RAII variations in Smith-Magenis syndrome patients without 17p11.2 deletions

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Running title: RAI1 mutations in Smith-Magenis syndrome

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ABSTRACT

Introduction: Smith-Magenis syndrome (SMS) (OMIM #182290) is a mental retardation syndrome characterized by behavioral abnormalities, including self-injurious behaviors, sleep disturbance, and distinct craniofacial and skeletal anomalies. It is usually associated with deletion involving 17p11.2 and is estimated to occur in 1/25,000 births. Our laboratory previously identified dominant frameshift mutations leading to protein truncation in the retinoic acid induced 1 gene (RAI1) in 3 individuals who had phenotypic features consistent with SMS. The RAI1 gene lies within the 17p11.2 locus, but these patients did not have 17p11.2 deletions. Subjects: We analyzed 4 individuals exhibiting features consistent with SMS who do not carry 17p11.2 deletions for variations in RAI1 using a PCR and sequencing strategy. Results: Two patients have small deletions in RAI1 that result in a frameshift and premature truncation of the protein. Missense mutations were identified in the other two patients. Orthologs across other genomes show these missense mutations occur in identically conserved regions of the gene. These mutations are de novo as all parental samples were normal. Several polymorphisms were also observed, including new and reported SNPs. Conclusion: The clinical features in these patients differ from 17p11.2 deletion patients by general absence of short stature and no visceral anomalies. All of these patients have developmental delay, reduced motor and cognitive skills, craniofacial and behavioral anomalies, and sleep disturbance. Seizures, not previously thought to be associated with RAI1 mutations, were also observed in one patient from this cohort. From these data, we conclude that haploinsufficiency of the RAI1 gene is associated with most features of Smith-Magenis syndrome, including craniofacial, behavioral, and neurological signs and symptoms.
INTRODUCTION

Smith-Magenis syndrome (SMS) is a multiple congenital anomalies and mental retardation syndrome usually associated with an interstitial deletion involving chromosome 17p11.2.[1] It is characterized by distinctive craniofacial anomalies,[2] delayed milestones, cognitive impairment, hearing loss,[3] myopia, and mild to severe mental retardation.[4] Behavioral abnormalities include significant sleep disturbances and maladaptive and self-injurious behaviors.[5][6][7] The facial appearance is characterized by a broad square-shaped face, brachycephaly, midface hypoplasia, and micrognathia in early infancy progressing to relative prognathism with age. Up-slanting palpebral fissures, deep-set eyes, short full tipped nose, tented upper lip and down-turned corners of the mouth are normally seen in the majority of SMS patients. Infancy and childhood are associated with hypotonia, sleep disturbances with inverted melatonin circadian rhythm, failure to thrive, and feeding difficulties. Neurobehavioral abnormalities become more pronounced with age and are characterized by hyperactivity, temper tantrums, attention seeking, self-hugging, polyembolokoilomania (insertion of objects into bodily orifices), and onychotillomania (picking and pulling-out of finger and toe nails). In addition, about 70% of SMS patients have elevated cholesterol levels.[8]

The incidence of this disorder has been estimated at 1:25,000 births[9] although with more awareness and proper diagnosis, the incidence is expected to be higher. About 75% of SMS patients with 17p11.2 deletions have a common deletion spanning ~3.5Mb, but the size of the deletion is variable from ~1.5 Mb to ~9 Mb.[10][11] Deletions associated with SMS are reported to result from unequal crossing-over mediated by SMS-repeat clusters.[12] We refined the minimal overlapping region common to all SMS patients carrying a 17p11.2 deletions to ~700 kb by analyzing patients with unusual 17p11.2 deletions using FISH.[13]

Further, studies in our laboratory found that a gene encoding the retinoic acid induced protein 1 (RAI1) carried small, intragenic deletions in patients who had no FISH-detectable deletion in the 17p11.2 region.[14] RAI1 (GenBank AY172136, AJ271790; OMIM*607642) maps to the central portion of the SMS critical interval, adjacent to SREBF1. It is formed by six exons generating an ~8.5 Kb mRNA and a 1906 amino acid protein. The RAI1 protein contains a bipartite nuclear localization signal, polyglutamine and polyserine tracts, and a PHD/zinc-finger domain at the C-terminus.[15] Murine Rai1 (originally reported as GT1) is up regulated in mouse embryonal carcinoma cells following retinoic acid treatment to induce neuronal differentiation.[16] We describe the sequencing analysis of RAI1 in 4 non-deletion patients with SMS and compare their phenotypic data with 17p11.2 deletion patients. A comprehensive study of the structure of this gene in humans and its conserved sequences in mouse, rat, and chimp genomes is also provided to facilitate the analysis of RAI1 mutations in SMS.

MATERIALS AND METHODS

Patients and Samples

Patients having the physical and neurobehavioral characteristics diagnostic of SMS, were referred from genetics clinics from various parts of United States and Europe. Informed written consent was obtained from the parents. This study was approved by the Michigan State University Committee on Research Involving Human Subjects and the Institutional Review Board of the Virginia Commonwealth University. About 7-10ml of blood was drawn by cubital venopuncture following sterile procedures. At times where blood could not be obtained, buccal
brush samples were collected. The molecular evaluation protocol followed is illustrated in Fig 1.

**Fluorescence in situ hybridization**

All patients were evaluated for 17p11.2 deletions by fluorescent in situ hybridization (FISH) to metaphase chromosome spreads using PAC probe RP11-253P07, representing the RAI1 locus. A commercially available nick-translation kit was utilized to incorporate Spectrum Green or Spectrum Orange dUTP following the manufacturer’s instructions (Vysis, Downers Grove, IL). A 17q-tel probe (RP1-314M5) was used as a control. Metaphase chromosomes were prepared for hybridization by incubating at 37°C in 2X SSC for 30 min followed by dehydration through an ethanol series and air-drying. After overnight hybridization at 37°C, slides were washed, and then counterstained using Vectashield antifade with DAPI (Vector Labs, Burlingame, CA). Analyses of FISH experiments were carried out on a Zeiss Axioplan IE microscope and photographed with Axion MR black and white camera using Zeiss Axio-vision software version 4.2 (Carl Zeiss, Thornwood, NY).

**PCR and Sequencing**

DNA from whole blood was isolated by the phenol-chloroform method. Standard, lab-optimized protocols were followed to isolate DNA from buccal smears and cell lines. PCR was performed to amplify patient DNA using overlapping RAI1 primers covering the entire coding region (Genbank AY172136), spanning exons 3–6 (see Table 1 for primers and annealing temperatures). PCR was performed with 100 ng genomic DNA, 20 pmol each of forward and reverse primers, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 200 μM dNTPs, and 1 U Taq DNA polymerase in a total reaction volume of 25 μl. Reactions were held at 94°C for 5 min followed by 30 cycles of denaturing at 94°C, 1 min; annealing at 55-62°C (see Table 1), 1 min; and an extension at 72°C, 1 min, followed by a final extension at 72°C, 10 min. Reactions were held at 4°C until use. PCR products (5μl) were either purified by digesting with 2 U of shrimp alkaline phosphatase and 10 U of exonuclease I (USB Corp, Cleveland, OH) at 37°C, 15 min followed by 80°C, 15 min to remove excess primers or were gel purified using a Qiagen gel extraction kit (Qiagen Inc, Valencia, CA). Sequencing was performed at either the Virginia Commonwealth University Sequencing Core or at the Michigan State University Genomics Technology Support Facility.

Cycle sequencing was done using 10 ng/μl of the purified PCR product, 10 pmol of each primer, and 4 μl of the ABI Prism BigDye terminator cycle sequencing ready reaction mix (Perkin Elmer, Applied Biosystems) in a 10 μl final volume for 25 cycles. Following cycle sequencing, the samples were purified with ethanol, precipitated, resuspended in formamide, denatured at 95°C for 5 minutes and loaded onto an ABI 3700 Genetic Analyzer (Applied Biosystems/Hitachi). Sequencing was initially done with the forward primer and the presence of any DNA variation confirmed by sequencing in the reverse direction. Available parental samples were evaluated for all identified mutations. The chromatograms and the sequence data were aligned to the RAI1 mRNA database sequence at the NCBI (GenBank AY172136) using Clustal X (version 1.83).
Table 1. *RAI1* PCR primers and annealing temperatures.*

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Product Size (bp)</th>
<th>Annealing Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>SHE 323: TCTGAGGCAAAAGGAAGTGG</td>
<td>SHE 324: GACTGGGAATGCTGAGGAAA</td>
<td>770</td>
<td>62°C</td>
</tr>
<tr>
<td>3</td>
<td>SHE 325: AAAGGCAGAAGCTGCAAGAC</td>
<td>SHE 326: CAGTGCTGGCTTTATGCTG</td>
<td>875</td>
<td>62°C</td>
</tr>
<tr>
<td>3</td>
<td>SHE 327: TGTCAGAGACCTCGCTGCAAGAC</td>
<td>SHE 328: GGGAAACAGTCAAAGGCTGC</td>
<td>777</td>
<td>60°C</td>
</tr>
<tr>
<td>3</td>
<td>SHE 329: TCTCGCTGGAGAACCACAG</td>
<td>SHE 330: AACCGACTCGTGGACCTTTGA</td>
<td>904</td>
<td>59°C</td>
</tr>
<tr>
<td>3</td>
<td>SHE 331: CACTCATCTGCACCAAGGAG</td>
<td>SHE 332: ATGAAGGCGACTCTCTTTTT</td>
<td>1000</td>
<td>60°C</td>
</tr>
<tr>
<td>3</td>
<td>SHE 333: CCAAGAAGCTCCTCGACAAC</td>
<td>SHE 334: TTCCGGCTCCTCTTTTTAGG</td>
<td>763</td>
<td>59°C</td>
</tr>
<tr>
<td>3</td>
<td>SHE 335: AAACCGAGGCTCCTACATC</td>
<td>SHE 336: CACACTCGGACCTTTGAGT</td>
<td>964</td>
<td>60°C</td>
</tr>
<tr>
<td>3</td>
<td>SHE 339: CTACCCCTGAACACTGCTCC</td>
<td>SHE 340: AGAGGCAATGGAGACAGGAA</td>
<td>887</td>
<td>60°C</td>
</tr>
<tr>
<td>4</td>
<td>SHE 341: CCAAGGCTGGATACCAACTCC</td>
<td>SHE 342: GGCAAGAGGAGGGAAACT</td>
<td>420</td>
<td>60°C</td>
</tr>
<tr>
<td>5</td>
<td>RA 45: GGAATGGAGAGTGAGTGAGGGAGG</td>
<td>RA 46: GGAATGGAGAGTGAGTGAGGGAGG</td>
<td>310</td>
<td>62°C</td>
</tr>
<tr>
<td>6 + 3'- UTR</td>
<td>SHE 345: ACTGAGGAGCTCGAGGTTGTG</td>
<td>SHE 346: GACTGGGAAGGGAGCAAAACA</td>
<td>816</td>
<td>55°C†</td>
</tr>
</tbody>
</table>

*Primers cover the coding region of *RAI1* and are based on Genbank #AY172136.
†Amplification requires Invitrogen® 10x PCR enhancer.
RESULTS
We were referred 4 patients with clinical symptoms of SMS who were initially evaluated by FISH analysis at commercial laboratories for 17p11.2 deletions but were negative for any 17p11.2 deletion. All patient samples were evaluated in this laboratory by FISH using an RAI1 specific probe. None of these patients carried a deletion of the 17p11.2 region; thus all were further screened for variations in the RAI1 coding region. Significant nucleotide changes were identified in exon 3 of the gene. In this report, we describe four novel de novo mutations that support a diagnosis of SMS in these patients. Each of the patients presented also carries inherited RAI1 polymorphisms; some are documented in the NCBI SNP database and some are reported for the first time in this study (Table 3).

SMS153
SMS153 is a 19 y old female of European decent with developmental delays and self-destructive behaviors who was initially evaluated at the age of 14 (Fig. 2a). She is the only child of a G1P1 mother with artificial insemination (known healthy sperm donor). SMS153 was born at 42 weeks after an induced labor followed by C-section. She was 4.3 Kg and was noted to have floppy muscle tone, upslanting palpebral fissures, and midface hypoplasia as a neonate and was initially diagnosed with Down syndrome. All laboratory studies were negative for trisomy 21.

Some developmental milestones were normal, but she was significantly delayed in motor skills and language development. Long tantrums, attention seeking, and repetitive behaviors began ~18 months of age. Enuresis was a problem until age 12. Tonsils and adenoids were removed in early childhood due to frequent ear infections. She was thoroughly evaluated for pseudohypoparathyroidism, given her history of mental retardation and brachydactyly of fourth metacarpophalangeal joint, but no significant biochemical findings were found. At 15 y, she had a developmental age of 8-10 y with IQ of 67 (Wechsler scale). Her facial and behavioral features are consistent with that of SMS (Table 2). She has a waddling gait, loud and hoarse voice, decreased sensitivity to pain, and short fingers and hands. She has low-set ears, clino-brachydactyly of the 5th fingers bilaterally, and prognathism. This patient has significant sleep disturbance (including frequent napping and multiple night awakenings) that has improved some with melatonin supplements. Skin-picking, onychotillomania (toenails), and polyembolokoilomania (mouth) are constant issues for the family. Over-eating and weight management have always been difficult. At age 19, weight is >97th centile, height is ~75th centile, and BMI (34) is >97th centile. She has carried a variety of psychiatric diagnoses throughout life, including attention-deficit disorder, obsessive-compulsive disorder, pervasive developmental delay (not otherwise specified), and more recently has exhibited bipolar episodes. She has taken a variety of medications to manage her behaviors.

Additional laboratory studies include a normal karyotype, negative fragile X studies, normal FISH for del(22)(q11.2), and normal FISH for del(17)(p11.2). SMS153 has a heterozygous deletion of 19 bp starting at nucleotide 253 in exon 3 of RAI1 that causes a frame shift mutation leading to misincorporation of 60 amino acids followed by a stop codon (Table 3, Fig. 2a, Fig. 4). This change was not seen in her mother’s DNA nor was it identified in >110 normal chromosomes.
SMS188
SMS188 is a 14 y old male of European decent who was evaluated in the genetics clinic between the ages of 4 – 14 y (Table 2). The boy is the third child of healthy, unrelated parents. He also has three healthy younger half-siblings. Family history is negative with regard to mental handicap or congenital malformations. Pregnancy was normal, but he was noted to be a very active baby already before birth. As an infant, he was very restless, cried excessively, and slept poorly. He could walk at 18 m, and spoke the first words at a normal age. Starting from infancy, his behaviour was remarkable. Sleep was severely disturbed, typically, he went to sleep around 23.00 h, and woke up around 3.30 h. There was head-banging and occasionally self-mutilation. He destroyed his toys and the furniture in his bedroom. From the age of 5 y, residential care in a school for children with developmental delay and behavioural difficulties was necessary. His intelligence at the age of 9.5 y was evaluated by WISCR and showed a full scale IQ of 73, verbal IQ of 85 and performance IQ of 65.

On clinical examination at age 4 y 10 m, weight was 20.7 kg (75th centile), length 109.5 cm (25th – 50th centile) and head circumference 52.5 cm (75th centile). Facial features included brachycephaly, midface hypoplasia, a tented upper lip and a broad, square face. He has epicanthic folds and an internal strabismus of the left eye. The hands are broad and short with a transverse palmar crease on the right hand. He was hyperactive, constantly moving around, and on excitation, he clapped his hands. On several occasions, foreign bodies needed to be removed from the ears and nose. He also had a hoarse voice. At the age of 12 y 10 m, weight was 51.8 kg (75-90th centile), length 156.9 cm (75-90th centile) and head circumference 55 cm. Puberty progressed normally. The sleep disturbances were slightly improved, but he remained very active and his behaviour was uncontrolled, including sexual obsessions.

Laboratory findings include a normal karyotype and normal FISH for 17p11.2 deletion. SMS188 carries a deletion of a single cytosine in exon 3 at nucleotide position 3801 on one allele of RAI1 (Table 3, Fig. 2b, Fig. 4). This deletion results in a frame-shift starting at amino acid 1267 leading to misincorporation of 46 aa and a downstream stop codon. Neither parent nor siblings carry this DNA variation, nor has it been observed in >100 normal chromosomes.

SMS195
SMS195 is a 17 y old adopted male of northern European and Jewish ancestry (Table 2). Biological parents reportedly have 3 normal children. Other family history is not available. He was delivered full-term at 3.86 Kg and 53.3 cm in length. Neonatal jaundice, sleep disturbance, and mildly delayed motor and cognitive milestones were the major complaints during early childhood. Medical history is notable for arthritis of the 5th digits and the knees bilaterally. He has high myopia, a loud and hoarse voice, a waddling gait, pes planus, and dry skin. Alternating diarrhea and constipation has continued since early childhood. At 15 y, he was prepubertal and Tanner stage II and sought care for evaluation of delayed puberty. After treatment with testosterone injections for 8 m, he was Tanner stage III-IV and had developed pubic and axillary hair. Treatment was discontinued due to aggressive behaviors.

His behaviors are notably significant and include sleep disturbances (hypersomnia as an infant which moved to frequent and early awakenings and daytime napping from ~age 4 to present), reported bipolar episodes, head-banging, tantrums, and aggressive and intrusive
behaviors. He also has deep scarring from obsessively picking his skin. Behaviors necessitated several psychiatric hospitalizations, and he is currently seeking placement in a residential treatment facility. He has been prescribed numerous psychiatric medications since age 6. At age 14, he had a developmental age of a 9 y old with IQ of 89.

His early childhood weight was <25th centile but gradually increased to 75th centile. Some improvement in height was seen with hormone therapy. Height is currently below the 3rd centile and weight is 75th centile. Weight for height (BMI) remains at greater than the 95th centile.

Laboratory findings include a normal karyotype and normal FISH for del(17)(p11.2). SMS195 has a heterozygous G5423A mutation causing a serine to asparagine change at amino acid 1808 in the RAI1 protein (Table 3, Fig. 2c, Fig. 4). He was adopted at 3 months of age; thus, parental DNAs were not available for sequencing. This mutation was not identified in >100 normal chromosomes.

SMS175

SMS175 is an 11 y old white female with mental retardation, progressive speech delays, stereotypic behavior, intractable complex seizures, and facial dysmorphisms. She was delivered vaginally at 42 weeks gestation weighing 4.1 Kg to a 33-year old G3P2A1 mother with two normal, living offspring. Behavioral problems were first noticed at age 9 months with explosive tantrums when distracted from “autistic-like” activity. She sat at 6 m and walked at 12 m, but speech was delayed and regressed at age 4 y when seizures began. Seizures were complex and involved grand mal, Jacksonian, and petit mal episodes occurring 10-30 times per month. Language, psychosocial, motor and cognitive skills are below 7th centile with IQ of 57 (Wechsler scale). Her behavior includes self-hugging, twirling of objects, biting and picking of toenails until they bleed, and polyembolokoilamania of rectum, umbilicus, ears, and mouth. She was frequently irritable in infancy, often associated with sleep disturbances. She is currently sedated as a component of seizure therapy but has no apparent abnormal sleep patterns. EEG showed 2.5-5 Hz delta activity with medium-high amplitude spikes, sharps and polyspikes seen over the right central parietal, right occipital and bifrontal regions, and at times diffusely over both sides. MRI showed a slight asymmetry in the gyral sulcation pattern along the left posterior frontal lobe in the precentral gyrus. She was diagnosed with microcephaly as an infant, with head circumference below 5th centile, but head growth gradually improved to the normal range. Height and weight remained at the 25th centile throughout early childhood, with gradual increases over time. Currently, on physical exam she is in the 75th centile for weight and height and the 50th centile for head circumference. She has a flat occiput and round face, like her mother’s. There is no midfacial hypoplasia (IC=3.3 cm; OC=6 cm; IP=5.5 cm). She has full lips with tenting of the upper lip. There are multiple small, non-pigmented papillae on her tongue. She is Tanner II-III in sexual development. She has mild acne of the face, two 2x3 cm café-au-lait spots on her trunk, and eczema behind the ears. She demonstrates self-stimulating and exclusionary concentration on coloring or twirling objects with oppositional behavior, including biting, if interrupted.

Laboratory data include a normal 46, XX karyotype with no evidence of 17p11.2 deletion. Total fasting cholesterol is 142mg/dl and triglyceride 155 mg/dl. Her RAI1 gene has a heterozygous adenine to guanine transition at nucleotide 4685 producing a missense substitution of an arginine
for a glutamine at amino acid 1562 (Table 3, Fig 2d, and Fig. 4). This Q1562R mutation was not seen in either parental DNA, nor has it been observed in more than 100 chromosomes.
Table 2. Phenotypic features of SMS patients with RAI1 mutations compared to those with 17p11.2 deletions.

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<thead>
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<tbody>
<tr>
<td>Mutation Identified</td>
<td></td>
<td>del(17)(p11.2)</td>
<td>RAI1 mutations</td>
<td>RAI1 mutations</td>
<td>253del 19 bp</td>
<td>3801delC</td>
<td>Ser1808Asn</td>
<td>Gin1562Arg</td>
<td>RAI1 mutations</td>
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<td>Craniofacial/skeletal</td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>Brachycephaly</td>
<td>89</td>
<td>3 / 3</td>
<td>1 / 2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>8 / 9</td>
<td>89</td>
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<td>Midface hypoplasia</td>
<td>93</td>
<td>0 / 3</td>
<td>2 / 2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>6 / 9</td>
<td>67</td>
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<tr>
<td>Prognathism (relative to age)</td>
<td>52</td>
<td>3 / 3</td>
<td>1 / 2</td>
<td>+</td>
<td>+</td>
<td>N</td>
<td>N</td>
<td>6 / 7</td>
<td>86</td>
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<tr>
<td>Tentated upper lip</td>
<td>73</td>
<td>3 / 3</td>
<td>2 / 2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>8 / 9</td>
<td>89</td>
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<tr>
<td>Broad, square face</td>
<td>81</td>
<td>2 / 3</td>
<td>2 / 2</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>8 / 9</td>
<td>89</td>
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<tr>
<td>Synophrys</td>
<td>62</td>
<td>2 / 3</td>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>3 / 7</td>
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<td>Cleft lip/palate</td>
<td>9</td>
<td>0 / 3</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0 / 9</td>
<td>0</td>
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<tr>
<td>Brachydactyly</td>
<td>85</td>
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<td>+</td>
<td>+</td>
<td>7 / 9</td>
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<td>Short stature (&lt;5th centile)</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>1 / 9</td>
<td>11</td>
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<tr>
<td>Scoliosis†</td>
<td>49-67</td>
<td>2 / 3</td>
<td>2 / 2</td>
<td>N</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4 / 8</td>
<td>50</td>
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<td>Otolaryngologic abnormalities</td>
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<tr>
<td>Chronic ear infections</td>
<td>85</td>
<td>2 / 3</td>
<td>1 / 2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4 / 9</td>
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<td>Hearing loss</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>1 / 9</td>
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<td>Hoarse, deep voice</td>
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<td>2 / 3</td>
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<td>+</td>
<td>+</td>
<td>6 / 7</td>
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<td>Neurological/behavioral</td>
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<tr>
<td>Variable mental retardation</td>
<td>100</td>
<td>3 / 3</td>
<td>2 / 2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>9 / 9</td>
<td>100</td>
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<tr>
<td>Speech delay†</td>
<td>&gt;90</td>
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<td>N</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>4 / 7</td>
<td>57</td>
</tr>
<tr>
<td>Motor delay†</td>
<td>&gt;90</td>
<td>1 / 3</td>
<td>N</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>4 / 7</td>
<td>57</td>
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<tr>
<td>Hypotonia</td>
<td>&gt;90</td>
<td>1 / 3</td>
<td>N</td>
<td>+</td>
<td>-</td>
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<td>+</td>
<td>4 / 7</td>
<td>57</td>
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<td>Seizures by history</td>
<td>11-30</td>
<td>1 / 3</td>
<td>0 / 2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>2 / 9</td>
<td>22</td>
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<tr>
<td>Sleep disturbance</td>
<td>70-100</td>
<td>3 / 3</td>
<td>2 / 2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>7 / 9</td>
<td>100</td>
</tr>
<tr>
<td>Self-hugging/hand-wringing‡</td>
<td>70-100</td>
<td>3 / 3</td>
<td>2 / 2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>9 / 9</td>
<td>100</td>
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<tr>
<td>Attention-seeking</td>
<td>80-100</td>
<td>3 / 3</td>
<td>2 / 2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>9 / 9</td>
<td>100</td>
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<tr>
<td>Self-injurious behaviors‡</td>
<td>78-96</td>
<td>3 / 3</td>
<td>2 / 2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>9 / 9</td>
<td>100</td>
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<tr>
<td>Onychotillomania</td>
<td>25-85</td>
<td>3 / 3</td>
<td>N</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>6 / 7</td>
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<td>Polyembolokoilomania</td>
<td>25-85</td>
<td>3 / 3</td>
<td>N</td>
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<td>+</td>
<td>+</td>
<td>7 / 7</td>
<td>100</td>
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<tr>
<td>Head-banging/face-slapping</td>
<td>71</td>
<td>2 / 3</td>
<td>N</td>
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<td>+</td>
<td>+</td>
<td>5 / 7</td>
<td>72</td>
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<td>77</td>
<td>2 / 3</td>
<td>N</td>
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<td>+</td>
<td>+</td>
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<td>Ocular abnormalities</td>
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<td>Myopia</td>
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<td>2 / 3</td>
<td>1 / 2</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>6 / 9</td>
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<td>Strabismus</td>
<td>50</td>
<td>2 / 3</td>
<td>1 / 2</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<td>Iris abnormalities</td>
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<td>+</td>
<td>-</td>
<td>1 / 9</td>
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<td>Other features</td>
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<td>Elevated cholesterol</td>
<td>&gt;50</td>
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<td>0 / 2</td>
<td>-</td>
<td>N</td>
<td>+</td>
<td>-</td>
<td>4 / 8</td>
<td>50</td>
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<tr>
<td>Structural cardiovascular anomaly</td>
<td>30</td>
<td>0 / 3</td>
<td>0 / 2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0 / 9</td>
<td>0</td>
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<tr>
<td>Structural renal anomaly</td>
<td>30</td>
<td>0 / 3</td>
<td>0 / 2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0 / 9</td>
<td>0</td>
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<td>Male</td>
<td>Male</td>
<td>Female</td>
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</tr>
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<td>Age at evaluation</td>
<td>14-19 y</td>
<td>4-14 y</td>
<td>14-17 y</td>
<td>11 y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

†Smith and Gropman 2005 (Ref 29).
‡Finucane 2001 (Ref 30).

+ Presence of clinical feature, – absence of clinical feature, N information is not available or child too young to evaluate.
Table 3. RAI1 mutations and polymorphisms identified in 4 SMS patients.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Nucleotide change*</th>
<th>Amino acid change</th>
<th>Polymorphisms†</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMS153</td>
<td>253del(19 bp)</td>
<td>deletion/frameshift</td>
<td>Pro664Pro‡ (homozygous) Ile1867Ile§ (heterozygous) (CAG)_{13}</td>
</tr>
<tr>
<td>SMS188</td>
<td>3801delC</td>
<td>frameshift</td>
<td>Gly90Ala** (heterozygous) Pro165Thr†† (heterozygous) Gln279Gln¶ (heterozygous) Pro664Pro (heterozygous) Arg1778Arg‡‡ (heterozygous) Ile1867Ile (heterozygous) (CAG)_{10/11}</td>
</tr>
<tr>
<td>SMS195</td>
<td>G5423A</td>
<td>Ser1808Asn</td>
<td>Pro664Pro (homozygous) Ile1867Ile (heterozygous) (CAG)_{10/11}</td>
</tr>
<tr>
<td>SMS175</td>
<td>A4685G</td>
<td>Gln1562Arg</td>
<td>Gln279Gln (heterozygous) Pro664Pro (heterozygous) Ile1867Ile (heterozygous) (CAG)_{9/10}</td>
</tr>
</tbody>
</table>

DNA changes with nucleotide number starting from the ATG in the coding sequence.
†NCBI SNP database: †SNP 8067439; ‡SNP 3818717; §SNP 11078398; ¶SNP 11649804; **SNP 3803763; ††SNP 11649804; ‡‡G to A change at nucleotide 5334, is a novel polymorphism not reported in the database. (CAG)_{X} represents the number of CAG repeats present on each allele.
DISCUSSION

We present 4 patients with mutations in the RAI1 gene each exhibiting features consistent with a diagnosis of SMS. These patients do not carry any detectable deletion involving 17p11.2 by FISH or G-banding analysis; however, all identified mutations in the RAI1 gene are de novo. Our original study identified 3 such patients [14], while work by Bi et al [28] studied 2 additional subjects bringing the total reported individuals with RAI1 mutations to 9. Even though the patients described have been evaluated by different clinicians from around the world, all individuals have very similar features and all were initially considered for a diagnosis of Smith-Magenis syndrome (see Fig. 1, Table 2, Fig. 4).

Consistent in the patients presented in this study and those presented previously [14] was a definitive delay in growth during early childhood, with weight and height for age gradually increasing to >50th centile in all the patients evaluated, with the exception of SMS195 who appears to be shorter for age. Sleep disturbances with a variety of manifestations, including difficulty getting to sleep, reduced REM sleep, frequent waking and increased daytime sleepiness and napping are noticed in all of the patients with RAI1 mutations. None of the patients evaluated had any systemic involvement, which still emphasizes our previous conclusion that haploinsufficiency of RAI1 is responsible for the behavioral, neurological, otolaryngological, and craniofacial features of the syndrome, but more variable features such as cleft lip/palate and heart and renal defects are probably due to hemizygosity of another gene(s) in the 17p11.2 region.[14] Some features, such as hypotonia, speech and motor delay, and hearing loss are observed less frequently or are less severe in the patients carrying RAI1 mutations than in those with 17p11.2 deletions (Table 2). It is important to note that even though all patients were previously evaluated for 17p11.2 deletions by G-banding techniques and FISH, we confirmed these reported findings with the RAI1-specific probe, as most commercially available probes do not include this gene.[13]

These patients carry de novo nucleotide changes that were absent in the parental DNAs, although germ-line mosaicism cannot be ruled out. The mutations reported cause a significant change in the amino acid sequence of the RAI1 protein that likely leads to the formation of abnormal or truncated protein or processing by nonsense-mediated decay. These data strengthen the hypothesis that haploinsufficiency and dosage imbalance of the RAI1 protein causes SMS. All mutations so far identified occur in exon 3 of RAI1 which codes for >98% of the protein, though a few polymorphisms are distributed in other exons. It is observed that the missense mutations alter amino acids in the highly conserved regions of the gene (Figs. 2, 3).

Except for a few polymorphisms that occurred in the homozygous state, all others occurred in heterozygous condition, and all of them have been previously reported in the SNP database (Table 3). The polymorphic CAG repeat region contained 10-13 repeats in this group of patients. The number of CAG repeats in RAI1 has been implicated in modulation of the susceptibility to schizophrenia, and one study showed correlation with the age of onset in spinocerebellar ataxia type 2 (SCA2).[17][18] The role of the polyglutamine repeat in any alteration of RAI1 function is unknown but may have its effects on normal neuronal development and/or function. Additional studies are required to fully understand any potential interaction. No polyglutamine expansion in RAI1 has been identified or reported.
RAI1 encodes a 1906-amino-acid protein with a predicted molecular weight of 203kDa (Fig. 3). The ~8.5 kb mRNA is expressed in all tissues studied so far and at high levels in the heart and neuronal structures.[14][15] This gene/protein is highly conserved through mammalian evolution (Fig. 3). The polyadenylation signal spans from nucleotides 7685-7641, and the gene has a large 3'-UTR. The polyserine signal at the carboxy-terminal end (residues 1628-1639) is composed of [S]3A[S]8, with stretches of 3 and 8 serines interrupted by an alanine (Fig. 3). This polyserine signal is similar to that found in both the DRPLA and the Drosophila hairless genes, both of which are involved in neuronal development.[19][20][21] These stretches of polyglutamine and polyserine have also been shown to be involved in transcription.[22] RAI1 has an extended PHD domain (residues 1823-1903) similar to that in the trithorax family of nuclear proteins which is involved in chromatin remodeling and transcriptional regulation (Fig. 3).[23][24][25] Further, amino acid sequence motifs representing 4 domains of the RAI1 protein are similar to the transcription factor stromelysin-1 platelet derived growth factor-responsive element binding protein, TCF20 (residues 1-6, 1315-1347, 1665-1710, 1823-1842; see Fig. 3).[19][26]

While it is postulated that RAI1 is a transcriptional regulator involved in neuronal development, its exact biochemical and functional role remains elusive. Studies in our laboratory (data not shown) indicate that RAI1-GFP fusion constructs are localized to the nucleus, consistent with a postulated function in transcription; however, further studies are required to fully understand its role in this process. A primary focus will be the identification of the pathways RAI1 modulates in the complex developmental and behavioral processes affected in Smith-Magenis syndrome.
ACKNOWLEDGEMENTS
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The authors declare no competing interests.

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This study was approved by the Michigan State University Committee on Research Involving Human Subjects and the Institutional Review Board of the Virginia Commonwealth University. Informed consent was obtained for each subject in this study. Permission to print photographs and to publish detailed patient information was obtained from the parents of each child prior to manuscript submission.
Figure legends.

Fig. 1. Flow chart for molecular evaluation of Smith-Magenis syndrome. Recommended procedure for diagnosis of Smith-Magenis syndrome is illustrated. In suspected cases of SMS, initial evaluation should include chromosomes, followed by FISH with an RAI1-containing probe. Non-deletion cases would then be referred for RAI1 sequencing analysis and any identified mutations would be confirmed de novo by investigation of parental samples.

Fig. 2. Smith-Magenis syndrome patients with RAI1 mutations. a. Photograph of SMS153, age 16 y. Electropherograms of both normal and mutant alleles are shown. The mutant allele contains a 19 bp deletion starting at nucleotide position 253. This mutation causes a frameshift mutation and truncation of the protein; b. Photograph of SMS188, age 12 y. Electropherogram shows 3801delC on one RAI1 allele, causing a frameshift mutation. c. Photograph of SMS195, age 14 y. Electropherogram shows a heterozygous transition mutation, G5423A, which causes a missense mutation, Ser1808Asn; d. SMS175, age 11 y. Electropherogram shows a heterozygous transition mutation, A4685G, causing a missense mutation, Gln1562Arg. Also shown for c and d are the conserved sequences across human, chimp, rat and mouse at the regions of the missense mutations illustrating that each missense mutation is located in highly conserved region of RAI1. This study was approved by the Virginia Commonwealth University Institutional Review Board, and informed consent was obtained for all subjects and permission granted for reproduction of photos.

Fig.3. RAI1 is highly conserved in mammalian species. Alignment of human RAI1 (NP_109590.3) chimp (Chr 19, uncurated sequence numbers from 17975106-18062618, UCSC Genome browser), rat (XP_220520.5) and mouse Rai1 (NP_033047.1) sequences. Human and mouse sequences are 84% identical and 88% similar, whereas the chimp sequence is 99.5% identical to human. Rat and humans share an identity of 84.4% and 88.5% in similarity. While the number of polyglutamines (***) ranges from 9-18 in humans (shown here at 14 repeats), mice and rats have 4 CAG repeats, while chimps carry 9 repeats. The polyserine repeats (……...), nuclear localization signal (-----), and the PHD domain (●●●●) are indicated. Sequences that are similar to the transcription factor, TCF-20 are indicated (+++ including amino acid residues 1823–1842 that lie in the PHD domain.

Fig. 4. Summary of mutations in RAI1 associated with Smith-Magenis syndrome. The primary RAI1 genomic structure with 6 exons is shown. All mutations reported to date are indicated. Mutations in this report¹ are depicted with previously reported mutations from Slager et al (2003)², and Bi et al (2004)³. The numbering of nucleotides is based on Genbank AY172136. The unfilled boxes represent non-coding regions, while the filled boxes represent the RAI1 coding region.
REFERENCES
19. Seranski P, Hoff C, Radelof U, et al. RAI1 is a novel polyglutamine encoding gene that is


Figure 1, Girirajan et al #031211

- **Characterization of deletion by FISH**
- **Clinical correlation**
- **Evaluation of parental samples**
- **Further clinical evaluation**
- **Sequencing of RAI1**
- **Mutation**
- **No mutation**

*RAI1* deletion

Patient sample

No deletion
a

SMS153

b

SMS188

c

SMS195

d

SMS175

Figure 2, Girirajan et al #031211

Normal allele

Deleted allele

253del 19

3801delC

G5423A

A4685G

H. sapiens 1803 RKHEC SKEAPAEPGG
P. troglodytes 1798 RKHEC SKEAPAEPGG
R. norvegicus 1783 RKHEC SKEAPAEPGG
M. musculus 1785 RKHEC SKEAPTEPGG

H. sapiens 1558 KRRRQQQVLPLDPAE
P. troglodytes 1553 KRRRQQQVLPLDPAE
R. norvegicus 1528 TRRRQQQVLPLDPAE
M. musculus 1527 TRRRQQQVLPLDPAE
Figure 4, Girirajan et al #031211

I II III IV V VI

ATG (CAG)_{9-18} 253del 191 1449del C^2 2773del 292 C2878T^3 3103ins C^3 3801del C^1 A4685G^1 5265del C^2 G5423A^1
RAI1 variations in Smith-Magenis syndrome patients without 17p11.2 deletions

Santhosh Girirajan, Louis J Elsas, II, Koenraad HMT Devriendt and Sarah H Elsea

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