

## Different Mechanisms and Recurrence Risks of Imprinting Defects in Angelman Syndrome

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### Summary

Angelman syndrome (AS) is a neurogenetic disorder that appears to be caused by the loss of function of an imprinted gene expressed from maternal chromosome 15 only. Approximately 6% of patients have a paternal imprint on the maternal chromosome. In a few cases, this is due to an inherited microdeletion, in the 15q11-q13 imprinting center (IC), that blocks the paternal→maternal imprint switch in the maternal germ line. We have determined the segregation of 15q11-q13 haplotypes in nine families with AS and with an imprinting defect. One family, with two affected siblings, has a microdeletion affecting the IC transcript. In the other eight patients, no mutation was found at this locus. In two families, the patient and a healthy sibling share the same maternal alleles. In one of these families and in two others, grandparental DNA samples were available, and the chromosomes with the imprinting defect were found to be of grandmaternal origin. These findings suggest that germ-line mosaicism or de novo mutations account for a significant fraction of imprinting defects, among patients who have an as-yet-undetected mutation in a *cis*-acting element. Alternatively, these data may indicate that some imprinting defects are caused by a failure to maintain or to reestablish the maternal imprint in the maternal germ line or by a failure to replicate the imprint postzygotically. Depending on the underlying cause of the imprinting defect, different recurrence risks need to be considered.

### Introduction

Angelman syndrome (AS) is a relatively frequent disorder of mental and motor development. Affected individuals invariably show severe mental retardation, delayed motor development, the almost complete absence of

speech, and movement or balance disorders, usually characterized by an ataxic gait. Most patients also present an atypical face with a wide and open mouth, protruding tongue, and prominent chin, and their behavior often is characterized by frequent laughter and inappropriate happiness. Furthermore, seizures and characteristic electroencephalogram findings are found in most older patients (Williams et al. 1995). In the urban area of West Berlin, the frequency of AS has been estimated to be 1 in 16,000 newborns (Reis et al. 1994b).

The genetic basis of AS is complex. At present, it is not known whether AS is caused by the loss of function of a single gene or whether several genes are involved. Approximately two-thirds of patients have a de novo deletion of chromosome-region 15q11-q13 (Kaplan et al. 1987; Magenis et al. 1987), which is always of maternal origin (Knoll et al. 1989). Approximately 1% of patients have uniparental paternal disomy, that is, both chromosomes are of paternal origin. The strict bias in the parental origin of these defects suggests that the AS gene(s) is imprinted and expressed from the maternal chromosome only. Genes in 15q11-q13 that are expressed from only the paternal chromosome appear to play a role in Prader-Willi syndrome (PWS; for a review on genomic imprinting in 15q11-q13, see Nicholls 1993). In some of the remaining patients, structural mutations affecting their *UBE3A* gene were found, indicating that *UBE3A* is the major AS gene (Kishino et al. 1997; Matsuura et al. 1997).

A small group of patients was found to have AS as the result of an imprinting defect (Glenn et al. 1993; Reis et al. 1994a). The patients have apparently normal chromosomes of biparental origin but have a paternal methylation pattern on both chromosomes and biallelic expression of paternal-only genes (Saitoh et al. 1996). In some of these patients, an inherited microdeletion 500–600 kb proximal to the AS gene was found in the maternal chromosome (Sutcliffe et al. 1994; Buiting et al. 1995; Saitoh et al. 1996). Buiting et al. (1995) proposed a mechanism by which genomic imprinting of 15q11-q13 is regulated, in *cis*, by a single genetic element in this region, termed “the imprinting center” (“IC”). In this model, the IC plays a central role in imprint switching in the germ line.

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Recently, the IC model was refined further. Dittrich et al. (1996) showed that the IC region encodes novel exons that are spliced to *SNRPN* exons 2–10. This alternative *SNRPN* transcript, the so-called *imprintor*, showed mutations in several AS imprinting-defect families. In contrast, PWS imprinting-defect patients were found to have a mutation affecting *SNRPN* exon 1, which is skipped in the *imprintor* transcript. The *SNRPN* exon 1 region was called the “imprint-switch initiation site.” Dittrich et al. (1996) have proposed that the paternal→maternal imprint switch in the female germ line requires the *imprintor* transcript in *cis* and a *trans*-acting factor specific for the female germ line. In the male germ line, the maternal→paternal imprint switch occurs by default or involves other factors. The model did not make any predictions regarding the fate of the paternal chromosome in the male germ line and the maternal chromosome in the female germ line. These imprints may be retained or may be erased and reestablished (Ferguson-Smith 1996).

We have investigated the segregation of 15q11-q13 haplotypes in nine AS imprinting-defect families, including one previously reported family (family D). Our results suggest that the imprinting defect can have different causes and recurrence risks.

### Subjects, Material, and Methods

Ten AS patients with an imprinting defect, including one sibship (family D), were identified. Seven of these patients are of German origin, and three are of Yugoslav origin. All patients were seen and clinically investigated by one of us, with their mothers present. Clinical details are published elsewhere (Bürger et al. 1996). Informed consent was obtained from the parents.

DNA was extracted, by use of standard procedures, from peripheral blood leukocytes. Methylation at the *D15S63* and *SNRPN* loci was investigated by use of Southern blot analysis, with probes PW71B (*CfoI*+*Bgl*III) (Dittrich et al. 1992) and kb17 (*Bgl*III+*Not*I or *Bgl*III+*Hpa*II) (Saitoh et al. 1997; K. Buiting and B. Horsthemke, unpublished data). Deletion screening was performed by use of quantitative Southern blot analysis, with a battery of probes from the IC (Buiting et al. 1995). Three micrograms of DNA were digested with the appropriate restriction enzymes (New England Biolabs or Boehringer Mannheim), were resolved on 0.7% or 1.0% agarose gels, and were analyzed by Southern blot hybridization. Probes were labeled by use of random oligonucleotide priming and [ $\alpha$ -<sup>32</sup>P]CTP (NEN Dupont). Autoradiography was performed at –80°C, with intensifying screens and Kodak XAR films.

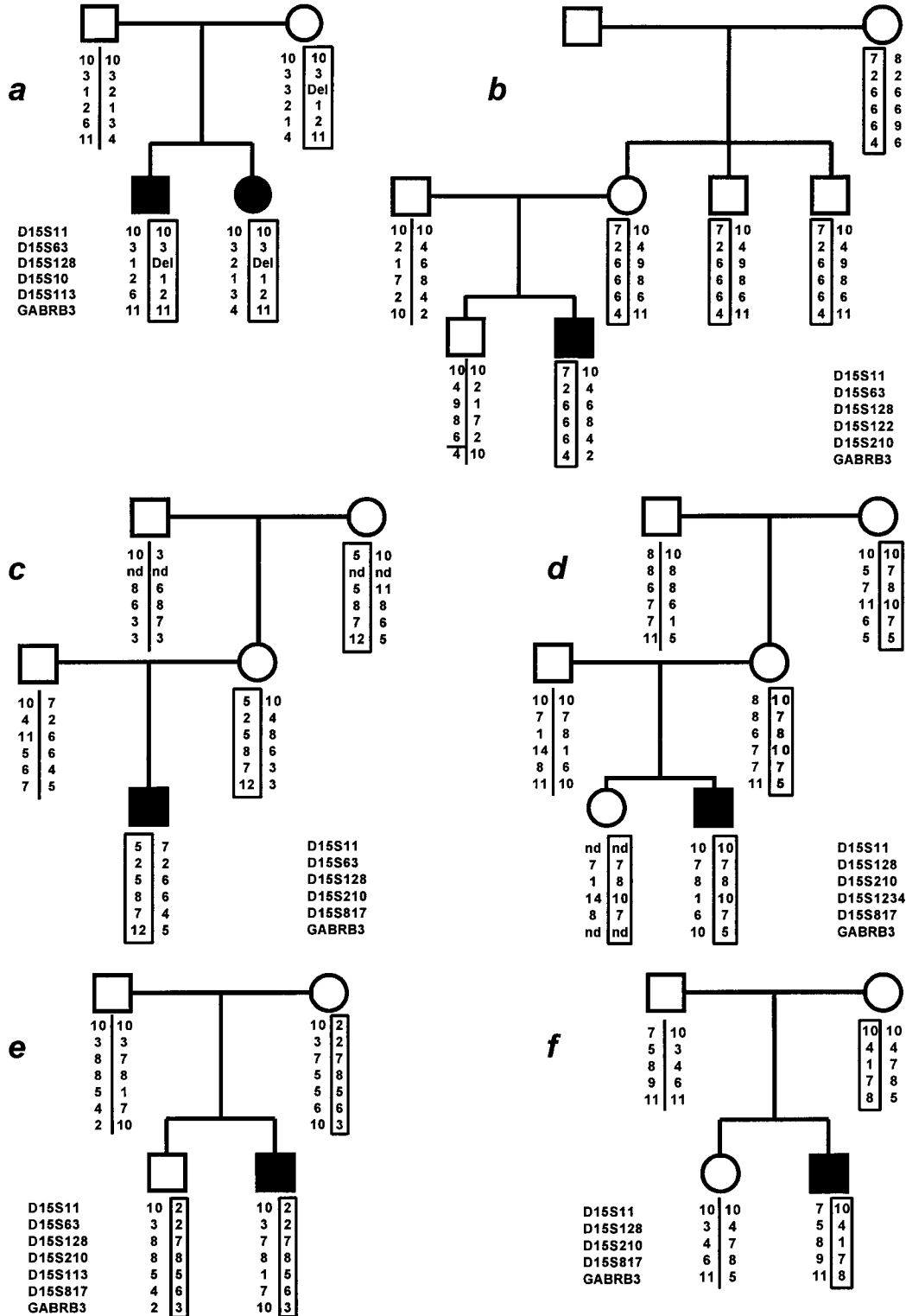
Genotypes were determined at the following microsatellite loci within the AS/PWS region: *D15S10* (Lindeman et al. 1991); *GABRB3* (Mutirangura et al. 1992b); *D15S11*

(Mutirangura et al. 1992a); *D15S63* (Wagstaff et al. 1993); *D15S113* (Mutirangura et al. 1993); *D15S128*, *D15S210*, and *D15S122* (Gyapay et al. 1994); and *D15S1234* and *D15S817* (Genome Database 1996). One of each primer pair was end labeled, either fluorescently (with FAM; TIB Molbiol) or radioactively, by use of [ $\gamma$ -<sup>32</sup>P]ATP (NEN Dupont) and T4 polynucleotide kinase (New England Biolabs). PCR was performed with 20 ng DNA, 10 mM Tris, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 100 or 200  $\mu$ M of each dNTP, 1  $\mu$ M of each primer, and 0.5 unit *Taq* polymerase (Perkin Elmer), in a total volume of 20  $\mu$ l. PCR was performed in a GeneAmp 9600 (Perkin Elmer), with an initial denaturation for 5 min at 94°C. Twenty-eight cycles were run, with denaturation for 20 s at 94°C, annealing for 20 s at a primer-specific temperature, and elongation for 20 s at 72°C. The final elongation at 72°C was 5 min. Annealing temperatures were 53°C for *D15S122*, 56°C for *D15S10*, *GABRB3*, *D15S11*, *D15S113*, and *D15S210*, and 59°C for *D15S63*, *D15S128*, *D15S1234*, and *D15S817*. Fluorochrome-labeled PCR products were run and analyzed on an automatic sequencer (A.L.F., Pharmacia) by use of the Fragment Manager software (Pharmacia). Radiolabeled PCR products were run on denaturing 6% polyacrylamide gels and, following autoradiography, were inspected visually.

Exons and flanking intronic sequences were PCR amplified and were sequenced on both strands. The following primers and conditions were used: exon BD1A, 5'-CAA GCG CAG TTG TAC CAT-3' and 5'-AAA CGA AAG TTG TAA GAC AAT-3' (annealing temperature 50°C); exon BD1B, 5'-CAA GCG CAG TTG TCC TCC-3' and 5'-GAA TGA AAG GCA TTA ATA TAC-3' (annealing temperature 54°C; 1% Triton-X100 was added to the PCR-reaction mixture, and the product was gel purified and reamplified); exon BD1B\*, 5'-GTT GGT GCT GAG GAC AAA AG-3' and 5'-GTG GTC ATG CAC GTA CAC TG-3' (annealing temperature 58°C); exon BD2, 5'-TTC CTT ACT ATG CAT TAA CAC-3' and 5'-AGG AAC ATA AGT GGA ACA G-3' (annealing temperature 50°C); and exon BD3, 5'-GTA CTT CTA TTT TGA ATG ACC-3' and 5'-ATG CAA GTG GAA GGT AAG-3' (annealing temperature 50°C; sequencing reactions were performed at 55°C and 45°C, respectively). PCR products were purified with Microcon-100 microconcentrators (Amicon) and were sequenced with fluorescence-tagged dideoxynucleotides and the *Taq* cycle-sequencing procedure. Sequencing reactions were analyzed on an ABI 373A DNA sequencer.

### Results

We identified 10 AS patients from nine families with an imprinting defect. Biparental inheritance of the AS/PWS region was shown by use of microsatellite genotyping (fig. 1). Since AS results from a mutation on the



**Figure 1** Segregation of chromosome 15q11-q13 microsatellite genotypes, in AS families with an imprinting defect. *a*, Family D. *b*, Family La. *c*, Family Ki. *d*, Family Gr. *e*, Family K. *f*, Family Le. The boxes indicate the haplotype associated with an imprinting defect in the patient. nd = genotype not determined; and del = deleted. The recombination site of the healthy sibling in family La (*b*) is at least 300 kb distal to the *UBE3A* gene (J. Bürger, H. C. Hennies, T. Wessel, E. Burkhard, K. Sperling, and A. Reis, unpublished data).

maternal chromosome, the segregation of the maternal allele of the index patient was analyzed after construction of haplotypes. Two independent methylation tests (with PW71B and *SNRPN*) demonstrated that both chromosomes have a paternal methylation pattern (not shown).

In one family with two affected sibs (family D), we observed a lack of a maternal allele at the *D15S128* locus (fig. 1a). As reported in a previous study, the mother and the two affected children have a deletion of 42 kb in the IC region (Buiting et al. 1995). Thus, *D15S128* is located within the IC. Fine-mapping studies indicate that this microsatellite maps 1 kb centromeric to exon BD2 (K. Buiting, B. Dittrich, and B. Horsthemke, unpublished data). In the other eight patients, no deletion or point mutation affecting the *imprintor* transcript was found.

In three families (W, Ge, and B), only the patients and their parents were available. In another family (Le; fig. 1f), the patient and his healthy sibling inherited different maternal alleles at the IC.

In families Gr (fig. 1d) and K (fig. 1e), an affected sib and an unaffected sib share the same maternal haplotype. Grandparental DNA was available from three families (La, Gr, and Ki). In all of them, the aberrantly imprinted chromosome was found to be of grandmaternal origin (fig. 1b, c, and d).

## Discussion

Imprinting defects offer a unique opportunity to dissect the imprinting mechanism. They contribute not only to AS and PWS (see Introduction) but also to Beckwith-Wiedemann syndrome (Reik et al. 1995), which maps to distal 11p15. The clinical effects of aberrant imprinting are similar to those of other genetic defects, as has been shown for AS and PWS (Bürger et al. 1996; Saitoh et al. 1997). Whereas imprinting defects seem to be rare among PWS patients (<1%), they are relatively frequent among AS patients. We estimate the rate of imprinting defects among all AS patients to be ~6%, since in our laboratory we have identified 10 such patients, as compared with 110 patients with a maternal deletion (J. Bürger, K. Tyler, K. Sperling, and A. Reis, unpublished data), which occurs in 60%–70% of AS patients. Despite important advances in the analysis of this new class of genetic defects—for example, the finding of *cis*-acting mutations in some PWS patients and some AS patients (Buiting et al. 1995; Dittrich et al. 1996; Saitoh et al. 1996)—we are still far from understanding the basic mechanism. Here, we have investigated the segregation pattern of aberrantly imprinted chromosomes in nine AS families.

### Segregation Analysis

In all the families studied, unambiguous microsatellite haplotypes could be constructed. In family D, the af-

ected sibs lack a maternal allele at the *D15S128* locus (fig. 1a). As shown in previous studies (Buiting et al. 1995; Dittrich et al. 1996), the patients and their mother have a deletion of 42 kb that affects two exons of the *imprintor* transcript. By use of methylation analysis with probe Y48.5, which detects one or more differentially methylated *HpaII* sites within the deletion region of family D, it was found that the mother has the deletion in her paternal chromosome (Buiting et al. 1995). These findings suggest that the deletion blocks the paternal→maternal imprint switch in her germ line (Buiting et al. 1995). No deletions or point mutations affecting the *imprintor* exons were found in the other patients. However, we cannot exclude the presence of point mutations, in the promoter region or in intronic sequences, that may impair the expression or splicing of the *imprintor*. The *imprintor* transcript could not be studied directly, because it was not detectable in the tissue samples that were available from the patients.

In two families (Gr and K), the patient and an unaffected sib share the same maternal haplotype (fig. 1d and e). This finding excludes the presence of a familial *cis*-acting mutation. The following explanations are possible:

- (1) The mutation is present in only a fraction of the germ cells carrying this haplotype (i.e., germ-line mosaicism).

- (2) The mutation has occurred in one germ cell only or during early postzygotic development (i.e., a *de novo* mutation). It is an as-yet-undetected structural mutation of the IC, an epimutation (Holliday 1987) resulting from an imprinting error, or a paramutation (Brink 1973) resulting from an interchromosomal transfer of epigenetic states. The latter process is mechanistically related to homologous recombination and probably involves hemimethylated chromatids (Holliday 1987; Colot et al. 1996). In this context, it is interesting to note that the maternal and the paternal chromosomes 15 pair in the late S phase of the cell cycle (LaSalle and Lalande 1996). This pairing occurs specifically at the imprinted 15q11-q13 region and may contribute to increased recombination.

- (3) The imprinting defect is caused by a mutation in *trans* that has impaired the mitotic replication of the maternal imprint, during the postzygotic development of the patient. It should be noted that the presence of a *trans*-acting mutation affecting the maternal germ line can be excluded in families Gr and K, because such a mutation should affect all maternal germ cells or all maternal germ cells carrying the same haplotype, if imprint switching and imprint maintenance involve different factors (see below). On the other hand, a *trans*-acting mutation impairing postzygotic replication of the maternal imprint should affect each of the two maternal

chromosomes—that is, one might expect to find two affected sibs with different maternal haplotypes. So far, the absence of linkage to 15q11-q13 has never been observed in any family with more than one AS patient.

In families La, Ki, and Gr, maternal grandparents were available for segregation analysis. In all of these families, the IC region clearly was inherited from the grandmother (fig 1*b*, *c*, and *d*). This is in contrast to family D and excludes a defect in the paternal→maternal imprint switch in the maternal germ line. As mentioned in the Introduction, the fate of the maternal chromosome in the maternal germ line is unknown. The imprint is either maintained or erased and reestablished. Recently, Szabo and Mann (1995*a*, 1995*b*) have shown in the mouse that *SNRPN* expression is only paternal from the four-cell stage on but is biallelic in the germ line, starting with the arrival of primordial germ cells at the genital ridge. Although these authors cannot exclude that the imprint was bypassed, their data suggest that the imprint was erased and reestablished. The finding of a paternal imprint on the grandmaternal chromosome in the La, Ki, and Gr patients suggests either that the grandmaternal imprint was lost in the maternal germ line or that it was erased and was not reestablished, in the maternal germ line. Both assumptions imply that the paternal imprint is the default setting. Otherwise one would have to assume that the maternal germ line is capable of generating a paternal imprint. Although we cannot exclude this possibility, we consider it to be less likely. The reasons for the imprint-maintenance defect or for the reestablishment defect are unclear, but, as discussed above, several causes are possible.

#### *Implications for Recurrence Risk, Genetic Counseling, and Prenatal Diagnosis*

Since the molecular mechanisms leading to aberrant imprinting seem to be heterogeneous, different recurrence risks need to be considered. Accurate genetic counseling is only possible if the causative mutation is known. Familial *cis*-acting mutations, such as the IC deletion in family D, have a recurrence risk of 50%, and direct detection of the imprinting mutation can be used for prenatal diagnosis (Saitoh et al. 1996). If the mother is a germ-line mosaic for normal and mutant alleles, the recurrence risk depends on the ratio of mutant to normal germ cells. Saitoh et al. (1996) demonstrated that germ-line mosaicism is relatively frequent: In six families with an IC deletion, two maternal germ-line mosaics were identified. Finally, a *de novo* mutation has no increased recurrence risk. Mutations of *trans*-acting factors may be autosomal recessive or autosomal dominant, with recurrence risks of 25% and 50%, respectively.

In many imprinting-defect families, the underlying defect is unknown. These families have a recurrence risk

within a range of 0%–50%, and accurate risk assessment is impossible. In these cases, prenatal diagnosis may be based on DNA methylation. *D15S63* methylation (with PW71) cannot be used for this purpose, because extraembryonic tissues are hypomethylated at this locus (Dittrich et al. 1993; Kubota et al. 1996). Recent data obtained by Kubota et al. (1996), however, suggest that methylation analysis, with *SNRPN*, of amniotic fluid cells and of chorionic villi samples may be a safe test for the prenatal detection of imprinting defects.

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