable and her parents were healthy. The height of her mother and father were 160 and 159 cm, respectively. The height of her paternal grandmother was 160 cm and that of her paternal grandfather 154 cm.

Materials and methods

CONVENTIONAL AND MOLECULAR CYTOGENETICS
Cytogenetic analyses of the patient and all family members were performed on cultures from peripheral lymphocytes using standard techniques. Preparations were RHG and CBG stained. 5-BrdU was incorporated (final concentration 60 µg/ml of medium) during the last seven hours of culture in order to identify the late replicated chromosome regions (RBG).

Probes with small insert size, pIR39 (D15S18), pTD3.21 (D15S10), and pTD189.1 (D15S13), were purchased from

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Figure 1 Partial karyotypes of chromosomes 15 from the proband (chromosome 15q+ is on the right). (A) Extra chromatin appears as a large positive R band after RHG banding. (B) Extra material appears late replicating after BrdU incorporation and RBG banding.

American Type Culture Collection (ATCC). They were tritium labelled by nick translation and hybridised as previously described.²¹ After coating with nuclear track emulsion (Kodak NTB2) the slides were exposed for 15 days at 4°C, then developed. R banding was performed by the fluorochrome-photolysis-giemsa method. One hundred metaphases were analysed for each probe. The percentage of silver grains on the 15q+ chromosome was compared to that observed on the normal chromosome 15, to determine the locus copy number.

When cosmids were available, fluorescence in situ hybridisation (FISH) was performed as recommended by the supplier (ONCOR Inc, Gaithersburg). Biotinylated cosmids corresponding to D15S11 and GABRB3 loci were detected by FITC conjugated avidin (Biosys Vector Laboratories cat A2011), as described by Pinkel *et al.*²² Digoxigenin labelled cosmid corresponding to SNRPN was detected by a mouse monoclonal antidigoxigenin antibody (Boehringer Mannheim cat 1333.062) and

fluorescein conjugated antimouse antibody (Boehringer Mannheim cat 1214.616). Two other probes were hybridised on metaphases of the proband: the biotinylated alpha satellite DNA probe D15Z1 and the specific chromosome 15 library (from ONCOR).

COMPARATIVE GENOMIC HYBRIDISATION (CGH)

CGH was performed according to the protocol of Du Manoir *et al.*²³ Briefly, test DNA and reference DNA were differentially labelled with biotin-14-dATP and digoxigenin-11-dUTP (Boehringer Mannheim cat 1558.706) respectively, using commercially available nick translation systems (Gibco, Life Technologies). A total of 200–400 ng of both DNAs was coprecipitated in the presence of 20 µg cotI DNA (Gibco, BRL), dissolved in hybridisation mixture, denatured at 70°C for 10 minutes, and preannealed at 37°C for one hour. Hybridisation was carried out on reference metaphase spreads for two to three days, at 37°C, in a moist chamber. Posthybridisation washes were done according to the regular FISH protocol described by Pinkel *et al.*²² For the detection of biotin molecules, we used FITC conjugated avidin (Biosys Vector Laboratories cat A2011). Digoxigenin labelled DNA was visualised after incubations with monoclonal antibody to digoxigenin from mouse-mouse hybrid cells (Boehringer Mannheim cat 1333.062), antimouse Ig-digoxigenin F(ab')₂ fragment (Boehringer Mannheim cat 1214.624), and antidigoxigenin-rhodamine Fab fragment (Boehringer Mannheim cat 1207.750). Images of the hybridised metaphases were evaluated with a digital image analysis system (Alcatel Answare TITN, France) based on a Zeiss fluorescence microscope and a cooled CCD camera (Hamamatsu).

DNA REPLICATION ANALYSIS BY FISH

FISH to interphase nuclei is a sensitive method for assaying replication of genomic sites.²⁴ Hybridised sequences appear as two single signals in cells that have not replicated (G1), as two pairs of doublets in cells in which both homologues have replicated (G2), and as a singlet and a doublet in cells where only one homologue has replicated (G1–G2 cells).

Interphase nuclei from our patient and two normal subjects were obtained from PHA stimulated lymphocytes without synchronisation, as described by Selig *et al.*²⁴ Nuclei preparations were treated with Proteinase K (100 ng/ml in Tris-HCl 20 mmol/l, CaCl₂ 2 mmol/l, pH 7.4) before hybridisation, and FISH with the digoxigenin labelled SNRPN cosmid was performed as recommended by the supplier (ONCOR, Gaithersburg). The hybridised signals were detected as described above. Over 200 nuclei were observed for each subject.

METHYLATION STUDIES

The methylation status was studied with the pW71 probe located at the D15S63 locus.¹⁷ DNA was extracted from the patient, her parents, and paternal grandparents by standard methods. DNA was digested with two restric-

Figure 2 Fluorescence in situ hybridisation with several biotinylated probes on metaphases of the proband (arrowheads indicate the chromosome 15q+). (A) Specific chromosome 15 library shows homogeneous painting on the whole abnormal chromosome 15. (B) D15Z1 probe detected normal signals on both normal and abnormal chromosomes 15. (C) D15S11 cosmid gives an identical signal on both chromosomes 15, but it appears shifted towards the middle on the 15q+. Cohybridised Myl cosmid gives a subdistal signal indicating the chromosomes 15. (D) Similar results are obtained with GABRB3 cosmid.

Figure 3 Comparative genomic hybridisation with proband's DNA on metaphase of a normal subject. Arrowheads show the amplified DNA in the juxtacentromeric long arm of both chromosomes 15.

tion enzymes, *Hind*III (a methylation non-sensitive enzyme) and *Hpa*II (a methylation sensitive enzyme), separated by 0.8% agarose gel electrophoresis, and transferred to nylon membrane (PALL) by the method of Southern.²⁵ DNA probe was labelled with α -³²P dCTP by the random primer method.²⁶

Hybridisation was performed at 65°C for 18 to 24 hours in a roller bottle hybridisation oven with 6 × SSC, 0.5% SDS, 2.5 × Denhardt's solution, 5% dextran sulphate, and herring sperm DNA (100 µg/ml). The filter was then washed at 65°C to a final stringency of 2 × SSC/0.1% SDS and exposed to x ray film.

When probe pW71 was hybridised with normal DNA, two bands were observed: one of 6.0 kb corresponding to the methylated allele (maternal), the other of 4.4 kb, corresponding to the non-methylated allele (paternal).

Results

CONVENTIONAL AND MOLECULAR CYTOGENETICS

Conventional cytogenetics performed in our patient identified an elongated chromosome 15, with positive R banded extra material in the proximal long arm (fig 1A). The extra material was not stained by the CBG technique (data not shown) but was shown to be late replicating by BrdU incorporation (fig 1B). Karyotypes of the proband's parents and paternal grandparents show that the abnormal chromosome 15 is present in both the father and the paternal grandfather.

FISH analysis with a chromosome 15 specific library probe showed homogeneous painting of the whole abnormal chromosome 15, excluding an insertion of material from elsewhere in the genome (fig 2A). FISH with biotinylated centromeric D15Z1 probe detected normal signals on both normal and abnormal chromosomes 15 (fig 2B).

Several cases of elongated chromosome 15 have been shown to result from duplication or triplication of the 15q11-q12 region involved in PWS and AS. To test whether a duplication of this region underlies the abnormal karyotype described in this study, in situ hybridisation was performed with various probes from the 15q11-q12 region; cosmids for D15S11, GABRB3, and SNRPN loci each showed only one signal on the elongated chromosome 15 (fig 2C, D), excluding a duplication or a triplication of these loci. Isotopic in situ hybridisation was performed with pIR39 (D15S18), pTD3.21 (D15S10), and pTD189.1 (D15S13) probes. From the 100 metaphases analysed with each probe, there was no difference in the hybridisation signal between the two chromosomes 15 and no silver grains were detectable in the extra material of the abnormal 15. Moreover, all the probes from the PWS/AS region gave a hybridisation signal shifted towards the middle of chromosome 15q+, confirming that the extra material is located proximal to this region.

COMPARATIVE GENOMIC HYBRIDISATION

As the abnormal chromosome 15q+ did not seem to result from an increase in the copy number of the PWS/AS region, we tried to define the origin of the extra material by comparative genomic hybridisation. CGH was performed using total DNA from the proband as a probe and gave a clear signal in the juxtacentromeric long arm of the chromosome 15 pair in a normal subject (fig 3). This result was in

Figure 4 Methylation studies with pW71 probe hybridised on HindIII/HpaII digested DNA from GF: grandfather, GM: grandmother, F: father, M: mother, and P: proband. There is no significant difference in intensity between the methylated band of 6.0 kb and the unmethylated band of 4.4 kb in the proband.

agreement with in situ amplification of a very proximal DNA sequence, located between D15Z1 and D15S18 loci, in our patient.

DNA REPLICATION ANALYSIS BY FISH

The amplification observed in the 15q+ chromosome gives rise to a late replicated homogeneous staining region (HSR), adjacent to a PWS region of paternal origin. As the SNRPN gene may be involved in the PWS,¹⁹ we investigated a possible decrease in its activity by studying the replication timing at this locus. Such a decrease would be expected to correlate with a delay in the replication timing of the expressed paternal allele, resulting in a reduced replication asynchronism. After in situ hybridisation with the SNRPN cosmid, 200 nuclei were scored from our patient and each normal subject. Nuclei with one single and one double signal were in a range of 30–34%, showing no significant difference between the three subjects, and values similar to those described by Kitsberg *et al.*¹⁵ and Knoll *et al.*¹⁶ Nuclei with two single signals were between 57 and 62% and nuclei with two pairs of signals were in the range of 7 and 12%. We conclude that the late replication of the extra chromatin has no effect on the replication timing of the nearby SNRPN locus.

METHYLATION STUDIES

In order to investigate further the possibility of functional modification in the adjacent PWS region, we studied the methylation status at the D15S63 locus. Analyses of DNA from our patient, her parents, and paternal grandparents were carried out using the pW71 probe. The results showed two bands in all tested subjects: a maternal methylated band of 6.0 kb and a paternal unmethylated band of 4.4 kb (fig 4). There was no difference in intensity of the two bands in the proband and her parents, in agreement with a normal methylation pattern of the D15S63 locus. On the other hand, in both grandparents of our patient, we observed a slightly decreased intensity of the methylated band which could be explained by a general demethylation of the genome with increased

age.²⁷ We conclude that the late replicated extra chromatin does not modify the methylation status at the D15S63 locus, and probably does not affect the activity of the paternal alleles in the PWS region.

Discussion

A 15 year old girl was referred for growth retardation that, associated with neonatal hypotonia and a tendency to hyperphagia, was evocative of some aspects of PWS. Cytogenetic analysis detected an abnormal 15q+ chromosome with extra material in the proximal region. Family study indicated that the elongated chromosome was inherited through the father and the paternal grandfather of the proband, who also had short stature. This observation raised two important issues: firstly, whether there was a correlation between the presence of the extra material and the phenotype of the proband, and, secondly, as the region involved is imprinted, whether there was any risk of this 15q+ being associated with a more severe phenotype in cases of maternal transmission.

Several cases of elongated chromosomes 15 have been published and interpreted as an insertional duplication or triplication from the proximal 15q region. However, no consistent phenotype has been associated with such a chromosomal abnormality; some 15q duplications appear to be phenotypically silent,^{28–32} others associated with atypical PWS^{29–33–36} or AS,^{37–38} and others with unexplained mental retardation^{32–39–40} or repeated miscarriages.^{30–32} Rare cases of triplication are associated with motor and mental retardation.^{8–9} It has to be noted that very few cases of so called “duplications” or “triplications” of the proximal 15q region have been analysed with molecular techniques and phenotypic discordances probably result from differences in the content of the extra chromatin. Using FISH techniques in our patient, we identified the extra material as resulting from in situ amplification of a DNA sequence lying between the D15Z1 and D15S18 loci, located proximal to the PWS/AS region. Some cases of supernumerary inv dup(15) chromosomes, containing a slight expansion in this region, are associated with a normal phenotype,^{4–6–41} suggesting the possibility that non-transcribed sequences are involved. Thus, we conclude that the abnormal 15q+ chromosome is probably not directly related to the phenotype observed in our patient.

Nevertheless, the late replication of the extra material, its location near an imprinted region, and also its large size, led us to search for a kind of “position effect” on adjacent regions. Such an effect has been observed in *Drosophila*, where euchromatic genes exhibit a decrease in expression when they are placed next to heterochromatin by chromosomal rearrangement.^{10–11} A similar phenomenon has been observed when mammalian autosomal genes are fused to the inactive X chromosome. We investigated a possible decreased expression of genes located in the adjacent PWS

region by two methods: replication timing analysis by FISH and methylation study.

Using FISH to interphase nuclei, we found no difference in the level of asynchronous replication of the imprinted SNRPN gene in our patient compared to normal subjects. Kawame *et al.*⁸² hypothesised that the apparent replication asynchrony by FISH analysis of imprinted loci reflects structural differences between the maternal and the paternal alleles, rather than differences in replication timing. Nevertheless, in such a situation, our results would indicate that the extra chromatin in our patient did not modify the chromatin structure at the SNRPN locus.

Secondly, we analysed the methylation status of the imprinted D15S63 locus,¹⁷ which maps 130 kb centromeric to SNRPN. The methylation pattern was normal in the patient and her parents, allowing us to conclude that the extra late replicated chromatin had no effect on the adjacent PWS region. In both grandparents (83 and 86 years old), a slight decrease of the methylated band was observed, possibly resulting from a general demethylation of the genome with increased ageing.²⁷

Finally, we conclude that the extra chromatin observed in our patient has no phenotypic effect in carriers. It could be compared to "homogeneously staining regions" (HSRs) that are frequently observed in the genome of tumour cells⁴³ and drug resistant cell lines,⁴⁴ but are generally not found in normal tissues.⁴⁵ A similar HSR, transmitted through the germline and associated with a normal phenotype, has however been described in *Mus musculus domesticus*.^{46 47}

In summary, our study highlights the necessity of determining precisely the nature of any extra chromatin in the proximal 15q region. Rare observations studied with molecular techniques^{8 9 38} (this observation) confirm the results obtained from small supernumerary inv dup(15) chromosomes. Any increased dosage of genes in the PWS/AS critical region causes an abnormal phenotype, dependent on the copy number and the parental origin. Nevertheless, increased copy number of sequences from the region proximal to the PWS/AS critical region seems to be associated with a normal phenotype.

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