ELECTRONIC LETTER

Molecular screening for Smith-Magenis syndrome among patients with mental retardation of unknown cause

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Smith-Magenis syndrome is a rare, multiple congenital anomaly/mental retardation syndrome (MCA/MR) associated with interstitial deletion of chromosome 17p11.2. Smith *et al*[1] first described this condition in two patients. To date, more than 150 cases have been described. Patients with SMS display a variable expression of subtle dysmorphic features, MR, short stature, brachydactyly, visual and auditory impairment, behavioural problems, sleep disturbance, and cardiac and renal malformations.[2 3] Almost all cases are de novo, non-mosaic, and of either maternal or paternal origin.[4 5] Although the abnormalities associated with SMS are well described, their subtlety and variable expression make clinical diagnosis often difficult, particularly in neonates and young infants.[6 7]

The SMS deleted region ranges in size from <1.5 Mb to 9 Mb and the majority of patients have a ~5 Mb deletion (~10-11% of chromosome 17).[4 8] In general, there is no obvious correlation between the size of the deletion and the severity of the phenotype.[8] In the majority of cases, this deletion is visible on careful routine cytogenetic analysis. Despite this, in several cases the deletion has been missed.[7 9] Using a FISH probe specific for SMS has enhanced detection of the syndrome, especially in equivocal cases.[10–12] Elsea *et al*[13] reported a patient with a typical SMS phenotype and a normal karyotype at 650 band resolution. This patient was subsequently diagnosed with SMS by FISH.[12]

Current estimates of the incidence of SMS, based on ascertaining cases in genetics centres, are between 1 in 25 000[4] and 1 in 50 000[14] births. Because the diagnosis of SMS can be missed both clinically and cytogenetically, it is generally believed that this syndrome might be underdiagnosed. In this study, we screened a large population of patients with MR/DD of unknown cause to determine the frequency of SMS in this group and to estimate its prevalence in the general population.

Key points

- Smith-Magenis syndrome (SMS) is a multiple congenital anomaly/mental retardation syndrome associated with deletion 17p11.2. The prevalence of this syndrome in the general population and its frequency among patients with mental retardation/developmental delay (MR/DD) is unknown. It is generally believed that this syndrome might be underdiagnosed because of its phenotypic and cytogenetic variability.
- In this study we screened a large number of patients with unexplained MR/DD to determine the frequency of SMS among this population. The screening involved Southern blotting and dosage comparison between non-polymorphic markers, SMS deletion specific and control probes. All samples suspected to have the SMS deletion were subjected to confirmatory testing.
- Of 1205 patients successfully screened, we detected two SMS patients. The prevalence of SMS in the general population is estimated.

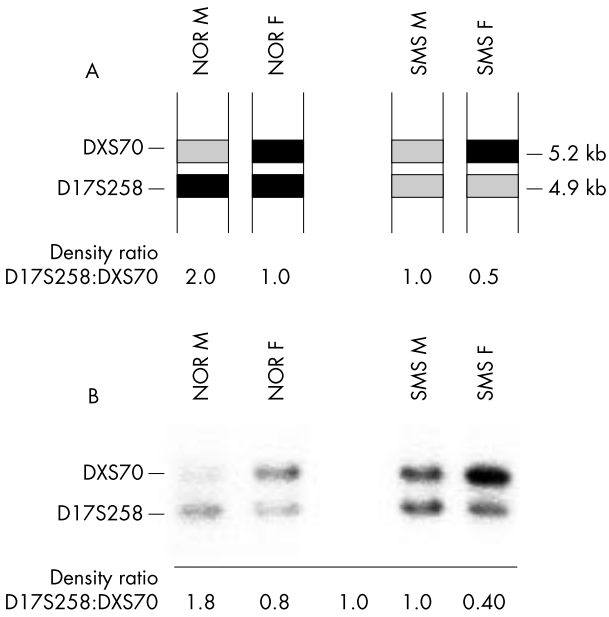


Figure 1 Densitometric analysis of D17S258:DXS70 allelic ratios of SMS and normal subjects. (A) Diagram of the predicted banding patterns using the two *Eco*RI non-polymorphic probes; the X chromosome control probe, DXS70, corresponds to the 5.2 kb band and the SMS deletion specific probe, D17S258, corresponds to the 4.9 kb band. Grey shading indicates a single copy of the probe, black shading is indicative of a double copy. The D17S258/DXS70 density ratio for each lane is shown at the bottom. (B) Southern blot of DNA from normal male and female controls and male and female SMS patients digested with *Eco*RI and cohybridised with DXS70 and D17S258 probes. The D17S258:DXS70 allelic ratios in normal males and females were approximately twice that of their SMS counterparts, illustrating the ability of this screening method to detect the SMS deletion.

MATERIAL AND METHODS

Study population

A total of 1618 DNA samples were screened in this study, which was approved by the Queen's University Research Ethics Board.[15] These samples were referred during the period between 1996 and 1999 for FRAX testing.

Screening strategy for detecting SMS deletion

A several stage screening strategy for detection of SMS was formulated using available patient material. The screening

Abbreviations: SMS, Smith-Magenis syndrome; MCA/MR, multiple congenital anomaly/mental retardation; MR, mental retardation; DD, developmental delay

Table 1 DNA markers used for molecular screening by Southern hybridisation

Probe	Locus	Location	Enzyme	Allele size (kb)	Heterozygosity
p1516-R4	D17S258	17p11.2	*Eco*RI*	4.9	0%†
p18-55	DXS70	Xp22.3	*Eco*RI*	5.2	0%†
pYNZ22	D17S5	17p13.3	*Eco*RI/*Bss*HII*	3.05–4.20	83.5%†

Data obtained from GDB™ Human Genome Database (database online).
*Additional digestion with *Bss*HII did not alter the allele size or heterozygosity.
†Present study.

Table 2 DNA markers used for PCR analysis of SMS deletion interval

Marker	Sequence name	Location*	Heterozygosity	Product size (bp)	Label
D17S1857	AFM357yg9a/m	Distal to common deletion	65.0%	177–187	HEX
D17S2206	YL63-64	Within deletion, distal to middle SMS-REP	91.7%*	141–165	6-FAM
D17S2207	YL601-602	Within deletion, distal to middle SMS-REP	42.0%*	135–155	HEX
D17S805	AFM234ta1a/m	Within deletion, proximal to middle SMS-REP	58.8%	216–228	6-FAM
D17S841	AFM238vb10a/m	Proximal to common deletion	65.4%	253–273	HEX

Data obtained from GDB™ Human Genome Database (database online).
*Potocki *et al*.[3]

involved dosage comparison between SMS deletion specific (D17S258) and X chromosome control (DXS70) markers. Initially, Southern blots, originally prepared for FRAX testing and containing *Eco*RI/*Bss*HII digested DNA were lightly stripped from the FRAX hybridisation and then cohybridised with radiolabelled D17S258 and DXS70 probes. Because these markers are non-polymorphic for the enzyme(s) used, each probe produced a single band. These probes were chosen because the resultant bands were close in size (D17S258 = 4.9 kb band, DXS70 =5.2 kb band), which facilitated dosage comparison. Normal males (46,XY) display two copies of the D17S258 band relative to a single copy of DXS70. Normal females (46,XX) display two copies of each band. SMS males would display only a single copy of the D17S258 allele and a single copy of the X chromosome allele, while SMS females would display a single copy of the D17S258 allele and two copies of the DXS70 allele. This method was validated before screening using normal male and female controls and SMS patients, equal dpm of each labelled probe, and variable exposures for each blot to ensure optimal representation of signal intensities (fig 1). For some experiments, another chromosome 17 probe (D17S5) was also included for dosage control.

In the second stage, patients who deviated from the expected banding patterns and were suspected of having the SMS deletion were subjected to a repeat of the screening assay, using their banked DNA and new Southern blots. Finally, patients consistently exhibiting suspicious results were investigated further using confirmatory testing by FISH and/or PCR microsatellite genotyping. Characteristics of the markers used in this study are listed in tables 1 and 2.

Southern blot analysis and densitometry

Southern blotting was performed as described previously.[16] A total of 50 ng of probe insert was labelled with 50 µCi α-(^{32}p) dCTP (3000 Ci/mmol) by random priming according to the manufacturer's instructions (Random Primers Labeling System, GIBCO BRL). Blots were hybridised in 1 mol/l NaCl, 1% SDS, 10% dextran sulphate, and 0.1 mg/ml salmon sperm DNA at 65°C overnight. Probe D17S258 required preassociation with human placental DNA (1 mg for each 50 ng labelling reaction) for one hour at 65°C.[17] Equal counts of each probe were added to the hybridisation mixture, to approximately 10^5-10^6 dpm/ml hybridisation solutions. Labelling reactions were scaled up depending on the number of blots to be hybridised. Blots were rinsed with 2 × SSC at room temperature, washed twice for 20 minutes each in 2 × SSC, 0.1% SDS at 65°C, followed by a stringent wash in 0.1 × SSC, 0.1% SDS at 65°C for 20 minutes.[17]

Band intensity indicating the number of copies for each probe was initially estimated visually. Densitometry was performed using scanned autoradiographs into Corel Photo-Paint software version 9 as described previously.[18] To estimate the number of copies of the SMS allele, the intensity of the autosomal band was divided by the intensity of the X chromosome band.

To minimise the risk of missing any SMS patients, all samples, which showed even minimal deviation from normal band intensity patterns, were selected for further evaluation.

Fluorescence in situ hybridisation (FISH)

Patients who consistently exhibited an unexplainable dosage difference on repeat screening were contacted through their referring physicians to obtain fresh blood samples for FISH. Metaphase FISH analysis was performed according to the manufacturer's protocols using a Vysis HYBrite™ Hybridization System and the Oncor D17S258 Smith-Magenis Chromosome Region Probe or the dual-colour Vysis Locus Specific Identifier Smith-Magenis Microdeletion Probe.

PCR analysis of DNA markers within and flanking the SMS deletion region

PCR heterozygosity analysis was performed for cases where a fresh blood sample could not be obtained, or repeat screening was not adequately performed owing to poor DNA quality. Fluorescent labelled primers for highly polymorphic sequences within and flanking the SMS common deletion were used for DNA amplification (table 2). Normal subjects were expected to display two alleles at most loci tested, while SMS patients were expected to be hemizygous for all loci within the deletion interval. Amplified fragments were electrophoresed on a 5% denaturing polyacrylamide gel in an ABI 373 DNA Sequencer and recorded by computer assisted laser. The forward primer of each pair, end labelled with either 6-FAM (blue) or HEX (green) dye, was synthesised by the University of Calgary Core DNA and Protein Services DNA Synthesis Laboratory (Calgary, AB). The unlabelled reverse primers were synthesised either by the same laboratory or by Cortec DNA Services Laboratory (Kingston, ON). DNA sequences for all primers were obtained from the Human Genome Database (GDB™ database online). Amplifications were performed in a PTC-100 thermocycler with a Hot Bonnet heated lid, in 1 × PCR buffer (200 mmol/l Tris-HCl, pH 8.4, 500 mmol/l KCl)

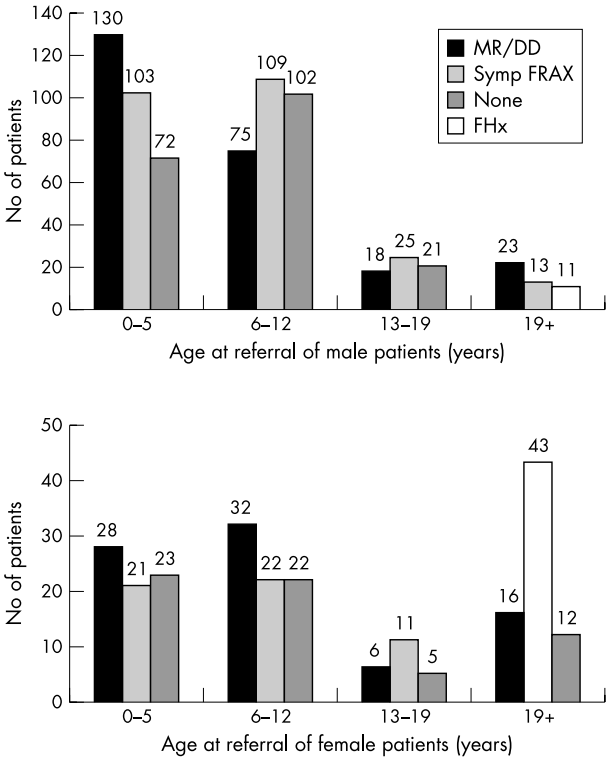


Figure 2 Reasons for referral for patients screened in this study by age and gender. MR/DD = mental retardation/developmental delay. Symp FRAX = symptoms of FRAX. None = no reason stated. FHx = family history of FRAX or mental retardation/developmental delay.

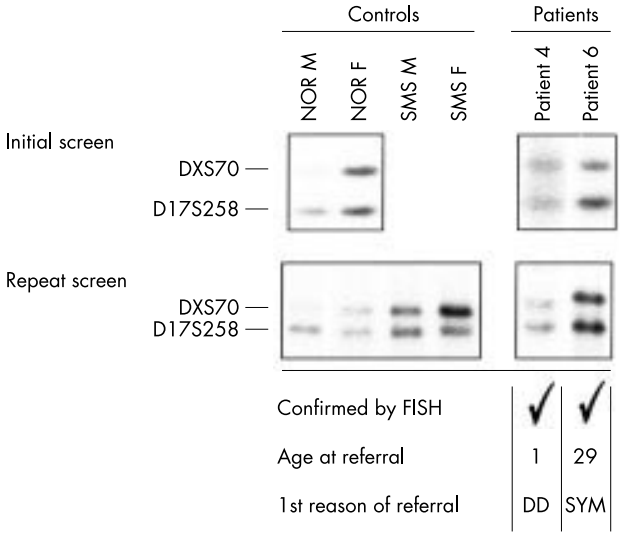


Figure 3 Smith-Magenis syndrome (SMS) patients detected in this study. Southern blots of DNA from SMS patients detected in this study digested with *Eco*RI +/- *Bss*HII and hybridised with DXS70 and D17S258 probes. Confirmatory testing performed and referral information is listed under the appropriate column at the bottom of the figure. NOR M = normal male. NOR F = normal female. SMS M = SMS male. SMS F = SMS female. SYM = symptoms of FRAX. DD = developmental delay.

with 1.5 mmol/l $MgCl_2$, 250 µmol/l each dNTP, 20 pmol of each primer, 30-200 ng genomic DNA, and 2.5 U of Platinum *Taq* DNA polymerase (Gibco BRL) in a 50 µl reaction volume. For D7S2206 and D17S2207 primers, 10% DMSO was added to the mixture. The mixture was denatured for five minutes at 95°C, then amplified for 10 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for one minute, followed by 15-25 cycles at 89°C for 30 seconds, 55°C for 30 seconds, and 72°C for one minute, and a final extension at 72°C for seven minutes. Amplifications were performed and stored at 4°C in the dark to prevent dissipation of fluorescent signals. PCR products that exhibited strong bands on a 2% agarose test gel were diluted 1:20 for 6-FAM and 1:10 for HEX as recommended by the manufacturers before electrophoresis. A DNA ladder, fluorescently labelled with ROX dye (red), was run in each lane to provide an internal size standard. Data were analysed using ABI GeneScan Analysis v2.1.

RESULTS

Age and sex of the screened population

A total of 1618 samples from 1278 patients were screened. Data on 73 patients could not be scored because of poor DNA quality. Of the 1205 patients successfully screened, 879 were male and 326 were female. Patient ages at the time of referral ranged from under a year to 74 years. There was no information about date of birth available for 35 males and 36 females.

Reasons for referral

During the initial screening, no information, with the exception of gender, was known about these patients. After screening was completed, reasons for referral were collected from the referral sheets that contain very limited information. MR/DD was the most common reason for referral among all age groups and both sexes, with the exception of females over 19 years of age, for which FRAX carrier testing was the main reason for referral. The reason for referral was not provided in 27% of cases. Fig 2 summarises the most common reasons for referral of the screened population by gender and age group.

Molecular analysis

Hybridisation with D17S258 and DXS70 probes

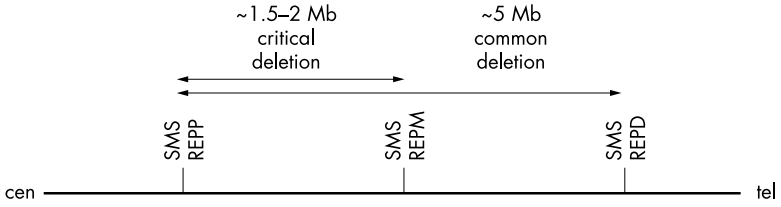
Both D17S258 and DXS70 markers proved non-polymorphic for *Eco*RI/*Bss*HII, producing a single band each. Evaluation of D17S258:DXS70 ratios identified 111 patients (60 males and 51 females) who deviated from the expected sex related patterns and they were selected for further evaluation.

Hybridisation with D17S258 and D17S5 probes

To confirm the dosage imbalance detected in the previous screening, we rehybridised the Southern blots with the SMS specific probe D17S258, and a different control probe D17S5. We observed that the D17S5 probe is highly polymorphic with *Eco*RI/*Bss*HII. The observed polymorphism was a variable number tandem repeat with alleles ranging in size from 3.05 to 4.2 kb and 83.5% heterozygosity (among 278 chromosomes tested). This marker, because of its variable allele size and intensity, was not suitable for dosage comparison with the SMS allele.

Repeat screening of suspicious samples using newly prepared Southern blots

Of the 51 females selected for retesting, two were excluded as their samples showed normal ratios on several other repeated blots. A third DNA sample was resistant to *Eco*RI digestion and was held for evaluation by PCR. Of the 60 male samples identified, 14 were excluded from further screening, eight because of a finding of an alternative diagnosis for the dosage imbalance, two because of having normal results on other repeated blots, and four because no DNA samples were available. The remaining 94 patients (46 males, 48 females) were rescreened. Seventy-two cases clearly exhibited normal patterns and were excluded from further testing. Fifteen samples were uninterpretable owing to poor DNA quality, seven of them were from asymptomatic patients who were referred for FRAX carrier testing, and the remaining eight were held for



Sample	D17S1857	D17S2206	D17S2207	D17S805	D17S841
Normal male	180	149 151	127 145	216 222	264 268
Normal female	180	145 155	127	224 226	264 268
SMS male	182	155	127	216	268 270
SMS female	184	155	127	224	266 270
Patient 6 (SMS)	180 182	–	145	216	264 268
Patient 4 (SMS)	182	151	127	224	268
Patient 1	182 184	151 157	127	224	268
Patient 2	180 182	147 153	147 159	226	268 270
Patient 3	184	159 153	127	224 228	264 268
Patient 5	178 180	151 153	145	–	268
Patient 7	178 182	145 151	127	216	266 268
Patient 8	178 180	145 153	–	216	268
Patient 9	180	149 151	127	224	266 268
Patient 10	–	147 153	127	216 224	266
Patient 11	178 180	147 151	127	222 224	268
Patient 12	–	145 155	145	216 224	–

Figure 4 Alleles detected at loci within and flanking the SMS deletion for normal and SMS controls and 12 patients screened.

PCR analysis. Only seven of the 94 retested samples (four males, three females) were determined by dosage analysis to warrant further testing.

FISH for SMS deletion

FISH was performed on fresh blood samples obtained from five of the seven suspected patients. The diagnosis of SMS was confirmed in two patients (patients 4 and 6). Fig 3 summarises the results and referral information for these patients.

PCR analysis of DNA markers in SMS deletion region

A total of 16 DNA samples were investigated by PCR genotyping. All SMS cases showed a single allele for all markers within the deletion interval, whereas the normal controls showed heterozygosity of one or more markers within the SMS deletion region. All other patients showed allelic heterozygosity for at least one of the markers within that region, indicating that none of these patients had the SMS deletion, as it is currently defined. Fig 4 summarises these results and illustrates the detected alleles for all tested samples.

Other diagnoses identified among the screened population

Besides the SMS deletion, the dosage imbalance between the X chromosome and SMS markers could be explained by other mechanisms, such as X chromosome aneuploidy, incorrect sex assignment, or rearrangement of the X or chromosome 17. In fact, eight patients with Klinefelter syndrome (47,XXY)[19] and one patient with incorrect gender assignment, because of clerical error, were identified among the screened population. Figure 5 summarises the results of our screening protocol.

DISCUSSION

Our novel screening method was based on dosage comparison between the SMS specific allele and a control allele on the X chromosome. Although it had its limitations, this method was significant not only in defining the frequency of SMS among patients with MR/DD of unknown cause, but also because it identified other genetic abnormalities among this population.[19]

Frequency of SMS among patients with MR/DD

Barnicoat *et al*[20] reviewed 544 cases referred over a four year period for FRAX cytogenetic testing and found one case of

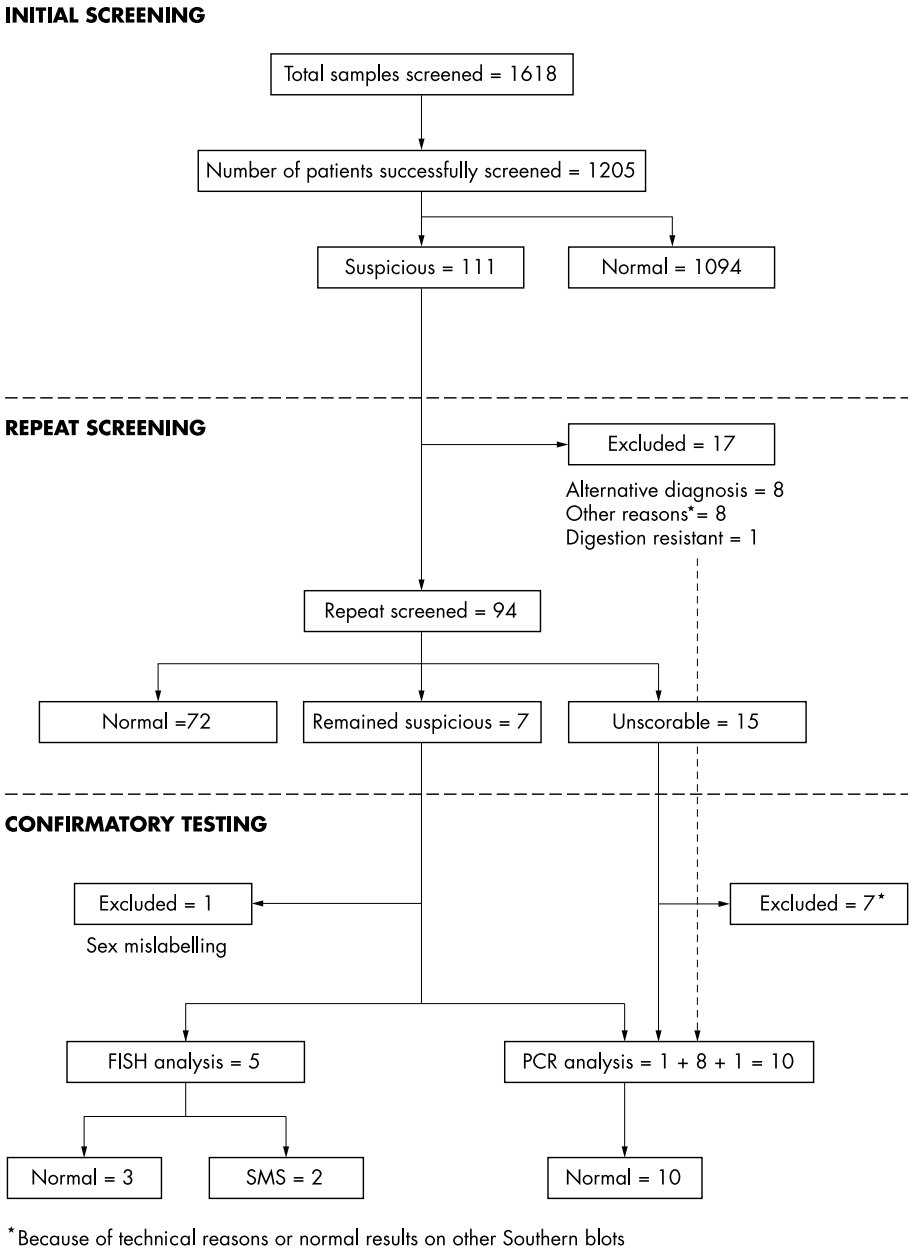


Figure 5 Summary of Smith-Magenis syndrome (SMS) screening protocol. Flow diagram summarising the results of the three stages of the molecular screening of 1618 DNA samples for SMS. A total of 1205 patients were successfully screened for the SMS deletion by dosage comparison between SMS specific and control probes. Repeated hybridisation and confirmatory testing (including FISH and PCR deletion analysis) identified two cases of SMS, eight cases of Klinefelter syndrome, and one sex mislabelled case.

SMS. Behjati *et al*,[21] in the same centre, detected four SMS cases among 1672 of these patients. No details were mentioned about these patients. Both of these estimates were based on retrospective evaluations of previous FRAX cytogenetic analyses and none of them was confirmed by FISH or other methods.

In our study, if we exclude the 67 normal subjects who were referred for FRAX carrier testing, we have detected two SMS patients among 1138 subjects with MR/DD, giving a frequency of 1 in 569. This is consistent with the previous estimates reported by Barnicoat *et al*[20] and Behjati *et al*[21] and suggests that cases of SMS undetectable by careful cytogenetic evaluation are probably rare. However, based on previous reports and our own experience, cases of SMS can still be missed by routine karyotype. In fact, both patients detected in this study had been missed on both clinical and cytogenetic evaluations. This might suggest that SMS patients could be missed early in life when features are subtle and are more likely to be detected later with the evolution of the phenotype.

Prevalence of SMS among the general population

As the prevalence of mental retardation/developmental delay among the general population is approximately 3% and 30-50% of patients with MR/DD are without a specific diagnosis,[22–29] our 1138 patients with MR/DD of unknown cause would be drawn from a general population of approximately 75 866 to 126 443 subjects, making the prevalence of SMS among the general population between 1 in 37 933 and 1 in 63 222. This is consistent with previous estimates.[4 14]

If we exclude the 27% of the samples tested with no reason for referral, the prevalence of SMS among the general population and its frequency among the MR/DD population would be substantially higher. However, we believe this would be an

artificial inflation, as over 85% of these patients are children under the age of 12, and they would not have been referred for FRAX testing if they were asymptomatic. Still, one must consider the possibility that our results are underestimates of the prevalence of SMS. It is possible that we did not detect SMS cases with atypical deletions that did not include D17S258, our screening probe. However, this is highly unlikely since only a single such case has been reported to date.[13] A second possibility is that we may have failed to detect cases in our initial screen, that is, false negative results. Again we feel this is unlikely since we intentionally lowered the threshold of selecting suspicious samples significantly to include even those with slight deviation from the expected dosage pattern, as shown by the large number of samples selected from the first screening that were eliminated by repeat testing. More importantly, we detected all cases of X chromosome aneuploidy in the screened population and a case of sex mislabelling that can also lead to deviation from the sex expected dosage pattern. This shows the ability of our screening to detect such dosage imbalances. We also acknowledge the fact that not every patient with MR/DD is sent for FRAX testing and that we may have missed SMS patients who were not obviously delayed and therefore not referred for FRAX testing.

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