LETTER TO JMG

Genotype–phenotype correlation of 30 patients with Smith-Magenis syndrome (SMS) using comparative genome hybridisation array: cleft palate in SMS is associated with larger deletions

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Background: Smith-Magenis syndrome (SMS) is rare (prevalence 1 in 25 000) and is associated with psychomotor delay, a particular behavioural pattern and congenital anomalies. SMS is often due to a chromosomal deletion of <4 Mb at the 17p11.2 locus, leading to haploinsufficiency of numerous genes. Mutations of one of these gemes, *RAI1*, seems to be responsible for the main features found with heterozygous 17p11.2 deletions.

Methods: We studied DNA from 30 patients with SMS using a 300 bp amplimers comparative genome hybridisation array encompassing 75 loci from a 22 Mb section from the short arm of chromosome 17.

Results: Three patients had large deletions (10%). Genotype–phenotype correlation showed that two of them had cleft palate, which was not found in any of the other patients with SMS ($p<0.007$, Fisher's exact test). The smallest extra-deleted region associated with cleft palate in SMS is 1.4 Mb, contains <16 genes and is located at 17p11.2-17p12. Gene expression array data showed that the ubiquitin B precursor (*UBB*) is significantly expressed in the first branchial arch in the fourth and fifth weeks of human development.

Conclusion: These data support *UBB* as a good candidate gene for isolated cleft palate.

Smith-Magenis syndrome (SMS) [MIM 182290] is a rare (prevalence 1 in 25 000) syndrome exhibiting multiple congenital anomalies and mental retardation, with distinctive behavioural characteristics, sleep disturbance and dysmorphic features, associated with a heterozygous interstitial deletion of chromosome 17p11.2.[1–3] Most patients have the same interstitial genomic deletion of around 4 Mb at chromosome 17p11.2, comprising 20 expressed genes.[4–6] Heterozygous frameshift mutations of the *RAI1* gene, leading to protein truncation, is likely to be responsible for the majority of the SMS features, but other deleted genes in the SMS region may modify the overall phenotype in the patients with 17p11.2 deletions.[7–9] In this study, we report comparative genome hybridisation (CGH) analysis of the short arm of chromosome 17 in patients with SMS.

METHODS

Patients

Patients with SMS were recruited by voluntary participation of members of the French Smith-Magenis Association (www.smithmagenis.com). All patients with SMS were diagnosed using commercial fluorescence in situ hybridisation probes (Oncor Inc., Gaithersburg, Maryland, USA and Vysis, Inc., Downers Grove, Illinois, USA) encompassing *FLII*. After informed consent was given, blood samples were obtained from 30 patients.

DNA preparation

Genomic DNA for CGH arrays was isolated from leucocytes from peripheral blood samples of each patient using genomic DNA columns (Qiagen Inc., Valencia, California, USA). DNA concentration was evaluated using a spectrophotometer (Nanodrop, Wilmington, Delaware, USA). Reference DNA was obtained in the same condition from a pool of 15 healthy men and women without cytogenetic abnormalities.

Design of the probes

We have made the choice of an ''in silico'' design based directly on an extraction of DNA sequences in the region of interest directly obtained from the Human Genome Browser Gateway (University of California Santa Cruz, Santa Cruz, California, USA; 24 April 2007; http://genome.ucsc.edu/cgi-bin/hgGateway?org=human) and a PCR-based production of the probes. For each of these sequences, primers were designed to obtain a nested PCR DNA fragment of 250–400 bp. This PCR fragment was tested to verify lack of redundancy, homology, freedom form repeats and specificity for the hybridisation. This process was fully automated design using bespoke software (available on our website at http://genopole-lille.fr) which combines BLAT results (http://www.genome.ucsc.edu/cgi-bin/hgBlat), identification of non-redundant sequences and absence of primer crosshybridisation.

All the primers had an identical melting point (mean (SD) 60 (1)℃), in order to have one GC clamp so that all primers could be used under the same PCR conditions. The 75 loci and the primers used are listed in a supplementary file available online at http://jmg.bmj.com/supplemental.

Array construction

PCR DNA products after purification were spotted at a concentration of 100 ng/μl in 3× SSC in triplicate onto Telechem Superamine glass slides by using a microarrayer (Eurogridderd; Eurogentec, Seraing, Belgium). The slides were heated at 80℃ for 10 min, and DNA fragments were cross-linked by UV light (2 × 150 mJ). Finally, the slides were stored at room temperature after denaturation (2 minutes in boiling water).

Abbreviations: CGH, comparative genome hybridisation; FISH, fluorescence hybridisation; HMM, hidden Markov model; UBB, ubiquitin B

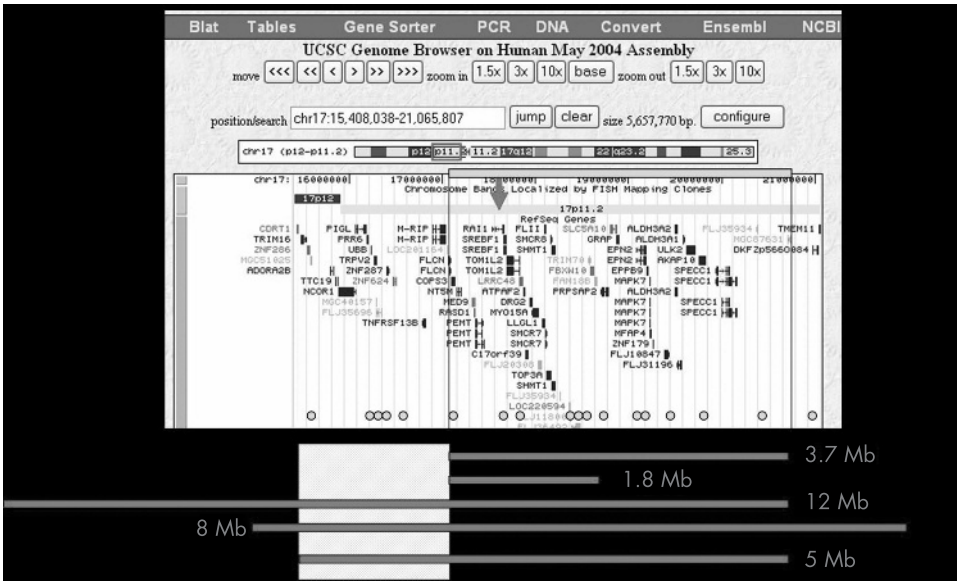

Figure 1 Genes in the 17p11.2–17p12 region (Human Genome Browser, http://genome.ucsc.edu/cgi-bin/hgGateway?org=human). Thick lines are the estimated locations of the deletions compared with the Genome Browser gene position. Spots are the estimated locations of the CGH array amplimer probes. The empty square is the estimated location of the smallest SMS extra-deleted region.

Array hybridisation, DNA test and reference labelling

The PCR primers allowed us to selectively amplify the region of interest in the test and reference DNA before the labelling to obtain more efficient hybridisation kinetics and a low complexity target. Very low amounts of genomic DNA (15 ng), were used in each multiplex. Test and reference muliplex-obtained DNA were fluorescently labelled by incorporation of CyTm3-conjugated and CyTm5-conjugated dUTP (Amersham Biosciences, Amersham, Buckinghamshire, UK) by random priming (Bioprime DNA labelling system; Invitrogen, Carlsbad, California, USA). Unincorporated nucleotides were removed using column centrifugation (YM30 Microcon membrane; Millipore, Billerica, Massachusetts, USA). For hybridisation, labelled test DNA and reference DNA in a ratio of 1:1 were coprecipitated and resolved in 200 µl of hybridisation buffer (Chipspread; Ventana, Tucson, Arizona, USA). After denaturation (85℃ for 5 min), the mixture was hybridised for 8 h at 42℃ then slides were washed automatically three times at 37, 50 and 55℃ in washing buffer (Ventana). Finally, slides were dried by centrifugation at 100 *g* for 5 min at 2500 rpm.

Image and data processing

Arrays were scanned using an 428 GMS scanner (MWG, Affymetrix, Santa Clara, California, USA). Fluorescent intensities were extracted after subtraction of local background using Jaguar software V.2.0. In addition, probes that we had identified in previous control hybridisations as inducing consistently weak signals or irreproducible or aberrant ratios were excluded from the analysis. The data normalisation was performed using an automatic algorithm added in the R package MANOR (Micro-Array NORmalisation; http://bioinfo-out.curie.fr/projects/manor, which is available from http://www.bioconductor.org). To determine gain and loss segments we used a hidden Markov model (HMM)-based method that assigns probes to different state (loss, normal or gain).[10] The HMM outputs are state medians weighted by the estimated probability of being in each state.

RESULTS

We studied deletion sizes from 30 patients with SMS using the 300 bp amplimer CGH array, encompassing 75 loci on a 22 Mb segment from the short arm of chromosome 17 (17 Mb). Medium resolution of this array was 300 kb. Three patients showed larger deletions (5, 8 and 12 Mb), and one also showed a smaller deletion (1.8 Mb) (fig 1). Genotype–phenotype correlation including mental retardation, craniofacial and skeletal anomalies, and behavioural abnormalities, showed that two of these three patients had cleft palate, which was not found in any other patient with SMS ($p<0.007$, Fisher's exact test).

The smallest extra-deleted region associated with cleft palate in SMS is 1.4 Mb (chr17:15 637 376 to 17 052 919). The telomeric breakpoint of this deletion was studied using fluorescence in situ hybridisation (FISH) analysis of bacterial and P1-derived artificial chromosomes and was estimated to be between chr17:15 700 000 and chr17: 15 800 000 (table 1). This region contains <16 genes and is located at the 17p11.2–17p12 junction. The first branchial/pharyngeal arch is a

Table 1 Results of the fluorescence in situ hybridisation of bacterial and P1-derived artificial chromosomes (BACs/PACs) for the smallest cytogenetic spreads associated with cleft palate in patients with CMS

BAC/PAC	Location	Molecular position		FISH result
RP11-90G21	17p12	15 321 344	15 511 681	Non-deleted
RP11-304M13	17p12	15 362 705	15 560 308	Non-deleted
RP11-273K13	17p12	15 398 210	15 560 308	Non-deleted
RP1-121M24	17p12	15 637 376	15 823 053	Non-deleted, asymmetrical signal
RP11-459E6	17p12-17p11.2	15 770 053	15 929 608	Deleted
RP1-188B3	17p11,2	15 995 972	16 077 445	Deleted
RP11-138I1	17p11.2	16 123 263	16 294 186	Deleted
RP1-77H15	17p11.2	16 289 474	16 438 473	Deleted

Table 2 Expression array data from the first branchial/pharyngeal arch at the fourth and fifth weeks of human development

		4th week, 1st pharyngeal arch		5th week, 1st pharyngeal arch	
Gene name	**Probe set**	**Call**	**Log average ***	**Call**	**Log average***
ADORA2B	36324_at	A/A	234/−301	A/A	7/92
TTC19	ND	ND	ND	ND	ND
NCOR1	ND	ND	ND	ND	ND
PIGL	34496_at	A/A	1691/−301	A/A	1968/296
PRR6(p30)	ND	ND	ND	ND	ND
UBB	1323_at	P/P	151075/92691	P/P	45433/79534
TRPV2	ND	ND	ND	ND	ND
MGC40157	39077_at	A/A	919/1372	A/A	316/676
FLJ35696	ND	ND	ND	ND	ND
LOC440403	ND	ND	ND	ND	ND
ZNF287	ND	ND	ND	ND	ND
ZNF624	ND	ND	ND	ND	ND
KIAA0565	35535_f_at	A/A	170/−39	A/A	60/−42
LOC96597	ND	ND	ND	ND	ND
TNFRSF13B	31410_at	ND	910/−2177	A/A	245/386
MRIP	38731_at	A/A	191/−302	A/A	25/−101

ND, data not determined.
*Positive/negative.

conserved embryonic structure that develops into the palate and jaw. Expression array data from the first branchial arch at the fourth and fifth weeks of human development allows the study of 7 of these 16 genes on an Affymetrix expression array (between *ADORA2B* and *MRIP*). These gene expression array data showed that only the ubiquitin B precursor (*UBB*) is significantly expressed in the first branchial arch at at the fourth and fifth weeks of human development (table 2). No such data were available for the nine other genes.

DISCUSSION

Smaller or larger deletions were seen in around 12% and around 10% of patients with SMS, respectively.[11] Genotype–phenotype studies of the patients with SMS showed no phenotype difference between deletions of 1.5–9 Mb in size.[12] However, because of less accurate cytogenetics techniques at the time, two of the nine patients with SMS and cleft palate reported by Smith and Magenis[1] may have had a larger 17p11.2 deletion. Cleft palate occurs in about 10% of patients with SMS but to date, there has been no evidence of a link between this 10% and the 10% with larger deletions.

Two linking studies suggest the presence of one gene on 17p implicated, directly or as a cofactor, in cleft palate: 17p13.1, near D17S974 and D17S1303[13] and 17p11.2–p11, near D17S1843 and D17S953.[14] These studies suggest that a gene at 17p11.2–11.1, together with the Van der Woude gene interferon regulatory factor 6 (*IRF6*) at 1q32, enhances the probability of cleft palate in an individual carrying these two risk genes.

In our study, the smallestextra-deleted region associated with cleft palate in SMS of 1.4 Mb contains <16 genes and is located at 17p11.2–17p12. Taking together the gene expression array data and previously published data, one gene, *UBB*, seems to be the best candidate to be implicated in cleft palate. This gene encodes ubiquitin, one of the most conserved proteins known. Ubiquitin is required for ATP-dependent, nonlysosomal intracellular protein degradation of abnormal proteins and normal proteins with a rapid turnover.[15] It is covalently bound to proteins to be degraded, and presumably labels these proteins for degradation (OMIM 191339, Genatlas). UBB is implicated in anomalies of the ubiquitin–proteasome system, particularly in neurodegenerative diseases.[16]

Several genes (*UFD1L*, *MID1* and *SUMO1*) implicated in the ubiquitin-mediated proteolysis pathways, are involved in syndromic or non-syndromic cleft palate. In Opitz G/BBB syndrome, a genetic disorder characterised by developmental midline abnormalities, *MID1* encodes a TRIM/RBCC protein that is anchored to the microtubules. The association of Mid1 with the cytoskeleton is regulated by dynamic phosphorylation through the interaction with the alpha4 subunit of phosphatase 2A (PP2A). *MID1* acts as an E3 ubiquitin ligase, regulating PP2A degradation on microtubules.[17 18]

In DiGeorge syndrome (DGS), *UFD1L* encodes the human homolog of the yeast ubiquitin fusion degradation 1 protein (UFD1p), involved in the degradation of ubiquitin fusion proteins. Even though its mechanism remains unclear, *UFD1L*, expressed in embryonic branchial arches and in the conotruncus, appears to play a prominent role in the pathogenesis of the 22q11.2 deletion syndrome.[19–21]

Another gene implicated in cleft palate, the small ubiquitin-related modifier *SUMO1*, reversibly modifies many proteins, including promoter-specific transcription factors. Msx1 is conjugated to *SUMO1*, and studies in both humans and mice indicate that the Msx1 transcription factor is associated with specific disorders, including cleft palate.[22 23]

CONCLUSION

Cleft palate in SMS is a rare event (10%) in a rare syndrome (prevalence 1 25 000). In our study, larger deletions were associated with cleft palate in SMS. Constitutional hemizygosity for *UBB* has a role in ubiquitin-mediated proteolysis and may act as a cofactor for cleft palate in patients with SMS. It may be a good candidate gene for non-syndromic cleft palate, especially Van der Woude syndrome modifier (OMIM 604547).

Electronic database information

- Human Genome Browser, http://genome.ucsc.edu/cgi-bin/hgGateway?org=human
- Expression array data from the first branchial arch: http://hg.wustl.edu/COGENE/
- Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM
- Genatlas, http://www.genatlas.org

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Supplementary file available on the JMG website — http://jmg.bmj.com/supplemental

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