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ARTICLE

Evidence from skewed X inactivation for trisomy mosaicism in Silver-Russell syndrome

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The finding of maternal uniparental disomy for chromosome 7 (matUPD7) in approximately 7% of Silver-Russell syndrome (SRS) cases has lead to the assumption that imprinted gene(s) on chromosome 7 are responsible for at least some cases. However, the observation in a familial case that both maternal and paternal inheritance of proximal 7p results in an SRS-like phenotype suggests that the causative genes may not be imprinted, and that an extra copy of genes within this region cause SRS. As all cases of complete matUPD7 could have arisen by trisomy rescue, it is possible that undetected trisomy 7 mosaicism contributes towards the phenotype of SRS, and that the matUPD7 seen in some cases is a consequence of trisomy rescue. Previous studies in cases of trisomy rescue for a number of autosomes have shown a strong association with skewed X inactivation in diploid tissues. Thus, we hypothesised that if trisomy mosaicism was involved in SRS, the frequency of skewed X inactivation should be increased in a population of non-matUPD7 SRS patients. Consistent with this hypothesis, results showed a significant increase in the frequency of completely skewed X inactivation in SRS patients (three of 29) when compared to controls (three of 270), suggesting the possible presence of undetected trisomy 7 in SRS patients and/or their placentas.
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Introduction

Silver-Russell syndrome (SRS) is a malformation syndrome characterised by severe pre- and post-natal growth retardation, asymmetry, craniofacial abnormalities, and other more variable features. Although the syndrome is probably heterogeneous in nature,[1] maternal uniparental disomy for chromosome 7 (matUPD7) is associated with approximately 7% of SRS patients,[2–4] leading to the assumption that one or more imprinted genes on chromosome 7 are responsible for at least a proportion of cases. More recently, the identification of duplications of proximal chromosome 7p in SRS patients[5,6] suggests specifically the over-expression of gene(s) located within this region as the pathogenic mutation. However, observations in a familial case that both maternal and paternal inheritance of the region 7p12.1–p13 results in an SRS-like phenotype suggests that the causative genes may not be imprinted.[5] Joyce *et al*.[5] therefore proposed that SRS might result from the presence of an additional copy of proximal chromosome 7p genes, either as a result of submicroscopic duplications of this region, or alternatively from undetected mosaic trisomy of chromosome 7.

Duplications involving a number of candidate genes within proximal 7p have been excluded from 87 SRS probands, (S Mergenthaler, personal communication)[5–7] and thus do not represent a common cause of the disease. In addition, mutation and imprinting analyses of numerous candidate genes have also failed to uncover any significant defects in SRS,[8–13] and thus the underlying genetic cause remains unknown. However, the alternative mechanism

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proposed by Joyce *et al*,[5] namely the possible involvement of mosaic trisomy 7 as a cause of SRS, has not been adequately addressed. Every case of complete matUPD7 could have arisen by trisomy rescue,[14] and trisomy 7 cells, apparently confined to the placenta, have been detected in five matUPD7 SRS cases.[14–16] Thus, it is possible that mosaicism for trisomy 7 contributes towards the phenotype in some cases of SRS, and that the occurrence of matUPD7 is an additional finding caused by the loss of the paternal chromosome 7 during trisomy rescue, which would be expected in one-third of such cases.[17]

Two previous reports have demonstrated that completely skewed X inactivation is frequently observed in the diploid tissues of individuals with mosaic trisomy that has originated from a trisomy rescue event.[15,18] In these cases, the detected trisomic cells are often confined to the placenta, and skewed X inactivation presumably occurs as a result of a reduction in the size of the disomic cell pool contributing to the developing foetus, because of either poor growth by, or selection against, the trisomic cells. Thus, skewed X inactivation can act as a marker of clonality following a trisomy rescue event which has occurred, perhaps in only a single precursor cell, during foetal development. As most cases of matUPD7 probably originate from trisomy rescue, it was hypothesised that if trisomy mosaicism was involved in the aetiology of SRS, the frequency of skewed X inactivation should be increased in a population of SRS patients when compared to controls.

In order to test this hypothesis, an analysis of the X inactivation patterns in 34 female SRS patients of unknown aetiology was performed, and the results compared to those obtained from a large population of normal females of similar age.

Materials and methods

The study population consisted of 34 unrelated female SRS probands of unknown aetiology, described previously.[4,14] The population had a mean age at sampling of 8.9 years, standard deviation 9.1, range 0.8–37, while the mean maternal age at birth was 28.6 years, standard deviation 5.5, range 19–39. A diagnosis of SRS was based on the following criteria: severe pre- and post-natal growth retardation, characteristic facial features, facial, trunk, or limb asymmetry, and a variety of other variable features.[19] High resolution cytogenetic examination of peripheral blood lymphocytes revealed a normal karyotype in each case. DNA was extracted from peripheral blood, and analyses of 18–20 polymorphic markers along the length of chromosome 7 in each proband and their parents found no evidence of UPD7, indicating normal biparental inheritance of chromosome 7 in each case.[4,14]

X inactivation ratios in peripheral blood were determined using the *AR* gene PCR assay as described previously,[20] which relies upon the differential methylation of the active and inactive X chromosomes at specific sites. Briefly, genomic DNA was digested with the methylation sensitive restriction enzymes *Hpa*II and *Cfo*I. Subsequent PCR using primers which span both a highly polymorphic repeat sequence and differentially methylated *Hpa*II/*Cfo*I sites in exon 1 of the *AR* gene only amplifies products from the undigested inactive X, and X inactivation ratios are then determined by measuring the relative intensities of the two alleles produced by PCR.

X inactivation ratios in 121 normal controls of similar age to the SRS cohort (mean age 12.1 years, standard deviation 8.8), and of a second elderly population (mean age 72.2 years, standard deviation 7.1) have been described previously.[20]

X inactivation ratios in two SRS patients with matUPD7 were also analysed. SR38 and SR57 were both heterodisomic, resulting from maternal meiotic non-disjunction of chromosome 7 followed by trisomy rescue.[3,4]

Fisher's exact test (StatXact v.3 software) was used to compare the proportion of females with extreme or completely skewed X inactivation ratios between test and control populations. As the hypothesis being tested predicted both the direction and magnitude (see Discussion) of the increased frequency of skewed X inactivation in SRS individuals compared to controls, a 1-tailed test was used to calculate *P*-values, although the use of 2-tailed tests gave similar results.

Results

Twenty-nine of the 34 SRS probands studied were informative for the *AR* assay. Extremely skewed X inactivation (ratios $\geqslant 90:10$) was observed in five of these 29 (17.2%), compared to eight of 121 (6.6%) controls of similar age (not significant, $P=0.079$). Additionally, completely skewed X inactivation (ratios 100:0) was observed in three of these five SRS probands (representing 10.3% of the cohort), compared to only three of 270 (1.1%) normal controls of all ages[20] (statistically significant, $P=0.014$). Each result was generated from a mean of two independent PCR reactions, which were closely concordant in most cases (mean difference 3.4%). Typical results of the *AR* assay are shown in Figure 1, while Figure 2 shows the relative distributions of X inactivation ratios obtained in non-UPD7 SRS patients and controls.

Analysis in the two SRS cases with matUPD7 showed X inactivation ratios of 86:14 and 85:15.

Discussion

Maternal uniparental disomy for chromosome 7 is associated with approximately 7% of SRS cases,[2–4] leading to the suggestion that one or more imprinted genes on chromosome 7 are responsible for the disease. However, as it is likely that most cases of matUPD7 arise by trisomy 7 rescue,[14] it is unclear whether the SRS phenotype is due to the presence of matUPD7 alone.

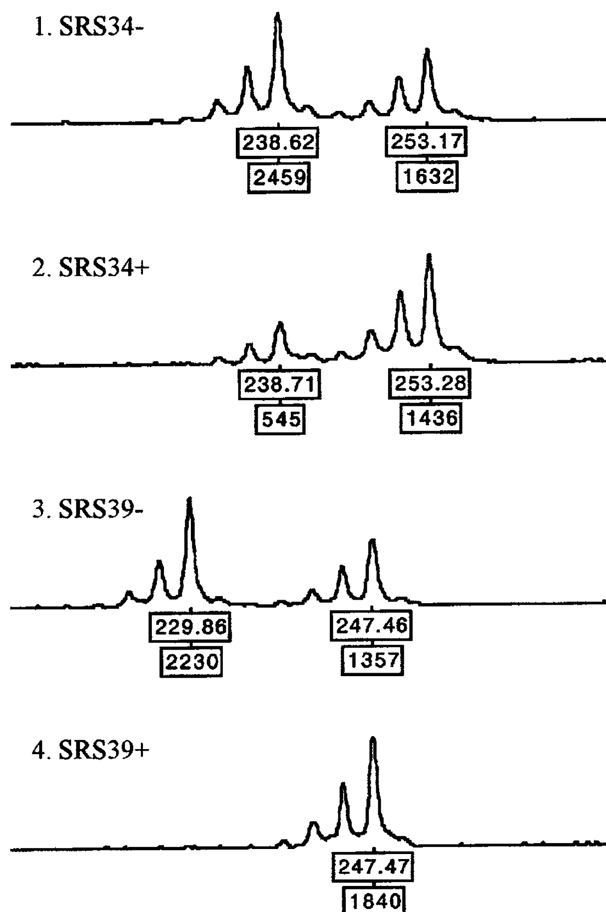

Figure 1 Typical results gained using the *AR* X inactivation assay. (−) denotes PCR of undigested DNA, (+) denotes PCR using DNA digested with *Hpa*II/*Cfo*I. SRS34 (tracks 1 and 2) has moderately skewed X inactivation (ratio 80 : 20), indicated by a reduced intensity of the smaller allele relative to that in the undigested track. SRS39 (tracks 3 and 4) has completely skewed X inactivation, indicated by the complete absence of one allele following digestion with *Hpa*II/*Cfo*I. Figures below each allele represent size in base pairs and peak height respectively.

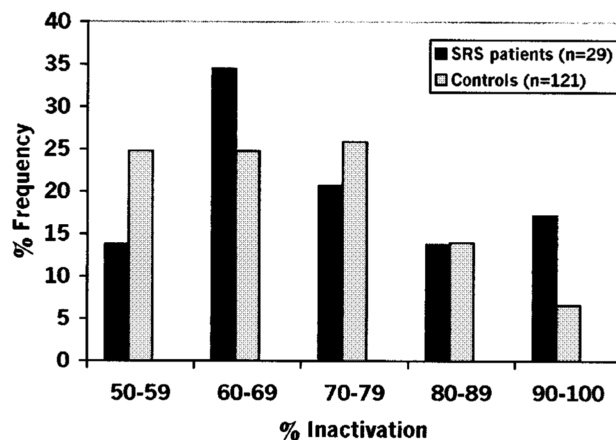

Figure 2 Relative distributions of the X inactivation ratios obtained in non-UPD7 SRS patients and controls. X inactivation patterns are expressed as the percentage ratio of the predominantly inactive allele to the predominantly active allele and are displayed in 10% intervals.

Based on previous reports demonstrating an increased frequency of skewed X inactivation in cases of trisomy rescue,[15,18] this study has set out to determine the possible role of trisomy 7 in SRS by analysing the frequency of skewed X inactivation in a cohort of 29 non-UPD7 SRS patients. Consistent with our hypothesis, results show an increase in the frequency of extremely skewed X inactivation in SRS (17.2%) compared to age-matched controls (6.6%), although this difference does not reach statistical significance (P=0.079). However, this is probably a result of the relatively small number of SRS cases analysed, and the low P-value is strongly suggestive that a significant effect would be observed had a larger cohort been studied. More-specifically however, the frequency of completely skewed X inactivation in the SRS population is some 10-fold higher than that found in controls (P=0.014), consistent with the possibility that in some of the cases analysed, trisomy rescue has occurred. Complete skewing in the diploid tissues of these individuals suggests that they are derived from a single, or very small number of, progenitor cells in which trisomy rescue occurred in the developing embryo, and predicts the presence of undetected trisomic cell lines, either confined to the placenta or within other somatic tissues.[15,18] These data are therefore consistent with the hypothesis that mosaicism for trisomy 7 exists in some cases of SRS, and suggests that the occurrence of matUPD7 seen in a proportion of cases is a consequence of trisomy rescue. However, these results do not exclude a role for imprinting in SRS, as indicated by a proband with segmental matUPD7 confined to 7q31-qter which would not be associated with trisomy 7,[21] and by several other SRS patients with abnormalities of chromosome 7 (D Monk, personal communication).

The proportion of SRS cases in which both extreme and complete skewing was observed is similar to that predicted from the 7% incidence of matUPD7 in SRS, as follows. As one-third of cases of trisomy rescue will result in UPD, it can therefore be estimated that trisomy 7 occurs in some 21% of SRS patients. This study has examined only those individuals with biparental inheritance of chromosome 7, representing 14% of total cases, and previous studies of trisomy rescue have found that approximately two-thirds of such cases where the trisomy is of meiotic origin show completely skewed X inactivation.[15,18] Approximately half of SRS cases with matUPD7 have arisen from a trisomy of meiotic origin.[2–4] Thus, some 5% of non-UPD SRS females should exhibit skewing as a result of trisomy rescue, in addition to the 'background' incidence of 7% found in age-matched

controls, totalling 12%, statistically similar to that observed here (17%). However, there did not appear to be an association of increased maternal age with SRS, which would be expected in those cases where the trisomy was of meiotic origin.[22] In particular, the mean maternal age for four of the five individuals with extreme skewing for whom data was available was 28 (range 21–34), although the small sample size made firm conclusions impossible. In addition, skewed X inactivation was not observed in three cases of maternal heterodisomy 7.[15] As these arose by trisomy rescue complete skewing might be expected in these cases, but once again firm conclusions cannot be drawn from such small numbers.

The possible association of trisomy 7 with SRS is similar to that seen in trisomy 16. All cases of trisomy 16 are of maternal meiotic origin,[23] and mosaicism for trisomy 16, often confined to the placenta, is associated with matUPD16 in many instances. However, in some cases there is poor correlation between matUPD16 and intrauterine growth retardation (IUGR) or abnormal pregnancy outcome.[18] Instead, it is likely that the IUGR and foetal abnormalities associated with matUPD16 are partly due to the effects of high levels of trisomy 16 in the placenta, and their possible persistence in some somatic tissues.[24]

Although the observed matUPD and structural abnormalities of chromosome 7 in SRS strongly suggest the specific involvement of chromosome 7, the finding of skewed X inactivation in SRS is also compatible with alternative explanations other than trisomy 7 rescue. Many X-linked syndromes are associated with skewed X inactivation, and X-linked inheritance has been suggested for some familial SRS cases.[25] While there is currently no direct evidence to support the involvement of the X chromosome in SRS, as the syndrome is undoubtedly genetically heterogeneous, our observations may also indicate an X-linked cause in a proportion of cases. Alternatively, the increased frequency of skewing in SRS could indicate the involvement of trisomy rescue for other autosomes, besides chromosome 7. A previous case of confined placental mosaicism for trisomy 16 showed asymmetric IUGR with a relatively large head, similar to that seen in many SRS cases,[26] and trisomy 18 mosaicism has also been reported in a child with features of SRS.[27] However, UPD for many autosomes, particularly those which are frequently observed as aneuploids, has previously been excluded from 70 SRS probands.[3,4] Thus, while trisomy mosaicism for chromosomes other than 7 may be responsible for a small number of cases, it seems unlikely to play any significant role in the disease.

Previous studies have failed to detect the presence of trisomy 7 cells in the blood or fibroblasts of SRS patients,[28] although the techniques used in some cases have been limited by low sensitivity.[3,4] In addition, high-level mosaicism or complete trisomy 7 is a lethal condition, with different phenotypic features from those seen in SRS.[29] Therefore, SRS might be characterised by distinct patterns of mosaicism for trisomy 7 cells, confined to certain tissues. Somatic mosaicism for trisomic cells might account for the asymmetrical growth and patchy skin pigmentation which is observed in some individuals with SRS, and mosaicism for a ring chromosome 7 has been reported in one case of SRS.[30] While such asymmetry might be predicted in individuals with mosaicism, only one of the three SRS individuals in which we found completely skewed X inactivation showed asymmetrical growth. In contrast to the formation of matUPD7, patUPD7, which is compatible with normal growth, probably results from monosomy rescue,[31,32] and would not be associated with trisomy.

In summary, the increased frequency of completely skewed X inactivation found in SRS patients with biparental inheritance of chromosome 7 is consistent with the possible presence of trisomy 7 confined to certain tissues. Although most probably rare, these data suggest trisomy 7 mosaicism may be present in some SRS patients. Whether this contributes to the phenotype of SRS requires further experimentation.

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