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ABSTRACT

Introduction: Smith-Magenis syndrome (SMS) is rare (prevalence 1/25.000) and associates psychomotor delay, a particular behavioral pattern and congenital anomalies. SMS is often due to less than 4 Mb chromosomal deletion at 17p11.2 locus, leading to haploinsufficiency of numerous genes. Mutations of one of them, *RAI1*, seems to be responsible of main criteria found in heterozygous 17p11.2 deletion.

Materials and Methods: We studied DNA from 30 SMS patients using a 300 bp-amplimers-CGH-array encompassing 75 loci on the 22 Mb from chromosome 17 short arm.

Results: Three patients showed larger deletions (10%). Genotype-phenotype correlation revealed that two of them had cleft palate beside none of other SMS patients ($p < 0,007$, Fisher's exact test). The smallest cleft palate SMS extra-deleted region of 1,4 Mb contains less than 16 genes and is located at 17p11.2-17p12. Among them, gene expression array data showed that Ubiquitin B precursor (*UBB*) is significantly expressed in first branchial arch at 4th and 5th of human development.

Conclusion: Therefore, all these data may support *UBB* as a good candidate gene for isolated cleft palate.

Keywords: Smith-Magenis, cleft palate, CGH-array

Smith-Magenis syndrome (SMS) [MIM 182290] is a rare prevalence (1/25.000) multiple congenital anomalies/mental retardation syndrome with distinctive behavioral characteristics, sleep disturbances and dysmorphic features, associated with a heterozygous interstitial deletion of chromosome 17p11.2.¹⁻³ Most patients have the same approximately 4 Mb interstitial genomic deletion within chromosome 17p11.2 with 20 expressed genes.⁴⁻⁶ Heterozygous frameshift mutations of *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 chromosome 17 short arm CGH analysis of SMS patients.

MATERIAL AND METHODS

Patients

SMS patients were recruited by voluntary participation of members of the french association Smith-Magenis France (www.smithmagenis.com/). All SMS patients were diagnosed using commercial FISH probes (Oncor™ or Vysis™) encompassing *FLII*. After informed consent was given, blood sample was 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™). DNA concentration was evaluated using Nanodrop™ spectrophotometer. Reference DNA was obtained in the same condition from a pooled of 15 healthy males and females individuals without cytogenetic abnormalities.

Design of the probes

We have made the choice of an in silico design based directly to an extraction of DNA sequences in the region of interest directly obtained from “Human Genome Browser Gateway, UCSC, april 2004” (<http://www.genome.ucsc.edu/cgi-bin/hggateway>) and a PCR based production of the probes. In each of those sequences, a couple of primers was designed to obtain a nested PCR DNA fragment sized between 250 and 400 bp. This PCR obtainable fragment was tested to verify lack of redundancy, homology, repeat free and specificity for the hybridization. All this operation is performed as a fully automated design with a home made software available on our website at <http://genopole-lille.fr>, which combine BLAT (Blat software UCSC (<http://www.genome.ucsc.edu/cgi-bin/hgBlat>), identification of non redundant sequences and absence of crosshybridization of primers.

All the primers have an identical Tm 60 +/- 1°C, in order to have one GC clamp, to be able to product all the fragment in the same condition of PCR.

Array construction

PCR DNA products after purification were spotted at a concentration of 100ng/μl in 3X SSC in triplicate onto Telechem™ Superamine glass slides by using the Eurogridder® microarrayer (Eurogentec™). The slides were baked at 80°C for 10 min, and DNA fragments were crosslinked by UV light (2X150 millijoules). Finally, the slides were stored at room temperature after denaturation (2 minutes boiling water).

Array hybridization, DNA test and reference labeling

Availability of PCR primers give us the ability to electively amplify region of interest in test and reference DNA before the labeling in order to obtain a more efficient kinetic of hybridization and a low complexity target. Very weak amounts of genomic DNA, as 15 ng, were used in each multiplex. Test and reference multiplex obtained DNA were fluorescently

labeled by incorporation of Cy^{tm3} and Cy^{tm5} conjugated dUTP (AmershamTM) by random priming (Bioprime[®] DNA labeling system, InvitrogenTM). Unincorporated nucleotides were removed through Microcon[®] membrane column centrifugation YM30 (MilliporeTM). For hybridization, labeled test DNA and reference DNA in a ratio of 1/1 were coprecipitated and resolved in 200 μ l of chipsread hybridization buffer (VentanaTM). After denaturation (85°C for 5 min), the mixture was hybridized for 8 h at 42°C then slides were washed automatically three times at 37, 50 and 55°C in wash buffer (VentanaTM). Finally, slides were dried by spinning for 5 min at 2,500 rpm.

Image and data processing

Arrays were scanned using an 428 GMS scanner (MWG[®], AffymetrixTM). Fluorescent intensities were extracted after subtraction of local background using JaguarTM 2.0 software. In addition, probes that we have identified in previous control hybridizations to induce consistently weakest signal or irreproducible or aberrant ratios were excluded from the analysis. The data normalization was performed using an automatic algorithm added in the R package MANOR (Micro-Array NORmalization) (<http://bioinfo.curie.fr/projects/manor>) which is available from <http://www.bioconductor.org>. To determine gain and losses segments we used a Hidden Markov model (HMM) based method that assigns probes to different state (ie 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 SMS patients using this 300 bp-amplimers-CGH-array, encompassing 75 loci on the 22 Mb from chromosome 17 short arm (17 Mb). Medium resolution of this array was 300 kb. Three patients showed larger deletions (5, 8 and 12 Mb), and one smaller deletion (1.8 Mb) (fig 1). Genotype-phenotype correlation including study of mental retardation, craniofacial and skeletal anomalies, behavioral abnormalities, showed that two of these three patients had cleft palate beside none of other SMS patients ($p < 0,007$, Fisher's exact test).

The smallest cleft palate SMS extra-deleted region size is 1,4 Mb (chr17:15,637,376-17,052,919). Telomeric breakpoint of this deletion was studied using BACs/PACs FISH and estimated between chr17:15,700,000 and chr17: 15,800,000 (table 1). This region contains less than 16 genes and is located at 17p11.2-17p12 junction. The first branchial/pharyngeal arch is a conserved embryonic structure that develops into the palate and jaw. Expression array data from the first branchial arch, at 4th and 5th of human development allows the study of 7 genes among these 16 on AffymetrixTM expression array (between *ADORA2B* and *MRIP*). These gene expression array data showed that only Ubiquitin B precursor (*UBB*) is significantly expressed in first branchial arch at 4th and 5th of human development (table 2). No such data were available for the 9 other genes.

DISCUSSION

Smaller or larger deletions were seen in approximately 12% and approximately 10% of SMS patients, respectively.¹¹ SMS patients genotype-phenotype studies showed no phenotype-difference between 1,5 to 9 Mb deletions.¹²

However, because of less accurate cytogenetics technics, two of the nine SMS patients published by Smith et Magenis¹ had a cleft palate may have larger 17p11.2 deletion. Cleft palate occurs in about 10% of SMS patients but until today no evidence was proved of the link between these 10% and the 10% larger deletions.

Two linking studies suggest the presence of one gene implicated, directly or as a cofactor, in cleft palate on 17p: 17p13.1 near D17S974 and D17S1303¹³ 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 (VWS) gene (*interferon regulatory factor 6 (IRF6)*) at 1q32, enhances the probability of cleft palate in an individual carrying these two at-risk genes.

In our study, smallest cleft palate SMS extra-deleted region of 1,4 Mb contains less than 16 genes and is located at 17p11.2-17p12. Joining literature and gene expression array data, one gene seems to be the best candidate to be implicated in cleft palate: *UBB*.

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 (OMIMTM, GenatlasTM). *UBB* is implicated in anomalies of ubiquitin-proteasome system especially in neurodegenerative diseases.¹⁶

Several genes implicated in Ubiquitin mediated proteolysis pathways, *UFDIL*, *MIDI* and *SUMO1*, are involved in syndromic or non-syndromic cleft palate.

In Opitz G/BBB syndrome, a genetic disorder characterized by developmental midline abnormalities, *MIDI*, 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). *MIDI* acts as an E3 ubiquitin ligase, regulating PP2A degradation on microtubules.^{17,18}

In DiGeorge syndrome (DGS), *UFDIL*, encodes the human homolog of the yeast ubiquitin fusion degradation 1 protein (UFD1p), involved in the degradation of ubiquitin fusion proteins. Even if it is remaining uncleared, *UFDIL*, 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 are associated with cleft palate in SMS. Constitutional hemizyosity for *UBB* has a role in Ubiquitin mediated proteolysis and may act as a cofactor for cleft palate in SMS patients: it may be a good candidate gene for non-syndromic cleft palate, especially in Van der Woude Syndrome Modifier (OMIM 604547).

Electronic-Database Information:

Accession numbers and URLs for data presented herein are as follows:

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 ManTM (OMIMTM),

<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM>

GenatlasTM, <http://www.genatlas.org/>

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Figure 1 Genes in the 17p11.2-17p12 region obtained by Genome browser® at Santa Cruz university website (Human Genome Browser, <http://genome.ucsc.edu/cgi-bin/hgGateway?org=human>). Red thick lines are figuring locations of the deletions compared to Genome browser® gene position. Green spots are figuring locations of the CGH-array amplimer probes. Blue empty square is figuring the smallest SMS extra-deleted region.

Table 1 Results of the FISH BACs/PACs on smallest cleft palate SMS extra-deleted patient cytogenetic spreads.

Table 2 Expression array data from the first branchial/pharyngeal arch, at 4th and 5th of human development. ND: data not determined.

UCSC Genome Browser on Human May 2004 Assembly

move <<< << < > >> >>> zoom in 1.5x 3x 10x base zoom out 1.5x 3x 10x

position/search chr17:15,408,038-21,065,807 jump clear size 5,657,770 bp. configure

chr17 (p12-p11.2) p12 p11.2 p11.1 p11.21 q12 22 q23.2 25.3

