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2009

RETINOIC ACID INDUCED 1 GENE ANALYSIS IN HUMANS AND ZEBRAFISH

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RETINOIC ACID INDUCED 1 GENE ANALYSIS IN HUMANS AND ZEBRAFISH

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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August, 2009

Acknowledgement

I would like to thank all the people who have supported and motivated me throughout my graduate studies. I had the great opportunity to work in Dr. Sarah Elsea's lab and I sincerely thank her for her willingness to help, patience and encouraging me after my failed experiments. I am also greatly thankful to Dr. Jim Lister for guiding and helping me every step of the way in finishing my zebrafish project. I also thank my third committee member, Dr. Andrew Davies for his project consultations, helpful alternatives and encouragement.

To all my lab members in Dr. Elsea's as well as Dr. Lister's lab, who have made these two years a fun experience, I have enjoyed getting to know you all. Without them, it would have been difficult and nearly impossible to bear all the failed experiments. I also thank Dr. Ying-Ping for all her help with troubleshooting the sequencing problems faced during the patient sequencing project.

Finally, my special thanks go to my family who has played the most important role in my life. Their constant help and unconditional love have brought me this far in my life and this would not have happened without their support. My parents, Dr. Akshay and Dr. Rama Vyas, sister Dr. Sejal Khilji and my fiancé, Saurav Bhatia have been source of inspiration and guidance in my life.

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Abstract

RETINOIC ACID INDUCED 1 GENE ANALYSIS IN HUMANS AND ZEBRAFISH

By Bijal Akshay Vyas, B.S.

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2009

Major Director: Dr. Sarah H. Elsea
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Smith-Magenis syndrome (SMS) is a complex mental retardation syndrome caused by deletion of 17p11.2 region or mutation of the *RAI1* gene (retinoic acid induced 1*).* Individuals with SMS typically exhibit speech and motor delays, mental retardation, characteristic craniofacial and skeletal anomalies, and a distinct neurobehavioral

phenotype that includes sleep disturbances, stereotypes, and maladaptive and self-injurious behaviors. RAI1 is thought to be a transcription factor modulating the expression of genes involved in a variety of cellular functions. Previous studies have shown the *RAI1* gene being induced by retinoic acid (RA), a derivative of vitamin A. RA plays a significant role in many processes such as immune function, neurogenesis and reproduction, and deprivation of RA causes craniofacial defects. We hypothesized that RA could be inducing *RAI1* which then acts as a transcription factor in modulating the expression of multiple genes. To understand the consequences of clinical variation of *RAI1* gene, we performed mutation screening and identified the first case of SMS without mental retardation. Using a zebrafish model, full-length *rai1* gene was cloned and spatial and temporal expression of *rai1* by *in-situ* hybridization was evaluated and the effect of RA on *rai1* expression was subsequently analyzed. The data show *rai1* expression in forebrain (diencephalon) and midbrain. A *rai1* antisense morpholino will eventually be created to perform knockdown studies and rescue experiments. These studies will help in determining the significance of the *rai1* gene, and its interacting molecular pathways responsible for growth, development, and behavior.

CHAPTER 1: Introduction

Smith-Magenis syndrome

Smith-Magenis syndrome (SMS, OMIM #182290) is a multiple congenital anomalies and mental retardation syndrome caused by an interstitial deletion of chromosome 17p11.2 including the retinoic acid induced 1 (*RAI1*) gene, or mutation of *RAI1*. Patients are diagnosed with SMS by their typical characteristics such as mental retardation, sleep disturbance, craniofacial and skeletal abnormalities, neurological and behavioral abnormalities, and ocular and otolaryngological abnormalities. Approximately 90% of cases have a fluorescent *in situ* hybridization (FISH) detectable 17p11.2 microdeletion ranging from ~1.5 to 9 Mb, while the remaining 10% have a mutation in *RAI1*. Almost all cases are sporadic. The incidence rate of this neurodevelopmental disorder is estimated to be 1:15,000-25,000 births, but it seems likely that these figures represent an underestimate (10).

Craniofacial/ Skeletal abnormalities

SMS patients exhibit distinctive craniofacial and skeletal anomalies that include brachycephaly, a broad square-shaped face, midface hypoplasia, synophrys, upslanting palpebral fissures, deep set eyes, everted upper lip with tented appearance, micrognathia

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in infancy, relative prognathism with age (1), brachydactyly, short stature and scoliosis. Short stature ($< 5^{th}$ percentile) is observed in SMS patients during their childhood but with time, most individuals reach 10th-25th percentile during adulthood (10, 15). Other skeletal abnormalities include 5th finger clinodactyly, 2-3 toe syndactyly, polydactyly and vertebral anomalies. (22, 29, 43)

Neurological/ Behavioral abnormalities

All individuals with SMS show hypotonia in infancy and childhood, motor and speech delay, and ultimately some degree of mental retardation (usually in the mild to moderate range). The behavioral phenotype is also very characteristic and includes twirling objects, hand-wriggling, hyperactivity, attention-seeking, temper tantrums, outbursts, aggression (7, 41) and self-injurious behaviors such as self-biting, polyembolokoilamania (insertion of foreign objects into body orifices), onychotillomia (pulling out fingernails and toenails), skin-picking, finger-chewing and head-banging (10). Specific stereotypes of the syndrome are the spasmodic upper body squeeze or "self-hugging" and the "lick and flip" behavior that is hand-licking and page-flipping (7, 12). Moreover, one of the most telling clinical features is the presence of sleep disturbances with inverted circadian rhythm of melatonin (36). The sleep problems experienced by SMS patients include difficulties falling and staying asleep, early awakenings, excessive daytime sleepiness, daytime napping, snoring, bed-wetting, and diminished rapid eye movement (REM) sleep (8, 20, 36).

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Ocular/ Otolaryngological abnormalities

Ocular abnormalities such as strabismus, myopia, microcornea, iris abnormalities are observed in SMS patients. Along with these, otolaryngological abnormalities that include hearing loss, hoarse deep voice, chronic ear infections and vocal cord nodules and polyps are also quite common (10, 22). Abnormalities such as hearing loss, which varies from being mild to moderate, could be a result of the ear infections seen in these patients (21, 22).

Systemic defects and other manifestations

Other features noted in SMS patients include cardiovascular anomalies, renal abnormalities, pes-planus (flat feet), hypercholesterolemia, immunoglobin deficiencies (IgA, IgG and IgE), and dental abnormalities (8, 10, 22). Cardiovascular anomalies seen in 30% of the SMS patients include ventricular and atrial septal defect, mild stenosis, tricuspid stenosis, aortic stenosis, pulmonary stenosis, tricuspid and mild regurgitation, mitral valve prolapse, tetrology of Fallot, and total anomalous pulmonary venous return (8, 11). SMS patients also reported with renal abnormalities such as enlarged kidneys, duplication of the collecting duct, ectopic kidneys, kidney stones and urinary tract infections (8, 22). These individuals have been reported to have dental abnormalities such as taurodontism and tooth agenesis (45).

Retinoic acid induced 1

Role of the *RAI1* gene

RAI1 (Genbank AY172136; OMIM*607642) maps to the central portion of the 17p11.2 region, which is defined as the SMS critical region (Fig. 1). The *RAI1* gene is

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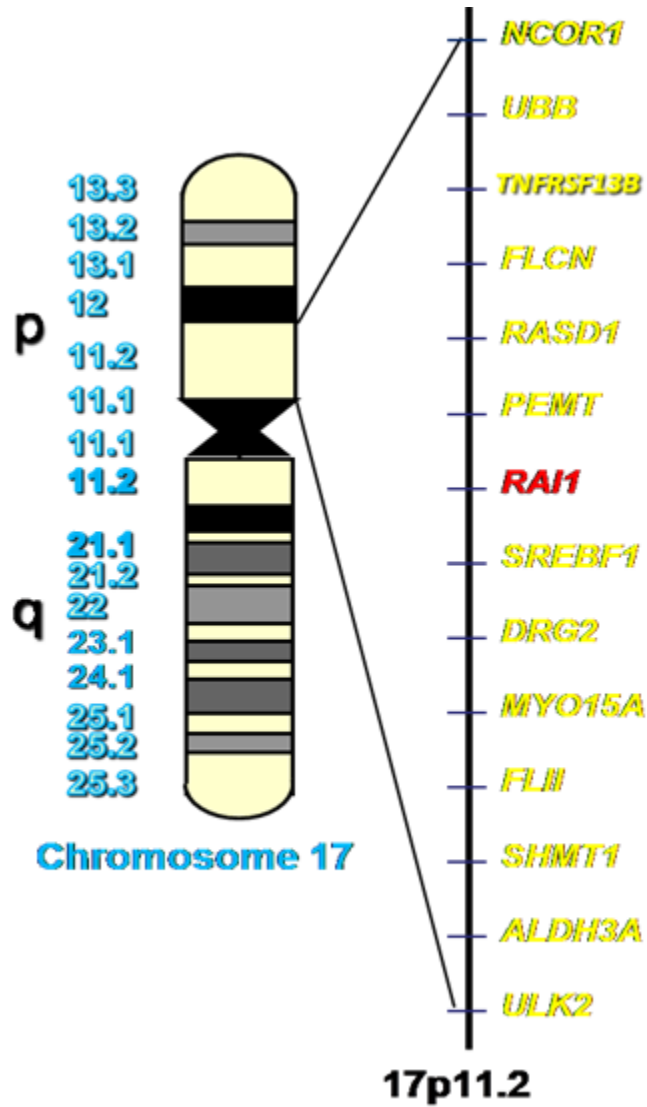
Fig.1 Schematic of chromosome 17. Map of the SMS critical region, including the *RAI1* gene. Deletion of the 17p11.2 region or mutation of the *RAI1* gene causes Smith-Magenis syndrome.

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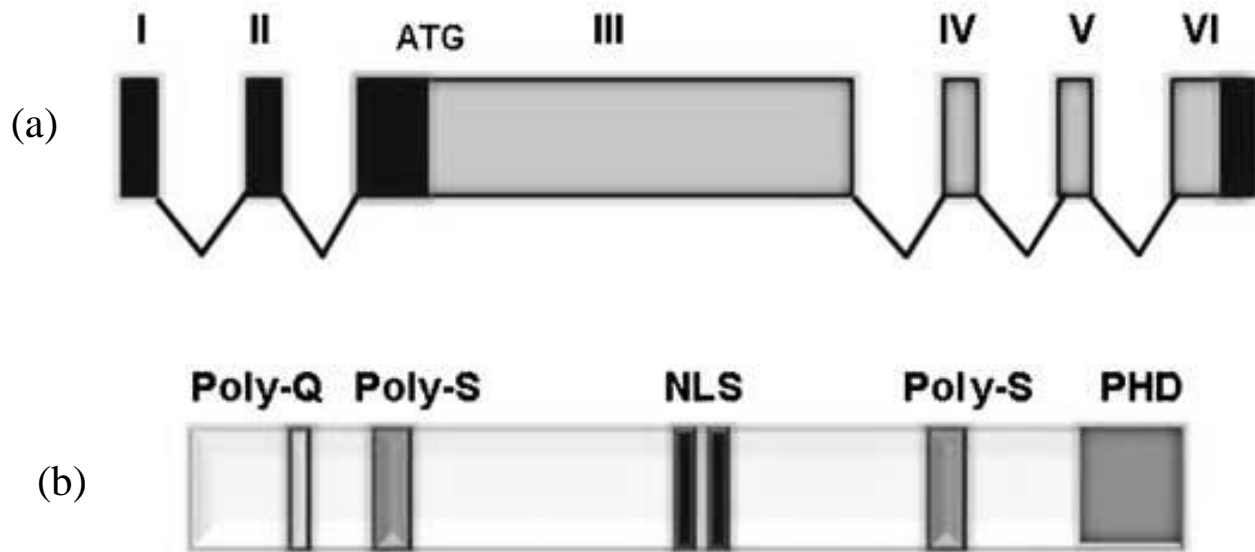
Fig.2 Genomic and protein structure of *RAI1*. (a) The genomic structure of the *RAI1* gene which consists of 6 exons, of which 3-6 are coding exons. *RAI1* gene is composed of ~5721 bp. (b) The protein structure of *RAI1* composed of 1906 amino acids and includes a polyglutamine (Poly-Q), polyserine (Poly-S), a bipartite nuclear localization signal (NLS), and C-terminal plant homeodomain (PHD).

Source: Elsea and Girirajan 2008

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made up of 6 exons, of which 3-6 are coding exons (Fig. 2). The *RAI1* gene generates ~7.6 kb mRNA and encodes a 1906 amino acid protein with a molecular weight of 203 kDa (Fig. 2). This gene was first identified in mouse pluripotent embryonic carcinoma cells (P19). These cells were treated with retinoic acid (RA) which lead to neuronal differentiation and also induced the upregulation of the *Rai1* gene (26). The alignment of the human *RAI1* gene sequence with other mammalian species showed the highly conserved nature of this gene (15). Comparing human with mouse indicated *RAI1* to be a homologue of the mouse *Rai1*, sharing 84% identity which suggests that human *RAI1* could be induced by RA in similar fashion and this induction contributes towards the neuronal development seen in SMS.

Furthermore, this gene contains a polyadenylation signal and a large 3'- UTR. The RAI1 protein contains a bipartite nuclear localization signal, polyglutamine and polyserine tracts and a plant homeodomain (PHD)/ zinc finger domain at the C-terminus (10, 46) (Fig. 2). The polyserine tract at the carboxy terminal is composed of [S]3A[S]8 and is similar to the motifs found in *DRPLA* and *Drosophila* hairless genes (10). These motifs have been shown to be involved in development of the nervous system and also in transcription of genes (32, 39). The PHD domain of the RAI1 protein is also similar to the trithorax family of the nuclear proteins which is involved in chromatin remodeling and regulating transcription. In addition to this, the amino acid sequence motifs such as bipartite nuclear localization signal and PHD domain are found to be related to the transcription factor stromelysin-1 platelet derived growth-factor-responsive element binding protein (TCF20) (10, 39). This suggests that RA could be inducing the *RAI1*

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gene which then acts as a transcription factor in modulating the expression of genes involved in a variety of cellular functions (2).

Retinoic acid

Retinoic acid (RA) is a derivative of vitamin A and plays an important role in cellular growth, differentiation, embryogenesis, vision, immune function and reproduction (30, 38). Plants and microorganisms synthesize and store carotenoids, a precursor of vitamin A. Animals obtain these precursors by the intake of plants and convert them into retinal using retinol dehydrogenases, and then oxidize retinal into RA irreversibly using retinal dehydrogenases (Raldh) (6).

These RA molecules bind to the transcription factors/receptors known as retinoic acid receptors (RARs) as they are not capable of binding directly to the gene (38). The receptors then become activated by the binding of all-trans retinoic acid and this RA/RAR complex regulate gene expression by binding to short DNA sequences (enhancer elements) in the vicinity of the target gene (13, 33, 35). This hypothesis was illustrated in an experiment done by Imai et al. in 1995, where the *Rai1* gene was induced in P19 mouse cells upon RA treatment. The upregulation of the *Rai1* gene and differentiation of neurons in mice by the induction of RA suggests that RA could be playing an indirect role in causing neurological features seen in SMS.

CHAPTER 2: *RAI1* variations in SMS patients and refinement of sequencing methodology

Introduction

Smith-Magenis syndrome was diagnosed in a patient for the first time by Ann Smith and Ellen Magenis in 1986 (43). This syndrome is caused by an interstitial deletion of 17p11.2 region or a mutation in the *RAI1* gene. The deletion was identified using a standard cytogenetic Giemsa staining (G-banding) technique. Although with time, techniques such as fluorescent *in-situ* hybridization (FISH) came into practice which made the identification of this deletion possible. With this technique, it was possible to map the deletions of various sizes on the 17p11.2 region and delineate the SMS critical region. The size of the deletions seen in SMS patient varies from ~1.5 Mb to ~9 Mb (48). Approximately, 75% of these patients have a common deletion of ~ 3.7 Mb. The remaining patients have smaller or larger deletions. The SMS critical region was further delineated to ~650 kb (47) that contained about 25 genes, including *RAI1*.

Along with this cohort of patients with a detectable 17p11.2 deletion analyzed by FISH, there were a few patients that were negative for the deletion but exhibited SMS phenotype. In 1995, Imai *et al.* had shown upregulation of the *Rai1* gene (GT2) and differentiation of neurons by the induction of RA, suggesting that the absence of RA

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could be a cause of suppression of the *RAI1* gene and the neurological features seen in SMS. As the *RAI1* gene lies in the SMS critical region, it was hypothesized that a deleterious change of nucleotide in this gene could result in SMS. Several patients with no 17p11.2 deletion but with SMS features were identified in Slager *et al.* (2003), Bi *et al.* (2004), Bi *et al.* (2006), Girirajan *et al.* (2005) and Girirajan *et al.* (2006), Lily Truong. (2008), GeneDx, and in the present report (Table 1). These patients were then analyzed by sequencing the *RAI1* gene to detect the variations as compared to the normal *RAI1* sequence. Sequence alignment identified point mutations in all of these patients. *RAI1* mutations are usually *de novo* as they are not present in the parental samples. If the mutations identified in SMS individuals are also present in their parents, then they are considered to be familial changes. Seven cases of familial changes have been previously identified (Table 2). Although the SMS individuals have inherited the changes maternally or paternally, there is no expressivity of SMS symptoms in their mother/father. We are unsure if these nucleotide changes are a result of reduced penetrance, mosaicism present in the parents, or a mutation in another locus that could be contributing in causing SMS. Hence, further functional studies are required to address these questions.

All the mutations identified until now are located on exon 3 of the *RAI1* gene. This exon is the largest exon in this gene and encodes > 98% of the RAI1 protein (Fig. 2a). Any point mutation or microdeletion in *RAI1* gene usually results in a frameshift mutation which produces a truncated and non-functional protein, which is probably degraded by a post-transcriptional process called nonsense mediated decay. This reveals

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Table 1. Summary of mutations in the *RAI1* gene.

Nucleotide Change	Amino Acid Change	Remarks
c.253del19	p.Leu85fsX60	Deletion/frameshift
c.1119delC	p.Ser373fsX65	Deletion/frameshift
c.1449delC	p.Pro483fsX34	Deletion/frameshift
c.1974G>A	p.Trp658X	Nonsense mutation
c.2773del29	p.Val925fsX8	Deletion/frameshift
c.2878C>T	p.Arg960X	Nonsense mutation
c.3103insC	p.Gln1035fsX30	Insertion/frameshift
c.3103insC	p.Gln1035fsX30	Insertion/frameshift
c.3103delC	p.Gln1035fsX28	Deletion/frameshift
c.3103delC	p.Gln1035fsX28	Deletion/frameshift
c.2966delAAGA	p.Lys989fsX38	Deletion/frameshift
c.3634A>G	p.Ser1212Gly	Missense mutation
c.3801delC	p.Pro1267fsX46	Deletion/frameshift
c.4649delC	p.Ser1550fsX36	Deletion/frameshift
c.4685A>G	p.Gln1562Arg	Missense mutation
c.4813A>T	p.Arg1604X	Nonsense mutation
c.4933delGCCG	p.Ala1645fsX35	Deletion/frameshift
c.5423G>A	p.Ser1808Asn	Missense mutation
c.5265delC	p.Pro1755fsX74	Deletion/frameshift
c.5280C>T	p.Arg1604X	Nonsense mutation

Table 2. Summary of familial changes in the *RAI1* gene.

Nucleotide change	Amino-acid change	Parent of Origin	Affected siblings
c.1142C>T	p.Ala381Val	Father	Sister
c.1867G>A	p.Asp622Asn	Mother	
c.3634A>G	p.Ser1212Gly	Father	Sister
c.5412T>C	p.Ser1648Pro	Father	
(PolyQ)$_{18}$	18 CAG repeats	Mother	
c.3781delGAG	p.Glu1261del	Mother	Brother
c.4051G>T	p.Ala1351Ser	Father	
c.4685A>T	p.Glu1562Leu	De novo/Pat DNA not tested	
c.3781delGAG	p.Glu1261del	De novo/Pat DNA not tested	
c.223G>T	p.Ala75Ser	Mother	

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the importance of the functional *RAI1* gene and its potential dosage dependent effect that could result in SMS features.

Materials and methods

Patient ascertainment and samples

Twelve patients exhibiting SMS phenotypes were referred through geneticists from various parts of the world and their DNA/blood/saliva samples were sent to Dr. Sarah Elsea's laboratory for research purposes. The Institutional Review Board of the Virginia Commonwealth University approved this study. Informed consent for genetic testing was obtained from the patient and from the patient's parents. A clinical survey was also requested that covered clinical and physical examination results such as individual's height, weight, head circumference, ENT reports, spine X-ray, podoscope of hands and feet, immunological tests, echocardiography, and renal ultrasound. Most of the information regarding the behavioral features was obtained from the parental survey while the clinical information was based on the geneticist reports. Short stature was measured relative to their parents or siblings height. If their height measurement was less than 5th percentile, then the SMS individual was considered to have a short stature. The algorithm followed for the diagnosis of SMS is illustrated in Fig. 3.

DNA isolation from whole blood

Genomic DNA was extracted from peripheral blood samples using the DNA mini-kit by Qiagen (Valencia, California, USA). The isolated DNA was quantified by a spectrophotometer at the absorbance ratio of 260/280.

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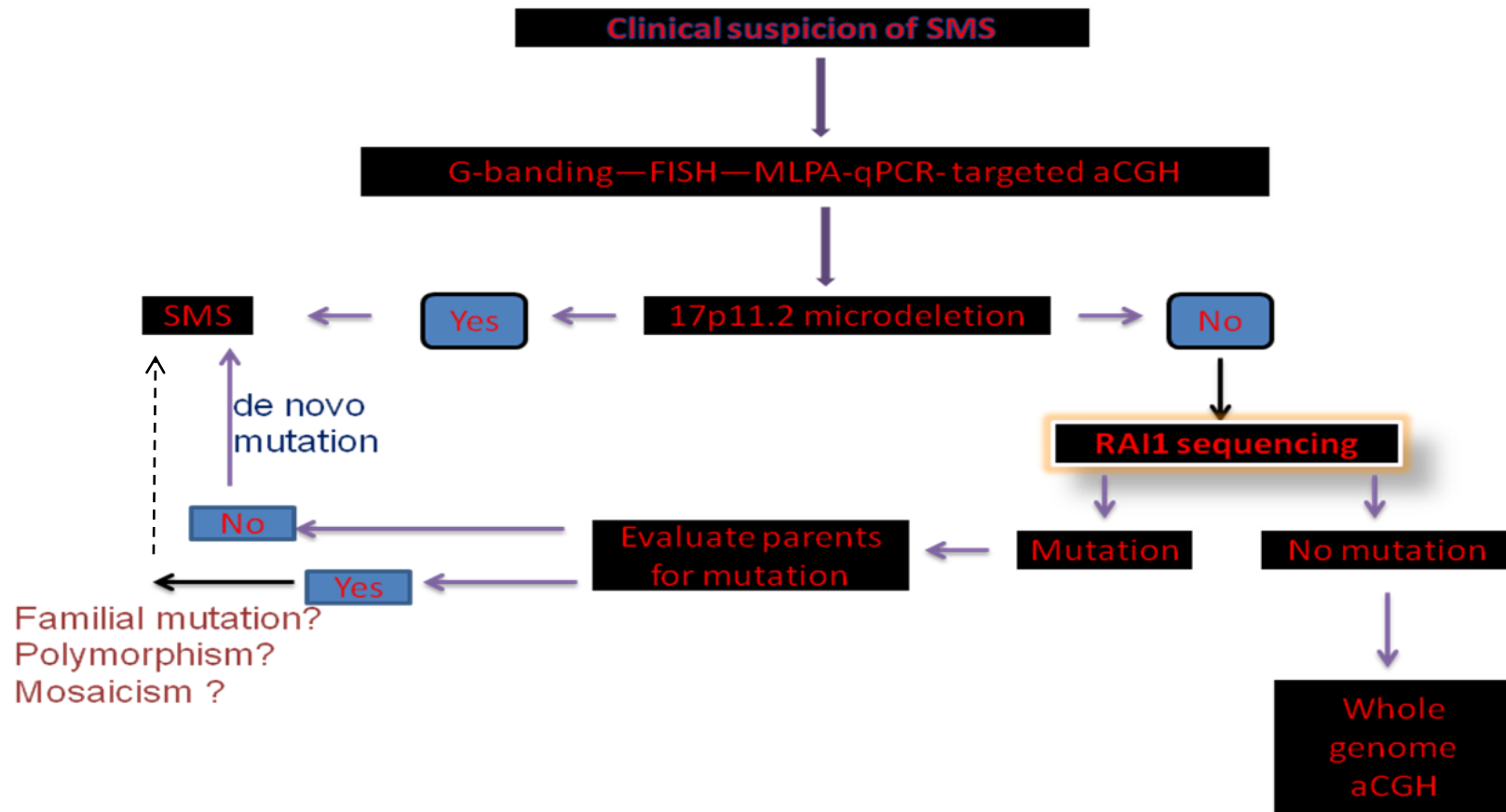
Fig. 3. Algorithm used for the diagnosis of SMS. A flow chart illustrating the procedure used for the diagnosis of SMS. Patients with clinical suspicion of SMS were evaluated for 17p11.2 deletions. Non-deletion cases were referred for *RAI1* mutation screening and if mutations were identified, their parental samples were evaluated to define the mutation as *de novo* or familial.

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DNA isolation from saliva

Genomic DNA was isolated from patient saliva samples using the protocol for manual purification of DNA from saliva by Oragene®. The isolated DNA was measured by a spectrophotometer at the absorbance ratio of 260/280.

Polymerase chain reaction

Polymerase chain reaction (PCR) was carried out on patient DNA by using overlapping primers, spanning exons 3-6 of the *RAI1* coding region, as shown in Table 3. PCR reactions contained the following: 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl and 1.5 mM $MgCl_2$), 20 pmol of each forward and reverse primers (Table 3), 200 μM dNTPs, 1 U of Taq DNA polymerase, and 50 ng genomic DNA in a total reaction volume of 25 μl. Cycling conditions were as follows: initiation at 94°C, 5 min, followed by 35 cycles of denaturing at 94°C, 1 min, annealing at 55-66°C, 1 min (Table 3), and extension at 72°C, 1 min, followed by a final elongation step at 72°C for 10 min. PCR products were run on a 1% agarose gel to assess quality. PCR products were either purified by gel extraction using the Qiagen Gel Extraction kit (Qiagen Inc, Valencia, California, USA) or by digesting with 2 U of shrimp alkaline phosphatase (SAP) and 10 U of exonuclease I (Exo) for a combined 7 μl reaction volume (USB Corporation, Cleveland, Ohio, USA) at 37°C for 30 min, followed by an 80°C incubation for 15 min to inactivate ExoSAP-IT.

Sequencing

DNA sequencing of these products was performed at the Virginia Commonwealth

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Table 3. PCR primers for *RAI1* sequencing.*

Exon	Forward primer	Reverse primer	Product size (bp)	Ann. temp.
Start E3	SHE323: TCTGAGGCAAAAGGAAGTGG	SHE324: GACTGGGAATGCTGAGGAAA	770	62ºC
3	SHE505: CCCGAGTCATGCAGTCTTTT	SHE506: TGGTAATGGAGGGTTTCCTG	925	60ºC
3	SHE507: GGTCCAGAATCTTCATGCCTA	SHE508: CATGTCGTCAGGAGAGGTCA	947	62ºC
3	SHE509: CTGCCAGGGTCAACAGCAAC	SHE510: CTCCTCCTCAGCTTCCAGGT	995	64ºC
3	SHE511: AGGAGGACCTGGAAGCTGAG	SHE512: GTGACCGAAGGATCATGGAG	861	66ºC
3	SHE513: GCTCCAAGACCAAGGAGACA	SHE514: GCCTCGGTTTTGAAACAGTCA	910	62ºC
3	SHE515: GGCCTCTCCTGGTAATCCTC	SHE516: GCAGCAAACAAGGCAAGAGGT	1010	64ºC
3	SHE335: AAACCGAGGCCTTCACATC	SHE336: CACACTCGGGACCTTTGAGT	964	60ºC
end E3	SHE339: CTACCCTGAACACTGCCTCC	SHE340: AGAGGCAATGGAGACAGGAA	887	60ºC
4	SHE527: CCAGCCTGTAAAGCTTGAGG	SHE528: GGAATGCCTCATTTCCATGT	362	62ºC
5	RA46: GGAGTGGAGTGGAGTGTGGAGG	RA45: GGACTGTGAAGGAGGTGCGAG	310	64ºC
6+3'UTR	SHE345: ACTGTGAAGTCCGAGGTCGT	SHE346: GACTGGAAAGGGGACAAACA	766	62ºC[+]

*Primers cover the coding region of *RAI1 isoform a.*
[+]Amplification requires Invitrogen® PCR enhancer.
PCR, polymerase chain reaction; Ann. temp., annealing temperature; UTR, untranslated region.

University Sequencing Core by cycle sequencing. Purified PCR product (10 ng/µl), 20 ng/µl of each primer, and 4 µl of the ABI Prism terminator cycle sequencing ready reaction mix (Applied Biosystems, Foster City, California, USA) were combined in 10 µl final volume for 25 cycles. The samples were then cleaned with filter paper, resuspended in formamide, denatured at 95°C for 2 min, and loaded onto an ABI3730 genetic analyzer (Applied Biotech/Hitachi).

Sequence alignment and analysis

The chromatogram results were received from the sequencing core and analyzed by comparing patient sequencing results with the normal *RAI1* sequence visually by looking for heterozygosity and sequence quality and also by aligning the patient sequence with the *RAI1* mRNA database sequence at the NCBI and Genome Browser using Clustal X and Bioedit. Forward and reverse primer sequencing results were used to confirm the presence of any variation identified in the patient. Parental samples were evaluated for any identified mutation.

Results

Clinical and molecular features of patients with *RAI1* variations

Twelve patients with negative FISH results for 17p11.2 deletion were tested for *RAI1* variations such as SNPs and mutations by sequencing of the *RAI1* gene. Most of them were identified with SNPs that were known and listed in the database. Four of these patients were identified with novel SNPs, and two of these SNPs were located in the intronic region and are of unknown significance (Table 4). These polymorphisms

have been identified in at least 1% of the normal population and are considered to be non-disease causing. However, we are unaware of their potential effect on SMS phenotype and hence, further studies have to be performed to study their role in SMS. Besides the identification of novel SNPs, we also identified the first SMS patient with an *RAI1* gene mutation that does not show frank mental retardation. Detailed molecular and clinical information of these patients are mentioned below.

Table 4. Novel *RAI1* mutations and polymorphisms identified in present study.

Patient	Novel Mutation	Amino acid change	Novel SNPs	Amino acid change
SMS339	**c.2966delAAGA**	**p.Lys989fsX38**	**c.2773C>A**	**p.Val925Ile**
SMS349			**c.3357C>T**	**p.Ser1119Ser**
SMS346			**c.5710-22G>T**	**N/A**
SMS351			**c.5710-49C>T**	**N/A**

SMS331

SMS331 is a 7 year old male of African American descent and had been evaluated since he was 17 months old. He exhibited SMS features such as sleep disturbance, mental retardation, speech delay, development delay, hypotonia, broad nasal bridge, upturned nose, tented upper lip, strabismus, vision impairment and chronic ear infections.

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His self-injurious behavior included head-banging, face-slapping and self-biting. At age of 17 months, his weight was 21.3 kg and height was 113.2 cm, which is ~ 75th percentile of weight/height scale.

Laboratory findings show normal karyotype (46 XY), negative fragile X studies, normal FISH for del(17)(p11.2) and normal aCGH. *RAI1* sequencing identified four polymorphisms, three of them were in heterozygous state and all of them were reported in the SNP database (Table 5). These nucleotide changes were not deleterious and therefore, non-disease causing mutations. No other abnormalities in the *RAI1* gene were identified in this patient.

SMS332

SMS332 is 11 year old Caucasian female with a history of cognitive impairment, behavioral problems, and seizures. She was born at 37 weeks gestation by vaginal delivery with a birth weight of 5 pounds; 10 ounces (25th- 50th percentile), a birth length of 19 inches (50th – 75th percentile), and a head circumference of 12.5 inches (10th- 25th percentile). She had suffered from jaundice after birth. Her behavioral problems included polyembolokoilomania, repetitive movements, food obsession and sleep disturbances. She also exhibited other features consistent with SMS such as midface hypoplasia, a broad, square face, tented upper lip, mental retardation, speech and motor delay.

Her family history was quite complicated. She had two brothers and the older one was diagnosed with pervasive developmental disorder. The patient's mother had a sister whose son was diagnosed with Down syndrome. Her grandmother committed

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Table 5. Known polymorphisms identified in present study.

	c.269G>C	c.493C>A	c.837G>A	c.1992G>A	c.4311T>C	c.4530C>T	c.5334G>A	c.5601T>C	Poly Q Allele 1	Poly Q Allele 2
SMS331				2	1	1		1	14	14
SMS332	1	1	1	1			1	1	13	14
SMS336	1			2	1	1	1		13	14
SMS339	2	2	2						13	13
SMS343								2	14	14
SMS344	1	1	2						10	13
SMS345				2				2	14	14
SMS346				2				2	14	14
SMS348	1	1	1	1				1	13	14
SMS349				2	1	1		1	12	13
SMS351				1				2	14	14
SMS356	1	1	1	1				1	13	13

1 – SNPs present in the heterozygous state

2 – SNPs present in the homozygous state

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suicide and had a history of depression. Her maternal aunt was reported with myasthenia gravis. The patient's maternal grandfather had 2 brothers who died from heart problems. Her father's sister died of diabetes and renal failure. Her paternal uncle had a son with autism.

Laboratory data included normal female karyotype, normal FISH for del(17)(p11.2), normal aCGH and negative result for Prader-Willi and Angelman syndrome (tested by methylation specific PCR for *SNRPN* gene). Mutation screening analysis identified six polymorphisms in SMS332, and all of them were in heterozygous state (Table 5). No other *RAI1* variations were identified in this patient's sequencing results.

SMS336

SMS336 is 5 year old female of Caucasian descent. She was evaluated at 3.5 years of age due to her unusual growth delay features. She was born at 37 weeks via normal spontaneous vaginal delivery to her 21 year old G_1P_o mother. During pregnancy, her mother had suffered from frequent UTIs, hypertension, group B strep and decreased movement towards the end of pregnancy. Patient had experienced multiple episodes of otitis media which affected her hearing, and she required the placement of bilateral myringotomy tubes. She exhibited some SMS features such as sleep disturbances, aggressiveness, language delay, temper tantrums, licking her hands, and polyembolokoilomania. At age 3, height (35.4 inches) and weight (28 pounds) were below the 5th percentile. Her physical features included frontal bossing of the forehead, up-slanted eyes and up-turned nose.

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Laboratory data included a normal karyotype. *RAI1* sequencing identified five polymorphisms, and four of them were in heterozygous state (Table 5). Additional aCGH studies revealed a duplication of 4p16.1 region.

SMS339

SMS339, referred for genetic evaluation at 14 years of age, is a female of Caucasian descent (both parents originate from South Italy) (Fig. 4 and 5). She was the second child of healthy non-consanguineous parents, and she had a healthy female sibling. Her parents had experienced three episodes of spontaneous abortion, all during the 1st trimester of gestation. Due to the history of recurrent miscarriages, karyotype analysis was performed on her parents, and results were normal. The family history was otherwise unremarkable.

The patient was born at term by normal vaginal delivery. Birth weight was 3,660 g (75th centile), length 50.5 cm (25-50th centile) and OFC 35 cm (50th centile). Apgar scores were 8 at 1 min and 9 at 5 mins. She had mild hypotonia during neonatal period and early infancy but showed normal motor development. Daytime continence was achieved at 5 years, and nocturnal enuresis persisted until the age of 6. The patient suffered from recurrent episodes of acute otitis media until 6 years of age, when a tonsillectomy was performed. At age 7 years, an audiometric evaluation excluded the presence of hearing loss. At age 10 years, an episode characterized by jerking movements of the upper and lower extremities was interpreted as febrile convulsions. The body temperature was 39°C, and subsequent EEG tests showed no abnormalities. At

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the age of 11 years, she underwent orthodontic treatment to resolve dental crowding. She attained menarche at 13 years with regular periods since then.

Evaluation at age 14 years revealed a broad face, bushy eyebrows with mild synophrys, upslanting palpebral fissures, full-tipped nose, mild retrognathia, hoarse voice, dry skin, overeating and mildly overweight (body mass index = 25.9), tricuspid regurgitation, and a history of constipation and recurrent urinary tract infections. Her body weight fell between 75th and 90th centile (62.5 kg), height between 10th and 25th centile (155.4 cm), and head circumference was at +1.2 standard deviations (55.8 cm). Fig. 4 shows her SMS features observed since childhood, and Fig. 5 (A-D) shows whole body pictures and the facial appearance of the patient at age 14 years. Skeletal anomalies included brachydactyly of fingers and especially toes, proximal cutaneous syndactyly of toes 2-3 (Fig. 6A-C), pes planus (Fig. 6D), and dorsolumbar scoliosis (Fig. 6E). Scoliosis was diagnosed at 8 years of age and became more severe over time. The compliance of the patient with the physiotherapy was very low, and she firmly refused to wear the orthopedic brace.

The patient had been evaluated by psychologists on several occasions using the Wechsler Intelligence Scale for Children (WISC-R). At 10 years, 9 months of age, her IQ was 82, while at 11 years, 8 months of age, the IQ was 78. No differences in verbal or performance IQ were noted. However, a repeat examination at 11 years, 10 months revealed a total IQ of 91, with verbal IQ at 100 and performance IQ at 84. At the present age of 17 years, the patient was again evaluated by the WISC-R and had a total IQ score of 76 with no differences in verbal or performance IQ; however, the psychologist noted

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significantly reduced motivation and collaboration in this examination with concerns that the test results did not support her true abilities. Despite some learning disabilities, a certain weakness in sequential processing, and poor short-term memory, she was able to attend school until the age of 12 years without the help of dedicated persons. Other neurobehavioral features included weak relational and social skills, tendency to depressed mood, obsessive thinking, stereotypies, and maladaptive and self-injurious behaviors, which worsened with age (especially since the onset of puberty). The obsessive thinking consisted of constant fear to die, to be abandoned, and to have been adopted. The maladaptive behaviors included temper tantrums, disobedience, impulsivity, verbal aggressiveness, attention-seeking, and prolonged crying. She showed self-abusive behaviors such as skin-picking of the legs, nail-yanking, self-hugging, polyembolokoilamania, cutting locks of her own hair, tearing away her eyebrows, writing or painting the skin, mouthing of hands and objects, and grinding of the teeth. Moreover, the patient had significant sleep disturbance, which consisted of difficulties falling asleep, prolonged night-time awakenings, and excessive daytime sleepiness since the age of 1 year.

The patient's karyotype (high resolution G-banding) was 46, XX. Genetic testing for fragile X syndrome (GC-rich PCR and Southern blot analysis) and molecular cytogenetic analysis for the detection of SMS deletions (FISH, Cytocell probe) gave negative results. Screening for variations in the *RAI1* coding region identified a significant nucleotide change in exon 3 that included a deletion of 4 bp starting at nucleotide 2966 (Fig. 7). This heterozygous novel deletion resulted in a frameshift

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Fig. 4. The changing face of SMS339. Smith-Magenis syndrome (SMS) phenotype observed in SMS339 since childhood. **A.** 7 months old. **B.** 3 years old. **A.-B.** Note broad forehead, upslanted palpebral fissures, open mouth posture, tented upper lip, and full-tipped and up-turned nose. **C-D.** Note broad nasal bridge. **E.** Note mild brachydactyly. **F.** 10 y, note broad nasal bridge and full lips.
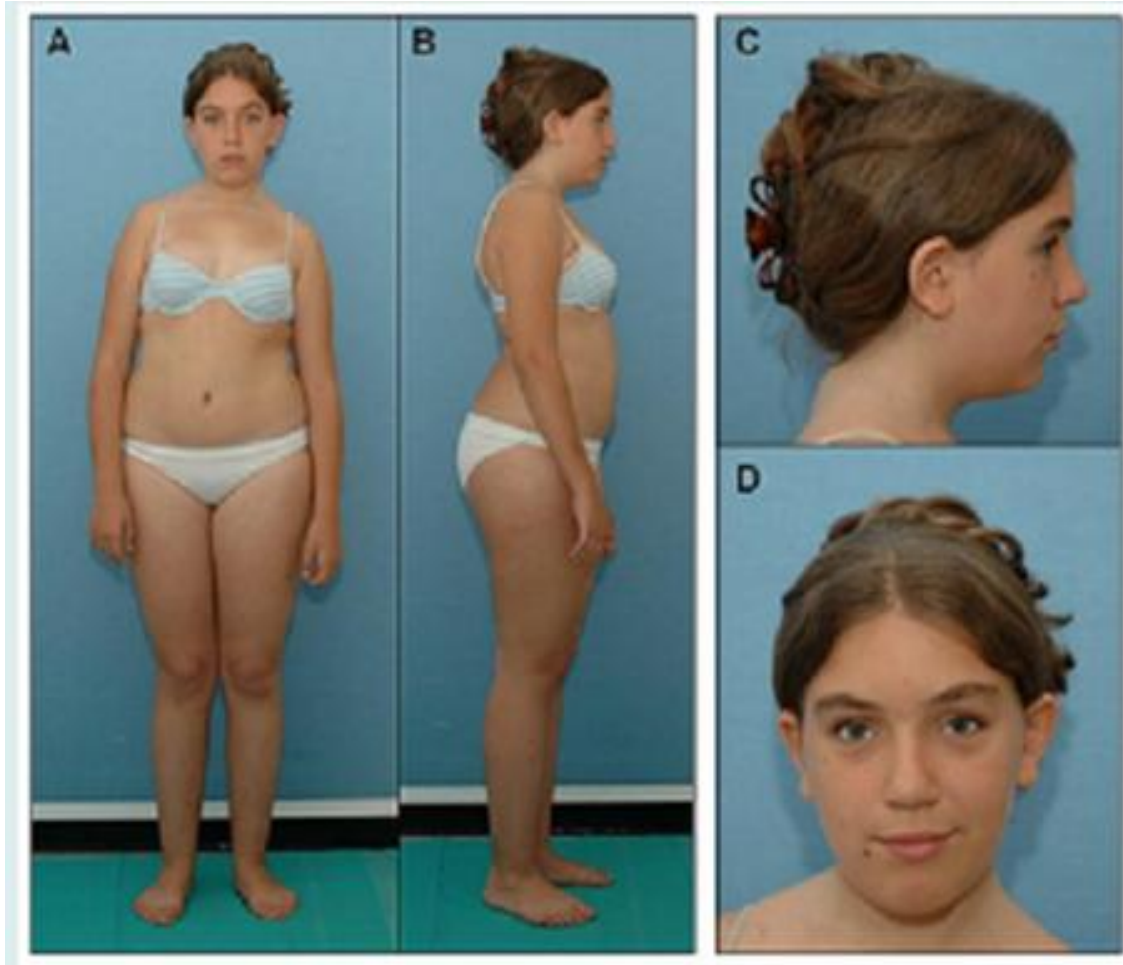
Fig. 5. Photos of SMS339 at age 14 years. A.-B. Whole body pictures of the patient.
(A) frontal view, note truncal obesity, lateral inclination of the trunk due to scoliosis, and
genu valgum; (B) side view, note again the truncal obesity and the lumbar hyperlordosis.
C.-D. Facial appearance at age 14 y. (C) side view, note the mild retrognathia and the
cervical kyphosis. (D) frontal view, note broad and coarse face, bushy eyebrows with
mild synophrys, and full-tipped nose.
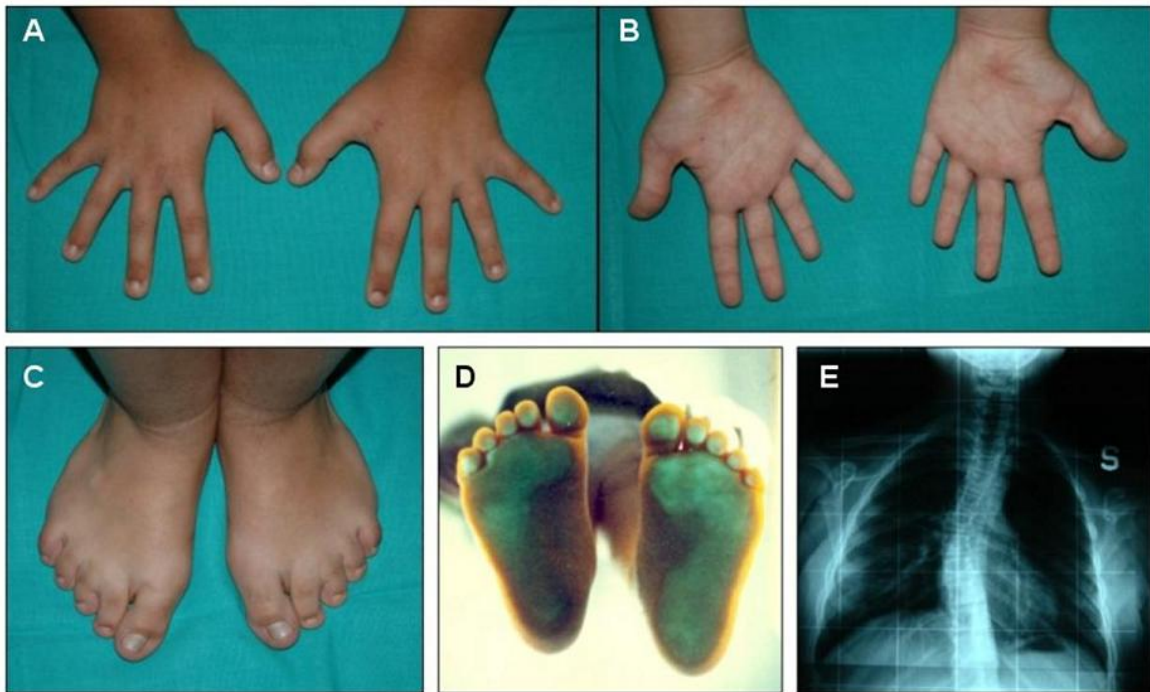
Fig. 6. Clinical features observed in SMS339. A.-B. Hands of the patient at age 14 y, note the broad palm and the mild brachydactyly. **C**. Feet of the patient at age 14 y, note the marked brachydactyly, proximal cutaneous syndactyly of toes 2-3, toenail hypoplasia, and mild hallux valgus. **D**. Plantar view on podoscope showing pes planus and brachydactyly. **E**. X-ray images showing dorsal scoliosis of the spine.
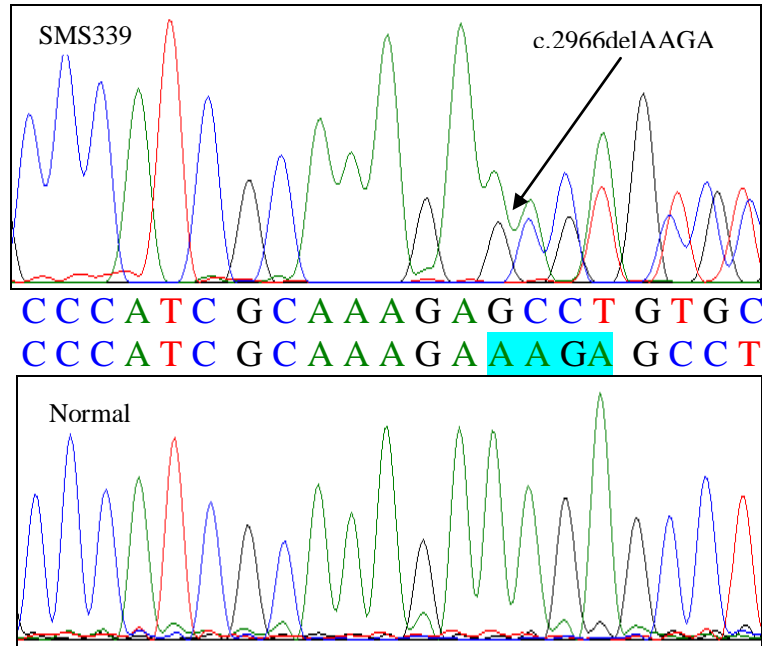
Fig. 7. *RAI1* **mutation analysis in SMS339. Top**: An electropherogram of both mutated and normal alleles from the patient, SMS339, is shown. The patient carries a 4 bp deletion (AAGA) starting at nucleotide position 2966 on one *RAI1* allele. This mutation causes a frameshift and truncation of the RAI1 protein. **Bottom**: normal *RAI1* sequence.

Table 6. *RAI1* variations in SMS339.

Nucleotide change*	Amino acid change	SNP ID
2966delAAGA	Frameshift/protein truncation	Novel mutation
Polymorphisms		
c.269G>C	p.Gly90Ala (homozygous)	SNP 3803763
c.493C>A	p.Pro165Thr (homozygous)	SNP11649804
c.837G>A	p.Gln279Gln (homozygous)	SNP 11078398
c.2773G>A	p.Val925Ile (heterozygous)	Novel SNP
PolyQ tract	(PolyQ)$_{13}$	

*DNA changes with nucleotide number starting from the ATG in the coding region.

mutation leading to misincorporation of 38 amino acids and truncation of the RAI1 protein. Parental DNA analyses were normal. This *de novo* mutation was consistent with the diagnosis of SMS.

The patient also carried several *RAI1* polymorphisms, including one novel SNP (Table 6). Out of the four polymorphisms identified in this patient, three were in the homozygous state and have been reported in the SNP database (Table 5). In addition, 13 polyglutamine repeats were present on each allele.

SMS343

SMS343 is a female patient of Caucasian descent referred for mutation screening of the *RAI1* gene. FISH analysis of the 17p11.2 region was negative by report. *RAI1* mutation screening identified one known polymorphism in the homozygous state located in the exon 5 region (Table 5). In addition to this SNP, 14 polyglutamine repeats were present on each allele. This patient was homozygous throughout the exons, which

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suggests the possibility of an internal deletion of the gene which is non identifiable by sequencing. Hence, additional studies such as aCGH should be performed on this patient sample to detect the real cause underlying these features.

SMS344

SMS344 is a 9 year old Caucasian female who was evaluated by geneticists between the ages of 4-8. She was born at 42-weeks gestation and weighed 7 pounds 4 ounces. At the age of 8, she weighed 92.2 kg ($> 97^{th}$ percentile), her height was 154 cm ($> 97^{th}$ percentile) and head circumference was 58 cm. She had experienced precocious puberty at the age of 7 and had suffered from severe obesity. She exhibited SMS features such as short, square shaped forehead, deep set eyes with horizontal palpebral fissures, prominent nasal bridge, small upturned nose, strabismus, hearing loss, speech and developmental delays. Some of other features included high-arched palate, sloping shoulders and mild waddling gait.

Laboratory data included a normal karyotype, negative FISH for 15q and17p11.2 deletion, Fragile X and Prader-Willi/Angelman syndrome and normal chromosomal microarray. *RAI1* mutation screening identified three known polymorphisms, with two of them in the heterozygous state (Table 5). No other deleterious nucleotide changes were identified in this patient.

SMS345

SMS345 is a Caucasian male patient exhibiting clinical signs of SMS. FISH analysis of 17p11.2 region as well mutation screening of *RAI1* gene did not identify any deletion. This patient carried two non-disease causing polymorphisms, both in the

homozygous state (Table 5). In addition, 14 polyglutamine repeats were present on each allele. This patient was homozygous throughout the exons, which suggests the possibility of an internal deletion of the gene which is non identifiable by sequencing. Hence, additional studies such as aCGH should be performed on this patient sample to detect the real cause underlying these features.

SMS346

SMS346 is 12 year old male of Caucasian descent, who was evaluated at age of 11 due to his history of developmental delay and dysmorphic features. In addition to these features, the patient also had some other features consistent with SMS such as aggressiveness, self-injurious behaviors including head-banging and hand-flapping, sleep problems, midface hypoplasia, tented upper lip, brachydactyly, and seizures. At the time of evaluation, his height was 127 cm (< 5th percentile), his weight was 48 kg (> 97th percentile) and head circumference was 54.5 cm.

Laboratory studies included negative results for Down syndrome and 17p11.2 deletion by FISH and normal aCGH. Sequencing of the *RAI1* gene identified four polymorphisms and 14 polyglutamine repeats on each allele. Three of the polymorphisms were located in the exonic region and two of these three were present in homozygous state (Table 5). The fourth polymorphism (c.5710-22G>T) present in heterozygous state was identified in the intronic region and is of unknown significance. Although this novel nucleotide change may cause alternate splicing of the *RAI1* gene which would disrupt a functional protein domain, additional studies are required to confirm this hypothesis.

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SMS348

SMS348 is 12 year old half Caucasian, quarter African-American and quarter Japanese male, who was born at 32 weeks gestation with a weight of 6 pounds 12 ounces. Since early childhood, he had suffered from behavioral disturbances, sleep problems, obesity, ear infections and speech delay. He was diagnosed with bipolar disorder and had been on medications which might have contributed to his obesity. The physical examination at the age of 11 showed his weight as 79kg ($> 95^{th}$ percentile), his height as 152 cm ($50^{th} - 90^{th}$ percentile) and head circumference as 55 cm. He also exhibited other features such as broad, square face, midface hypoplasia, broad nasal bridge, tented upper lip, flat face, synophyrs and brachydactyly that were also consistent with SMS. Family history was significant as the patient's father and two of his paternal half-siblings had bipolar disorder and mother had been diagnosed with Charcot-Marie-Tooth disease type 1A (CMT1a).

Laboratory data included normal karyotype and negative result for Fragile X syndrome. Although aCGH and *RAI1* mutation screening ruled out SMS, the patient was identified with duplication of chromosome 17p12 region that included *PMP22*. This finding is diagnostic for CMT1a and is consistent with the CMT1a diagnosis in patient's mother. *RAI1* mutation screening identified five known polymorphisms, and all of them were present in the heterozygous state (Table 5).

SMS349

SMS349 is 19 year old male of Latvian descent with features consistent with SMS. His craniofacial features included midface hypoplasia, brachycephaly, frontal

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bossing, broad nasal bridge, down turned upper-lip, up-slanting eyes and prognathism. His ocular/otolaryngological abnormalities included strabismus, myopia, and hoarse voice. He exhibited some other manifestations such as dental abnormalities, kidney abnormalities, obesity, pes planus, scoliosis, developmental delay, sleep disturbance and self-injurious behavior (nail yanking).

Laboratory data included normal aCGH and sequencing of the *RAI1* gene which identified four polymorphisms and three of them were in heterozygous state (Table 5). One of the four polymorphisms was novel and not listed in the NCBI SNP database. No other studies have been performed on this patient sample.

SMS351

SMS351 is 4 year old Caucasian male with broad nasal bridge, down-turned upper lip, brachydactyly, frequent ear infections, sleep disturbance, developmental delays and self injurious behaviors such as polyembolokoilomania, self-biting, and head banging in addition to other features consistent with SMS. He was evaluated at the age of 3, and his clinical examination shows that his weight was 17 kg, height was 95.4 cm and head circumference was 51.2 cm.

Laboratory findings included negative 17p11.2 deletion by FISH and normal aCGH results. Mutation analysis on the *RAI1* gene identified three polymorphisms (Table 5). Two of the polymorphisms were known and located in the exonic region, and one of these two SNPs was in homozygous state. Although the third polymorphism was novel and located in the intron 5 region, this variant was present in both his parents.

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SMS356

SMS356 is 11 year old female of Austrian descent with features consistent with SMS including neurological features (sleep disturbance, mental retardation, hypotonia, speech and motor delay), craniofacial features (brachycepahly, midface hypoplasia, broad-square face, and short stature) behavioral features (self-hugging) and self-injurious features (polyembolokoilomania).

Laboratory results included negative FISH for 17p11.2 deletion. On performing *RAI1* mutation screening, we identified five heterozygous polymorphisms, all reported in the SNP database (Table 5). Besides these changes, no other abnormalities were identified in SMS356.

Comparison between mutation and non-mutation cases

We compared thirty-two clinical features commonly associated with the *RAI1* mutation cases to the cohort of non-mutation cases (Table 7). All features were scored as present (+), absent (-) or data not available (N) (data not shown). These scores were then recalculated to obtain the percentages and compared with each other. The results (Table 7) indicated that all features were consistent between the two groups. These features included craniofacial/skeletal characteristics such as synophyrs, midface hypoplasia, tented upper lip, scoliosis and short stature. Neurological/behavioral similarities included mental retardation, developmental delay, hypotonia, sleep disturbance, self-hugging and seizures. Other features that showed consistency included self injurious behavior such as head-banging/skin picking/finger chewing; ocular abnormalities such as strabismus and

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myopia; otolaryngological abnormalities such as chronic ear infections and hearing loss; and lastly, pes planus (flat feet) and dental anomalies. Not many *RAI1* mutation patients have been reported with cardiovascular anomalies; SMS339 from present study was one of the exceptions. There were no significant differences that existed between the mutation and non-mutation cases and hence, their referral for *RAI1* sequencing was right.

Refine the sequencing methodology

RAI1 is a large gene consisting of 6 exons, of which 3-6 are coding exons (Fig. 2a). These coding exons encode a 1906 amino acid protein with the molecular weight of 203 kDa. Out of four coding exons, exon 3 is the largest exon composed of 5580 bp nucleotides and encodes > 98% of the protein. Exons 4, 5 and 6 are comparatively smaller exons composed of 93 bp, 49 bp and 12 bp respectively. Sequencing of the *RAI1* gene with twelve sets of overlapping primers was a tedious, time-consuming and expensive process. To make the sequencing methodology time- and cost-effective, the protocol was refined (Appendix A). Two rounds of sequencing with different sets of primers were planned (Fig. 8). The first round of sequencing included PCR amplification with primers (e.g. forward primer of 1st set and reverse primer of the 2nd set) covering big fragments (~ 2 kb) of the coding region and the amplimers sequenced with forward primer of 1st set and reverse primer of the 2nd set. This process saved time and cost of performing two PCR amplifications and sequencing with two sets of forward and reverse primers. If and only if the first round of sequencing did not cover the entire region, then it was followed by second round of sequencing which included individual sets of primers

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Table 7. Phenotypic comparison between mutation and 'SMS-like'$^{\$}$ cases.

SMS Features	% Non-mutations$^{\$}$ (n=11)	% *RAI1*-mutations[#] (n=14)	Fisher's exact test (p-value)
Gender (Male)	54 (n=11)	57 (n=14)	1
Craniofacial/Skeletal			
Brachycephaly	67 (n=9)	91 (n=11)	0.28
Midface hypoplasia	78 (n=9)	82 (n=11)	1
Broad, square face	78 (n=9)	92 (n=12)	0.55
Tented upper lip	67 (n=9)	75 (n=12)	1
Prognathism (relative to age)	34 (n=3)	87 (n=8)	0.15
Synophrys	45 (n=9)	33 (n=12)	0.67
Brachydactyly	60 (n=5)	73 (n=11)	1
Short stature (<5th centile)	33 (n=9)	25 (n=12)	1
Scoliosis	50 (n=8)	33 (n=12)	0.65
Neurological/Behavioral			
Mental retardation	100 (n=9)	86 (n=14)	1
Speech delay	100 (n=9)	100 (n=14)	1
Motor delay	100 (n=9)	100 (n=14)	1
Hypotonia	57 (n=7)	67 (n=12)	1
Sleep disturbance	100 (n=9)	100 (n=14)	1
Self-hugging	50 (n=8)	77 (n=13)	0.34
Seizures	43 (n=7)	33 (n=12)	1
Overeating	100 (n=4)	87 (n=8)	1
Self-injurious behavior			
Head-banging	45 (n=9)	77 (n=13)	0.19
Skin picking/finger-chewing	50 (n=6)	80 (n=10)	0.30
Onychotillomania	37 (n=8)	69 (n=13)	0.20
Polyembolokoilomania	62 (n=8)	77 (n=13)	0.63
Ocular abnormalities			
Strabismus	57 (n=7)	45 (n=11)	1
Myopia	75 (n=4)	64 (n=11)	1
Otolaryngologic			
Chronic ear infections	50 (n=8)	75 (n=12)	0.36
Hoarse, deep voice	50 (n=4)	83 (n=12)	0.24
Hearing loss	37 (n=8)	27 (n=11)	1
Other manifestations			
Cardiovascular anomalies	0 (n=7)	17 (n=12)	0.5
Pes planus	75 (n=4)	62 (n=8)	1
Renal anomalies	12 (n=8)	10 (n=10)	1
Dental anomalies	62 (n=8)	67 (n=9)	1
Overweight/Obese	86 (n=7)	87 (n=8)	1

(N) Information not available. [#] Includes previous Elsea lab cases + new mutation case.
$^{\$}$ Includes non-mutation cases from present study.

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covering an approximately 1 kb region of the coding exons and then sequencing with the forward or reverse primers that covered the remaining region of the exons. In addition to this, new internal primers were also designed to check for novel SNPs or for sequencing for the remaining uncovered region. Along with the new primers, novel SNPs and mutations have been added to the *RAI1* genomic sequence used for the sequence alignment and analysis (Appendix B). This new protocol of sequencing *RAI1* gene has greatly improved the screening technique for mutation analysis by making the process faster and cost effective.
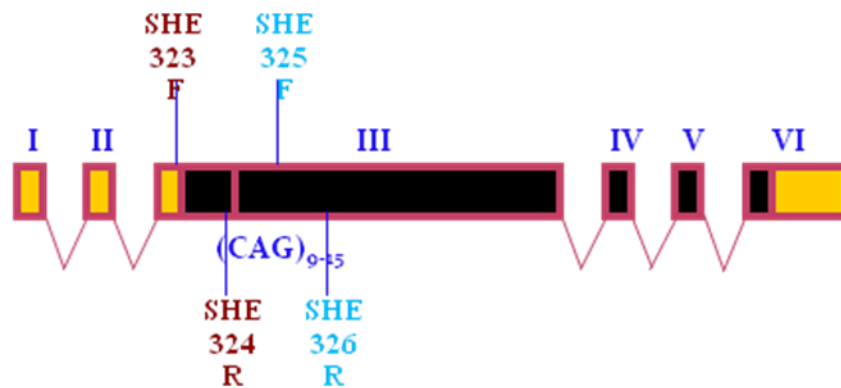
Fig. 8 Schematic explaining the two rounds of sequencing. The first round includes PCR amplification with SHE 323/324 set covering larger fragments. The second round includes PCR amplification with individual sets of primers (SHE323/324 and SHE325/326).

Discussion

Identification of mutations and polymorphisms

We reported twelve patients exhibiting SMS features but had a normal FISH result for 17p11.2 deletion. On analyzing their DNA sample by sequencing of the *RAI1* gene, only one of them was identified with a novel *RAI1* mutation. The novel mutation identified in our patient was *de novo* as it was absent in the parental DNA, although the possibility of germline mosaicism cannot be ruled out. This mutation caused a significant nucleotide change in exon 3 that included a deletion of 4 bp starting at nucleotide 2966 (Fig. 7). This heterozygous novel deletion should result in a frameshift mutation leading to misincorporation of 38 amino acids and truncation of the RAI1 protein. This novel *de novo* mutation was consistent with the diagnosis of SMS. Previous studies in our lab and others have identified several patients with *RAI1* mutations, which along with this patient, bring the total number of individuals with sporadic *RAI1* mutations to 19 (Fig. 9) (4, 8, 10, 15, 20, 40).

Our 17 year old patient was the first SMS case with a demonstrated *RAI1* gene mutation and absence of frank mental retardation. She showed a physical phenotype characterized by being mildly overweight, and craniofacial and skeletal anomalies evocative of SMS (broad face, bushy eyebrows, synophrys, upslanting palpebral fissures, full-tipped nose, mild retrognathia, dorsolumbar scoliosis, brachydactyly of fingers and toes, and pes planus). Other physical features were hoarse voice, dry skin, tricuspid regurgitation, and a history of constipation and, until early infancy, hypotonia. The clinical picture was, however, dominated by the behavioral abnormalities, which, like in

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most SMS patients, began in early childhood and increased in severity with progressing age. The patient displayed weak relational and social skills, obsessive thinking, tendency to depressed mood, stereotypies, significant maladaptive behaviors (temper tantrums, disobedience, impulsivity, verbal aggressiveness, attention seeking, and prolonged crying), self-injurious behaviors (skin-picking of the legs, onychotillomania, self-hugging, polyembolokoila-mania associated with recurrent urinary tract infections, cutting locks of her own hair, tearing away her eyebrows, and writing and painting the skin, mouthing of hands and objects, and grinding of the teeth), and sleep disturbances (difficulty falling asleep, prolonged night-time awakenings, and excessive daytime sleepiness).

The behavioral phenotype and the sleep disturbances of SMS patients can be considered as real diagnostic "handles" in the diagnosis of this syndrome. Also in our case, these cardinal signs, along with some physical features (mainly the brachydactyly of toes and the mild but suggestive facial dysmorphisms), were the pivotal clinical manifestations that pointed out the possibility of the patient having SMS.

It is important to note that, although the patient showed some learning disabilities and certain weaknesses in sequential processing and short-term memory, her IQ fell in the low normal range, and she did not have mental retardation. In the medical literature, no cases of SMS with IQ greater than 75 have been reported, and our patient was the first SMS case with an absence of frank mental retardation (10).

When compared with SMS patients that have deletions, the few reported subjects with *RAI1* mutations are more likely to exhibit overeating, overweight/obese habits, dry

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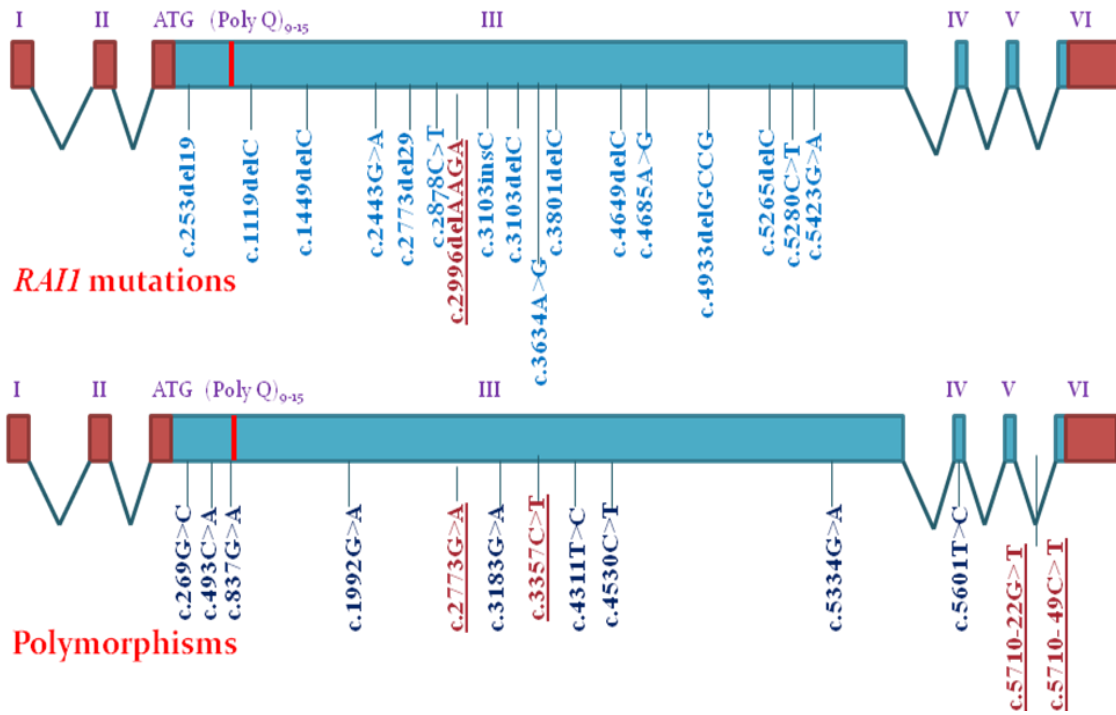
Fig. 9. *RAI1* **variations identified in SMS patients.** The schematic illustrates mutations and polymorphisms identified in *RAI1* gene. The coding exons are black and non-coding exons are orange. All the mutations are located in exon 3 which encodes >98% of the protein. The underlined variations were identified in the twelve patients evaluated in present study.

Source: Slager et al. 2003; Bi et al. 2004; Bi et al. 2006; Girirajan et al. 2005; Girirajan et al. 2006; GeneDx; **Present study 2009**

skin, muscle cramping, less severe motor delay, higher functioning, and self-injurious behaviors such as polyembolokoilamania, skin-picking and self-hugging. Moreover, they tend to not have organ system involvement. In particular, very few *RAI1* mutation patients have been reported to have cardiac or renal anomalies. Excluding muscle cramping, our patient showed all the clinical features typically associated with SMS due to mutations in *RAI1*. However, it is interesting to note that she was the first case with an *RAI1* mutation having a cardiac anomaly of mild tricuspid regurgitation. This finding partially enlarges the spectrum of symptomatic effects of *RAI1* mutations, which is clearly not yet completely known because of the limited number of recognized patients and an understandable selection bias toward the more severe and typical cases of SMS.

We also identified several polymorphisms in these patients. Most of them were known SNPs from the database, along with four novel SNPs, two of which were located in the intronic regions (Table 4). Along with these novel SNPs, the total number of SNPs identified in the coding sequence of the *RAI1* gene is 13 (Fig. 9). Except for a few that were in homozygous state, the majority of these SNPs were heterozygous. The intronic SNPs that were found in SMS346 and SMS351 are located in intron 5. At present, we are unaware of the potential cause and effect of these exonic and intronic SNPs and hence, further studies need to be performed to evaluate their role in causing SMS features.

Future studies to improve diagnosis of SMS

The incidence rate of this neurodevelopmental disorder is estimated to be 1:15,000-25,000 births, but it seems likely that these figures represent an underestimate

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(10). Many of these patients were initially diagnosed with some other syndrome, but eventually when tested for 17p11.2 by FISH or *RAI1* sequencing, they were identified with deletion or mutation, and were correctly diagnosed with SMS.

For example, our patient (SMS339) was initially diagnosed with mood disorder associated with behavioral anomalies, and the psychiatric signs were not recognized as part of a known syndrome. It is probable that other "psychiatric patients" actually have SMS, and it is believed that this condition could easily go undiagnosed when the cognitive impairment is lacking or the severity of mental retardation falls in the mild or borderline range. On the other hand, both the variable expression of the clinical picture and the still limited knowledge of the syndrome by clinicians are the principal reasons for considering the current incidence rate figures of SMS as an underestimate. Obtaining the correct diagnosis is of critical importance for the family and the patient, particularly so that the patient can obtain the necessary educational, therapeutic, and medical services needed for an individual with SMS.

Future studies are important also for those patients that are negative for deletion of 17p11.2 region or mutation of *RAI1* gene, but clearly exhibit features consistent with SMS. Some of these cases have been evaluated for the alternative diagnosis of SMS, as suggested by Elsea and Girirajan (2008) such as Prader-Willi, Angelman, Down, and Fragile X syndromes (Table 8), but the laboratory findings were negative. In my study, 11/12 patients belong in this category and are classified as "SMS-like" cases. There might be several possibilities that could lead to the appearance of 'SMS-like' phenotype.

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Table 8: Alternative diagnosis of Smith-Magenis syndrome.

Syndrome	OMIM No.	Clinical Features[a]
9q34 deletion syndrome	#61253, *607001	Mental retardation, brachycephaly, flat face, midface hypoplasia, tented upper lip, short nose, hypertelorism, synophyrys, prognathism, hypotonia, cardiac anomalies, seizures, hearing loss, sleep disturbance, obsessive-compulsive disorders, and stereotypic movement
Down syndrome (Trisomy 21)	#190685, *602917, *605296, *605298, *604829	Mental retardation, hypotonia, short stature, brachycephaly, epicanthic folds, upward slanting palpebral fissures, flat facies, congenital heart defects, Brushfield spots (iris), single palmar crease, brachydactyly, small and low-set ears, obtrusive sleep apnea, and cardiac and endocrine problems
Williams syndrome (del(7)q11.23)	#194050, *130160, *601329, *603432, *604318, *601679	Mental retardation, characteristic 'elfin' facies, supravalvular aortic stenosis, hypercalcemia, prenatal growth retardation, short stature, lack of depth perception, and friendly, loquacious personality
Prader-Willi syndrome (paternal del(15)q11-q13)	#176270, *182279, *602117	Mental retardation, speech and motor delay, brachycephaly, almond-shaped eyes, down-turned mouth with thin upper lip, hypotonia, short stature, gait abnormalities, hyperphagia, genital hypoplasia, and self-injurious and obsessive-compulsive behaviours
22q11.2 deletion syndrome (DiGeorge/ velocardiofacial syndromes)	#188400, #192430, *602054	Congenital heart disease, velopharyngeal insufficiency, cognitive and motor delay, feeding difficulties, immune deficiency, hypocalcemia, hearing loss, and skeletal abnormalities
Sotos syndrome	#117550, *606681	Mental retardation, hypotonia, cognitive and motor delay, feeding difficulties, tall stature, macrocephaly, malar flushing, frontotemporal hair sparsity, down slanting palpebral fissures, seizures, scoliosis, and cardiac and renal abnormalities

[a]Summarized from Online Mendelian Inheritance in Man (OMIM)

Source – Elsea and Girirajan, 2008

It is possible that RAI1 interacts with other genes or proteins via multiple pathways that could lead to an SMS or an SMS-like phenotype. The amino acid sequence motifs such as bipartite nuclear localization signal and PHD domain of the *RAI1* gene are found to be related to the transcription factor stromelysin-1 platelet derived growth-factor-responsive element binding protein (TCF20) which strongly suggests that RAI1 could be a transcription factor modulating the expression of genes involved in a variety of cellular functions. The 'SMS-like' phenotype showed by our patients confirmed from a clinical point of view that further studies are required to fully understand the role played by RAI1 in the biochemical and molecular pathways important for development, cognition, and behavior and thus, improve the diagnosis of SMS.

Conclusions

Smith-Magenis is a complex and contiguous gene syndrome affected by several genes in 17p11.2 region. *RAI1* gene is one of the 25 genes located in the SMS critical region and mutation screening by *RAI1* sequencing has proven that mutation in this gene causes SMS. *RAI1* mutations are mostly *de novo* as they are not present in the patient's parents. Including the cohort of patients with 17p11.2 deletion and *RAI1* mutations, there exist a group of patients with familial mutations in the *RAI1* gene. These familial mutations have complicated the diagnosis of SMS, due to their clinically normal parents and siblings. Besides the patients diagnosed with SMS, there is one more category of patients with 'SMS like' phenotype. Although these patients have SMS phenotypes, they

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are negative for 17p11.2 deletion or *RAI1* mutation, which suggests a role played by a different gene/modifier which might be upregulating or downregulating the *RAI1* gene and contributing to the phenotype. This dosage-sensitive effect of *RAI1* can be evaluated in zebrafish models by characterizing *rai1* gene expression in the presence and absence of its inducer.

CHAPTER 3: Cloning and sequencing of zebrafish *rai1* gene

Introduction - Zebrafish model

Zebrafish (*Danio rerio*), a tropical freshwater fish, serves as a very important model organism in the field of scientific research. It was first used as a model organism in the 1980's by George Streisinger, who induced mutations in mature germ cells of embryos by gamma-rays and opened the arena of studying mutagenesis and gene regulation by performing forward genetic screens in zebrafish (44). This model presents many advantages over other model organisms such as rapid generation time, high fecundity, external development, transparency of embryos, low cost and simpler staining techniques for manipulation of development at all stages. Comparative genomics shows the similarity between genomes of human and zebrafish. Humans and zebrafish have diverged ~ 420 million years ago but have similar skeletal cell structure, including neural crest cells (28). This makes zebrafish a useful resource in studying cranio-facial and neurological development observed in SMS patients.

Broad period of embryogenesis

The seven stages of zebrafish embryogenesis are as follows: zygote (0- 0.75 hours), cleavage (0.75 – 2.25 hours), blastula (2.25 – 5.25 hours), gastrula (5.25 – 10.33 hours), segmentation (10.33 – 24 hours), pharyngula (24 – 48 hours) and hatching (48 – 72 hours) (27). The zygote stage is 1-cell stage where the chorion swells and detaches from the newly fertilized embryo. The cytoplasm streams away from the vegetal pole and towards the animal pole to form a blastodisc. This defines the first stage of embryogenesis. In the second stage known as the cleavage stage, six cleavages (2 cells to 64 cells) occur every fifteen minutes in regular orientations giving rise to 3 tiers of blastomeres. Blastula stage is defined as the stage where the 3 tiers further divide to 11 tiers of blastomeres and the marginal blastomeres form rows of yolk synctial layer (YSL). The YSL continues to develop and the blastula flattens and forms a sphere between the yolk and blastodisc. This sphere begins to bulge towards the animal pole to form a blastoderm. By the end of the blastula stage, the blastoderm covers 30% of the yolk sac. In the gastrula stage, the blastoderm continues to cover a larger fraction of the yolk (~ 90%) forming a germ-ring and then an embryonic shield. By the end of this stage, the embryo develops 90% epiboly, brain and notochord rudiments. The fifth stage of development is marked by segmentation of somites (1 – 26+) which leads to the development of primordial regions such as hindbrain-midbrain boundary, trunk, tail, and lens. The pharyngula stage is an important stage for comparison of the morphologies of the diverse vertebrates according to Baer's famous law (25). At this stage, the embryo develops heartbeat, pigmentation, primary body organs such as brain and retina. By 48

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hpf (hours post fertilization), the differentiation of neurons and neural crest cells takes place which further differentiates into cartilage. The yolk size starts to reduce and the size of the head starts increasing. The final stage of zebrafish embryogenesis, the hatching stage is when pectoral fin buds and blades are formed, morphogenesis of the organs is completed, and the embryo hatches. At the end of the third day or hatching period and afterwards, they are known as 'larvae' and after 90 days post-fertilization, they are known as 'adults'.

rai1 gene in zebrafish

A single putative ortholog of the _rai1_ (XM 001333125, LOC793412) gene was predicted in zebrafish but not fully annotated (Fig. 10). This gene is a homologous to the _RAI1_ gene and conserved in human, chimpanzee, dog, mouse, rat and chicken (9). The putative gene maps to chromosome 3 and is made up of 3 exons composed of 5824 bp nucleotides and encodes a 1936 amino acid protein. Zebrafish putative gene sequences based on computation analysis are added to the NCBI HomoloGene database and Genome browser. Comparing the putative zebrafish _rai1_ gene sequence with available transcripts, an expressed sequence tag (EST) (clone 2691424) was identified to have the 5' end of the gene along with some UTR region, approximately 500 bp (Fig. 11). As this suggested the EST contained the full-length _rai1_ gene, it was bought from Open Biosystems and sequenced with overlapping forward (F) primers (Table 9). Sequencing the full-length clone was important because this clone was generated from a different strain than the sequence data in the Genome Browser, and accurate sequence is required
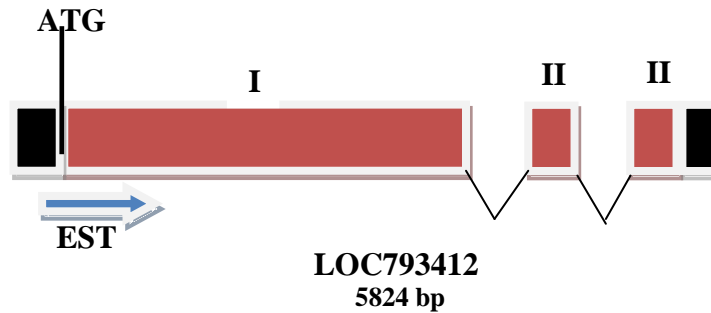
Fig. 10. Putative structure of zebrafish *rai1* gene. LOC793412 maps to chromosome 3 of the zebrafish genome and is composed of 3 exons. This putative structure is based on the computational analysis and projection studies performed by NCBI Homologene database.
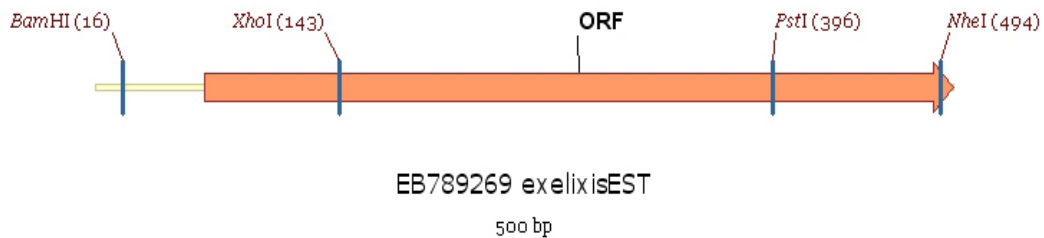
Fig. 11. Schematic of *rai1* Expressed Sequence Tag (EST). EST was identified to have a 5'end and some UTR region. This suggested that it contained the full-length *rai1* and was used for sequencing the 5'end of the *rai1* gene.

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to design an antisense knockdown (morpholino) and perform rescue experiments. Once the accurate sequence of zebrafish *rai1* gene is annotated, this model organism will serve as a unique and powerful system to study dosage sensitivity of *rai1* and identify new and existing gene networks and functions.

Materials and methods

Isolation of DNA from plasmid

DNA was isolated from the plasmid containing the full-length clone (EXELIXIS2691424) of zebrafish *rai1* using DNA midi kit (Qiagen, Valencia, California, USA). The DNA samples were treated with RNase to eliminate contamination by RNA. The isolated DNA was quantified by a spectrophotometer at the absorbance ratio of 260/280.

PCR and sequencing

PCR amplification of the plasmid DNA was performed initially by using two different combinations of the vector primers (M13F (gtaaaacgacggccagt) and M13R (caggaaacagctatgac); T7F (taatacgactcactatagg) and M13R (caggaaacagctatgac)). Several overlapping primers were also designed for sequencing the M13F/M13R PCR product to obtain the remaining gene sequence that was not covered by the initial set of primers (Table 9). PCR reaction mix and conditions were followed as described in Chapter 2 (polymerase chain reaction). Sequencing was performed at the Virginia Commonwealth University Sequencing Core using the protocol described in Chapter 2 (Sequencing).

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Sequence analysis

The chromatograms results were received from the sequencing core and the sequencing results from the forward primer were analyzed by comparing the results with the predicted *rai1* sequence visually by looking for variations (nucleotide change) and sequence quality. The predicted *rai1* sequence was obtained from Genome Browser and NCBI. A poly A tail was encountered at 3720 bp (after *Sal*I site) and sequencing results became unclear (Fig. 12).

Table 9. Overlapping primers for sequencing *rai1* gene

Forward primer	Sequence covered
M13: gtaaaacgacggccagt	490 bp
SHE608: gtacatgagcgaaggtcac	460 bp
SHE610: gtaaggtcgccggaacaatac	952 bp
SHE614: catggaccgagaggatg	828 bp
SHE633: atcatcacatcgattgt	656 bp
SHE635: agtccaaagccttgtccta	330 bp
JL502: caactgtggcatcagtagctaaaggc	1818 bp
SHE646: gcgcccagatctaagtgacg	897 bp

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Collect cultivated embryos

Overnight tanks that separated three male from three female fish were set-up in the afternoon. Mating took place in the morning and embryos were collected in a 10 cm petri dish after 1-2 hours. Each petri dish contained ~ 80 embryos and the dish water was maintained by removing empty chorions and deformed embryos.

TRIzol treatment

Collected embryos were incubated at 28.5°C until they reached the required stage. At 72 hpf, a total of 30 embryos were homogenized in 1 ml of TRIzol reagent and stored at -20°C until ready to isolate RNA.

RNA extraction

Total RNA was extracted from TRIzol treated (72 hpf) embryos using the RNA preparation kit (Invitrogen), according to the manufacturer's instructions.

Reverse-transcriptase PCR (RT-PCR)

First strand cDNA synthesis was performed on the extracted RNA from 72 hpf embryos according to the manufacturer's protocol (Stratagene Accuscript protocol) using 1 µl of 1-2 ug of total RNA, Accuscript RT buffer, Accuscript RT, oligo dT, DTT (0.1M), Rnasin and dNTP mix (10 mM).

PCR and sequencing

cDNA obtained from RT was PCR amplified with overlapping primers (Fig. 12) designed between the *Sal*I site and the 3' end of the putative *rai1* gene sequence (JL502F (caactgtggcatcagtagctaaaggc) and SHE647R (cgtggtatgcagtcctcagg); SHE646F

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(gcgcccagatctaagtgacg) and SHE650R (tcacacacttaccttgtgtttcgg)). PCR reaction mix and conditions were followed as described in Chapter 2 (polymerase chain reaction). Sequencing was performed at the Virginia Commonwealth University Sequencing Core using the protocol described in Chapter 2 (Sequencing).

Sequence analysis

The chromatograms results were received from the sequencing core, and the sequencing results were analyzed as described in Chapter 3 (sequence analysis).

Cloning *rai1* PCR products

PCR products of the correct size were purified by gel extraction using the Qiagen Gel Extraction kit (Qiagen Inc, Valencia, California, USA). Purified PCR products (~ 10 ul) were treated with 0.25 ul of Taq polymerase (5u/ul) and 0.25 ul of dNTP (10 mM) at 72° for 10 mins. These products were then TOPO® cloned into pCR®2.1-TOPO® [646/650] /pCR®4-TOPO® [502/647] vector using the TOPO® cloning kit by Invitrogen (CA) (Fig. 13). The protocol was revised as follows: the ligation reaction was incubated for 30 mins at 22°C, the TOPO- *rai1* construct was transformed into One-Shot® - Top F' chemically competent *E-coli* cells and incubated on ice for 30 mins, and 50 ul of transformation mix were spread on LB plate with 50 µg/ml of kanamycin and incubated at 37°C for overnight. Colonies were grown in a culture with 5 ml of LB media and 2.5 ul of kanamycin (100 µg/ml). The culture was used to isolate DNA using the DNA mini-prep kit by Fermentas (Burlington, Canada). PCR amplification of the isolated DNA was performed by using the *rai1* specific primers (JL502/SHE647; SHE646/650) to confirm

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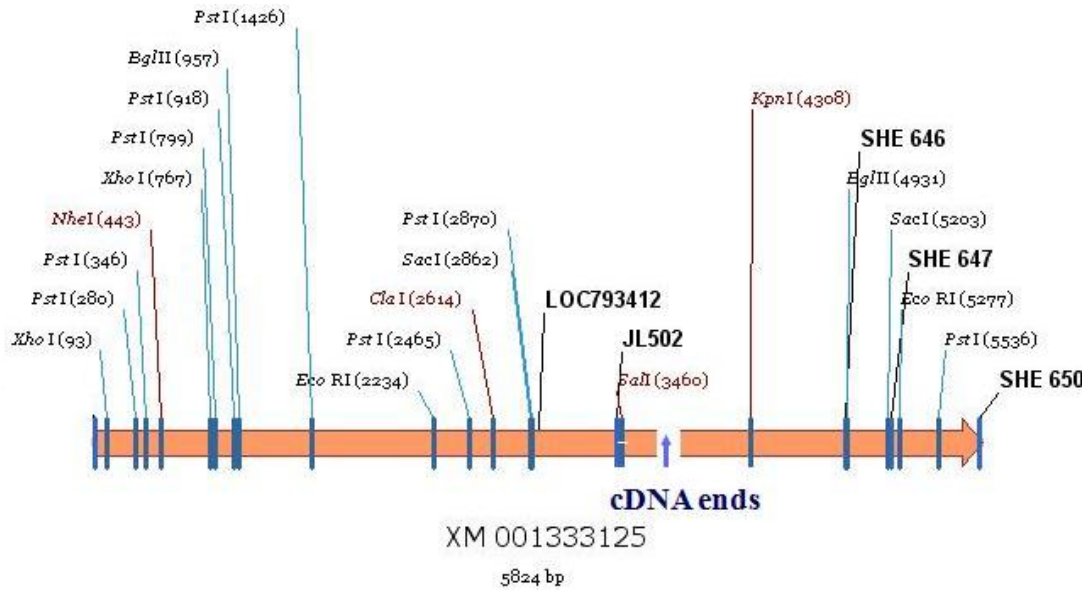
Fig. 12. Schematic illustrating the end of EST and overlapping primers used for obtaining 3' end. Poly A tail was encountered at 3720 bp (cDNA ends). Overlapping primers JL502/SHE647 and SHE646/ SHE650 were used for PCR amplification for obtaining 3'end of *rai1* gene.
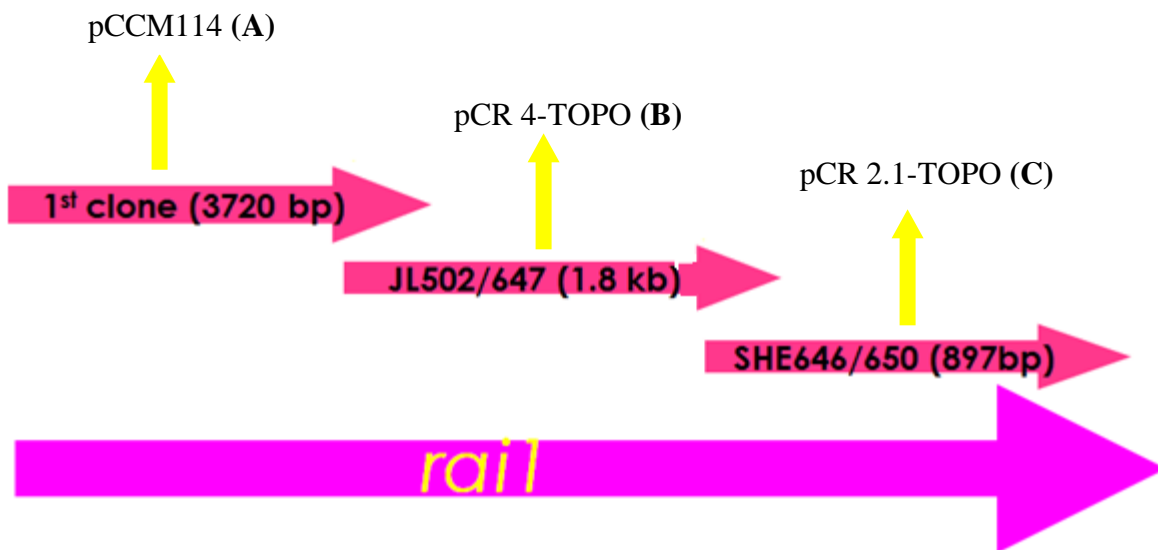
Fig. 13. Schematic representation of the three *rai1* clones. The three fragments of the zebrafish *rai1* gene were present in three different vectors. **(A)** EST containing the 5'end cloned into pCCM114 vector. **(B)** JL502/647 fragment cloned into pCR 4 –TOPO vector and **(C)** SHE646/650 fragment cloned into pCR 2.1- TOPO vector.

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the presence of the insert. DNA was also digested with restriction enzymes to check for the insert (*EcoR*I) and its orientation in the vector (*Sac*I – SHE646/650 fragment, *Spe*I and *Bgl*II – JL502/SHE647 fragment). Glycerol stocks were made of the *rai1* fragments for storage purposes.

Ligation of *rai1* cDNA fragments

The three *rai1* fragments in three different vectors were ligated together to create a full length *rai1* clone. The SHE646/650 and JL502/647 fragments present in two different vectors were double digested with *Bgl*II and *Spe*I enzymes. The digested products were gel purified using the Qiagen Gel Extraction kit (Qiagen Inc, Valencia, California, USA). Purified product (JL502/647) was treated with calf intestinal phosphatase (CIP) to prevent recircularization of the cloning vector. It was further treated with the Qiagen gel purification kit (Qiagen Inc, Valencia, California, USA). This was followed by the ligation of the JL502/647 fragment (JL502/647 + pCR 4 vector) and SHE646/650 (insert in pCR 2.1 vector) using T4 DNA ligase and incubating it overnight at 14° C (Fig. 14). Once the ligation was complete, the pCR 4.TOPO- JL 502/650 *rai1* construct was transformed into One-Shot® Top F' chemically competent *E-coli* cells and incubated on ice for 10 mins, heat shocked for 40 seconds at 42°C and incubated at 37° for 90 mins after the addition of LB. The transformations (~300 µl) were spread on LB plates with 50 ug/ml of kanamycin and incubated at 37°C overnight. Positive colonies were grown in a culture with 2 ml of LB media and 2.5 ul of ampicillin (100 µg/ml). The culture was used to isolate DNA using the DNA mini-prep kit by

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Fig. 14. Ligation of two *rai1* constructs at the 3'end. JL502/647 fragment (JL502/647 + pCR 4 vector) and SHE646/650 (insert in pCR 2.1 vector) were double digested with *Bgl*II and *Spe*I and ligated with T4 DNA ligase.
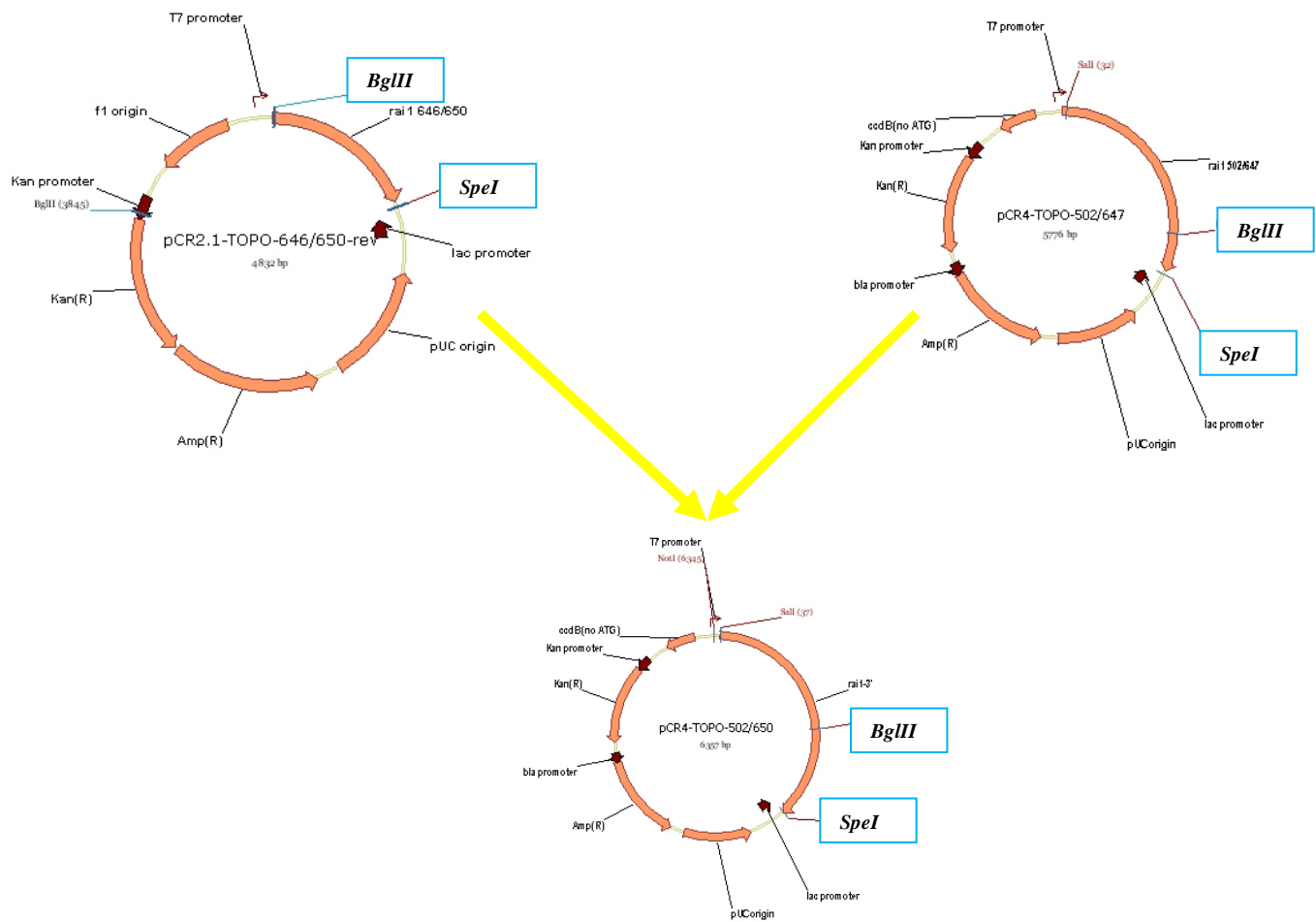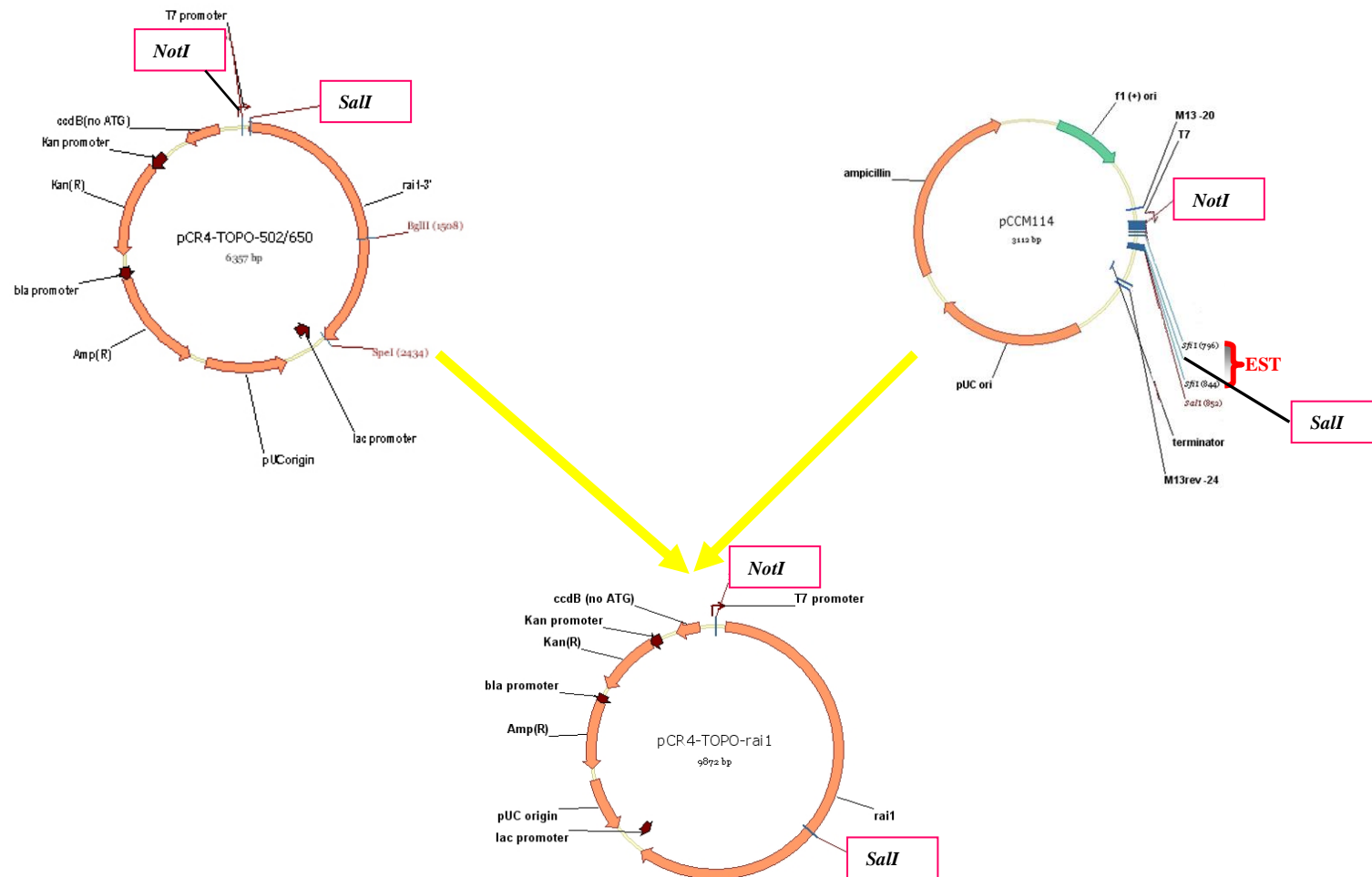
Fig. 15. Ligation of 5' end with 3'end. JL502/650 and pCCM114 containing the EST were double digested with *Not*I and *Sal*I enzymes and ligated with T4 DNA ligase.

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Fermentas (Burlington, Canada). The JL502/650 DNA was digested with *Not*I and *Spe*I to confirm the size and orientation of the inserts. This was followed by sequencing of the junction between JL502/647 and SHE646/650. The JL502/650 fragment was then ligated with the 5'end of the gene (1st fragment) in the pCCM114 vector (Fig. 15). The JL502/650 and pCCM114 constructs were double digested with *Not*I and *Sal*I enzymes. The JL502/650 + vector (6.4 kb) was also treated with CIP to prevent recircularization. These products were then gel purified using the gel purification Qiagen kit, followed by the ligation of the JL502/650 construct (JL502/650 + pCR 4 vector) and 5' end (insert in pCR 2.1 vector (~3720 bp)) using T4 DNA ligase and incubation overnight at 14° C (Fig. 15). Once the ligation was complete, the pCR 4.TOPO- full length *rai1* construct was transformed into *E-coli* cells as mentioned above. Colonies were grown in a culture with 2 ml of LB media and 2.5 ul of ampicillin. The culture was used to isolate DNA using the DNA mini-prep kit by Fermentas (Burlington, Canada). The full length *rai1* DNA was then digested with *Spe*I to confirm the size of the insert. This was followed by sequencing of the full length zebrafish *rai1* gene using *rai1* specific primers on the 5' end (JL502/ SHE650) to confirm the junctions.

Results

Full-length *rai1* gene sequence

The sequence obtained from the ligated *rai1* plasmid (pCR 4 vector) had 99.5% similarity with the putative *rai1* sequence published in the NCBI database. Although nucleotide changes were identified in our full-length r*ai1* clone, this could be the result of

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the different strain of fish used in our study or the putative sequence obtained from computational study was inaccurate. This full-length *rai1* sequence will serve as a reagent for morpholino studies to eventually provide a better understanding of the *rai1* gene and its role in causing cranio-facial and neurological defects.

Discussion

Analysis of rai1 in zebrafish

A full length cDNA encoding the *rai1* gene from zebrafish was cloned by RT-PCR of two fragments (3' end) and ligation of these fragments with an EST (5' end). Zebrafish *rai1* has an open reading frame coding for 1935 amino acids. The alignment of the zebrafish rai1 with human RAI1 amino acid sequence shows 27 % identity (Fig. 16). In regards to the polyglutamine region, human RAI1 is composed of fourteen glutamine repeats. But in all other species including mouse and rat, the polyglutamine region generally includes fewer repeats. As shown in Fig. 16, zebrafish rai1 contains two Qs in the vicinity of the human glutamine repeat, which is consistent with the reduced number of glutamines identified in other species. Some domains were conserved in zebrafish and show 100% similarity (Fig. 16). The conservation of these domains serves as evidence that the full-length *rai1* clone from zebrafish in our study is truly the ortholog of human *RAI1*.

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                10        20        30        40        50
          ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1     mqsfrercgfhgkqqnyqqtsqetsrlenyrqpsqaglscdrqrllakdy
zebrafish rai1 MQSFRERSGFHSDQHCYQQEPQELPRLESYRHHYSQSRQGYEPHALASAG

                60        70        80        90        100
          ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1     ynpqpypsyeggagtpsgtaaavaadkyhrgskalptqqglqgrpafpgy
zebrafish rai1 MSTAGSKDCYGQQNYPVYTSATQTKKPYRGGKTPNQHLQAGYSNHMGTGY

                110       120       130       140       150
          ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1     gvqdsspypgryageeslqawgapqppppqpqplpagvakydenlmkkta
zebrafish rai1 TAQYMSEGHLQQKWDESAQMTQFEQDMVGRLESGSSQYLEQNMLAISQSQ

                160       170       180       190       200
          ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1     vppsrqyaeqgaqvpfrthslhvqqppppqqplaypklqrqklqndiasp
zebrafish rai1 CHLPAQSSAPVYTSPHQQGIPPNPAPSPLMYPQGHMHFPQHSQTSSSPSS

                210       220       230       240       250
          ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1     lpfpqgthfpqhsqsfptsstysssvqgggqgahsyksctaptaqphdrp
zebrafish rai1 AYMDKCNTASHGYKGYGIPPNAQYGRQLSNHSSLKQSGYRPQNNYGYQQT

                260       270       280       290       300
          ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1     ltassslapgqrvqnlhayqsgrlsydqqqqqqqqqqqqqqalqsrhhaq
zebrafish rai1 PSRSGFEQGSLQGMSGTQESLQKFQYYNQTQQNYCITDISVRSPEQYYQT

                310       320       330       340       350
          ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1     etlhyqnlakyqhygqqgqgycqpdaavrtfpeqyyqtfspssshsparsv
zebrafish rai1 CSPSSSHSPARSVGRSPSYNSTPSPLMANADSFQYGQPPINPGASSSTGL

                360       370       380       390       400
          ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1     grspsysstpsplmpnlenfpysqqplstgafpagitdhshfmpllnpsp
zebrafish rai1 QNEQNMLMPPHTHSSSSVNQTQSFSGSMKERFSEKLLTNPSLWSLNALTS

                410       420       430       440       450
          ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1     tdatssvdtqagnckplqkdklpenllsdlslqsltaltsqvenisntvc
zebrafish rai1 QVENISNNVQQLLLSEALMASKKTSKRNHPKKGEDYRGQLEGMDESCPEN
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                            460        470        480        490        500
                     ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1           qlllskaavpqkkgvknlvsrtpeqhksqhcspegsgysaepagtplsep
zebrafish rai1       QHGPSPSDAYSIPRSMTAGLQEGGYSSSTEDQMERNYYYFGQEKGQGHTQ


                            510        520        530        540        550
                     ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1           psstpqsthaepqeadylsgsedplersflycnqargsparvnsnskakp
zebrafish rai1       THSRLSLDTVSTCSLNSADDVSVRSGDSVRSLQSVASEDNLNCDPRVQRA


                            560        570        580        590        600
                     ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1           esvstcsvtspddmstksddsfqslhgslpldsfskfvagerdcprllls
zebrafish rai1       LSEEEPDSSLCCIRDERSPIGVTAPSPMKQESDSPPDIKRSESTQKENFE


                            610        620        630        640        650
                     ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1           alaqedlaseilglqeaigekadkawaeapslvkdsskppfslenhsacl
zebrafish rai1       ESAWTERMADEDKTEQSKPSAEHDCKGEVTEKQEKWLEDEKGPALFQKIN


                            660        670        680        690        700
                     ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1           dsvaksawprpgepealpdslqldkggnakdfspglfedpsvafatpdpk
zebrafish rai1       KTVLEEGYSYETEENIYQGLQNKYDSEKENTTEIDCFAELNHKCDEDTEI


                            710        720        730        740        750
                     ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1           kttgplsfgtkptlgvpapdpttaafdcfpdttaassadsanpfawpeen
zebrafish rai1       KTEHFKSEPQTSVEMPTEDSPSNSATELCLPEKEEHSSSENSVETPTCTL


                            760        770        780        790        800
                     ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1           lgdacprwglhpgeltkgleqggkasdgiskgdtheasaclgfqeedppg
zebrafish rai1       EGPADTLEEQTSVLSHTSTEVGDEKDSILTSSEEVINNKTESQESFPDVC


                            810        820        830        840        850
                     ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1           ekvaslpgdfkqeevggvkeeaggllqcpevakadrwledsrhccstadf
zebrafish rai1       GTYEEAENMQQVNTETAESIAQDTPHRDSERRSVICDIAPQTHTAKSDFS


                            860        870        880        890        900
                     ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1           gdlpllpptsrkedleaeeeysslcellgspeqrpgmqdplspkaplict
zebrafish rai1       ALNEKTTPLAQARDHHIDCSDAKVLEPDSPQLPGKSILHSAPSWADTPPS
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                        910       920       930       940       950
               ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1     keeveevldskagwgspchlsgesvillgptvgteskvqswfesslshmk
zebrafish rai1 PKKADEDMEPGINCPSAVTPSAKPEAFTPSSHTRMFGKKHVRGRRRPMSS


                        960       970       980       990      1000
               ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1     pgeegpdgerapgdsttsdaslaqkpnkpavpeapiakkepvprgkslrs
zebrafish rai1 TAGIRRQLSIEGDTQTPNIPSSKTTDIVDQMETAHQNVSSQTPKLVTESL


                       1010      1020      1030      1040      1050
               ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1     rrvhrglpeaedspcrapvlpkdllllpesctgppqgqmegagapgrgase
zebrafish rai1 PSRMCTRSYGSQGSPKVSPQDRRKPGAKPAPKPSSKIAVKPGPKSNPKPA


               1060      1070      1080      1090      1100
               ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1     glprmctrsltalseprtpgppgltttpappdklggkqraafksgkrvgk
zebrafish rai1 PKHSPKSITKPGSKSVPKSEVMPSVKPGPKPGLKSTGSKESPKPCPKSGP


                       1110      1120      1130      1140      1150
               ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1     pspkaasspsnpaalpvasdsspmgsktketdspstpgkdqrsmilrsrt
zebrafish rai1 KSATKSGPKTASKPASKPGSVPGEILAPKSNQNTAESKPIVASVAKGPGR


                       1160      1170      1180      1190      1200
               ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1     ktqeifhskrrrpsegrlpncratkklldnshlpatfkvssspqkegrvs
zebrafish rai1 PKGISKNKQMKMKQDENVQTTTEHTKDTHCTQELNLQDVTTELALDTIVG


                       1210      1220      1230      1240      1250
               ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1     qrarvpkpgagsklsdrplhalkrksafmapvptkkrnlvlrsrssssssn
zebrafish rai1 VVDTTKGETVAPTLECTLDSSCLKSLGREQKSMVLRSRKQTKEKLTEEKE


                       1260      1270      1280      1290      1300
               ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1     asgnggdgkeerpegsptlfkrmsspkkakptkgngepatklpppetpda
zebrafish rai1 QNDDKSTKTPSAMVMQDVTVDQSCKTGPSNLELPKQKDVSTDSLQQPDEE


                       1310      1320      1330      1340      1350
               ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1     clklasraafqgamktkvlpprkgrglkleaivqkitspslkkfackapg
zebrafish rai1 VVSTKRKCSLPTSETKKRKKGRQDEVKAQDSPLDIVHIPKGKRKRGQRLL
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                            1360      1370      1380      1390      1400
                     ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1           aspgnplspslsdkdrglkgaggspvgveeglvnvgtgqklptsgadplc
zebrafish rai1       VESVSTTLTTDTLPTDDISDVPSVPPQCPTKTKYLPPRKGRGLKYEAMVQ


                            1410      1420      1430      1440      1450
                     ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1           rnptnrslkgklmnskklsstdcfkteaftspealqpggtalapkkrsrk
zebrafish rai1       KITSPGSKKQPVSNQADIALEESTLKPVPQVPDQKETTNAPEMPQKEGES


                            1460      1470      1480      1490      1500
                     ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1           gragahglskgplekrpylgpallltprdrasgtqgasednsggggkkpk
zebrafish rai1       TISTQEPENTEIQTPRKKRRKWATVESSDAPDVSLDGGSLIINTPRLAKQ


                            1510      1520      1530      1540      1550
                     ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1           meelglasqppegrpcqpqtraqkqpghtnyssyskrkrltrgrakntts
zebrafish rai1       RAIKNNHEMHLKQRKKRRKGTEPQENIPIVEQPESTQTEVLPTSPPPPET


                            1560      1570      1580      1590      1600
                     ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1           spckgrakrrrqqqvlpldpaepeirlkyissckrlrsdsrtpafspfvr
zebrafish rai1       LEQTECKEPPNEVCVPLMKPKRGRRPSLKKKQEELSKSQKEDENAIEKKK


                            1610      1620      1630      1640      1650
                     ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1           vekrdafttictvvnspgdapkphrkpsssassssssssfsldaagasla
zebrafish rai1       KTGPKKKCVDAVKVVVKRPKIKTNELSPTVQGTLSDLRPDLSDGKSSVRP


                            1660      1670      1680      1690      1700
                     ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1           tlpggsilqprpslplsstmhlgpvvskalstsclvcclcqnpanfkdlg
zebrafish rai1       YVHIDSSMDVASSCTIVNRPEEEQMLAQARTKSVPNIKNQAAKTGSNSSV


                            1710      1720      1730      1740      1750
                     ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1           dlcgpyypehclpkkkpklkekvrpegtceeaslplertlkgpecaaaat
zebrafish rai1       MLQGPLVNKSLSDRCLTCCLCGKPANYRELGDLCGPYYPEDCIPRKTLSL


                            1760      1770      1780      1790      1800
                     ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1           agkpprpdgpadpakqgplrtsarglsrrlqscyccdgredggeeaapad
zebrafish rai1       THRSEFRQNSNCANETESSSIVKNTQSVCEEAIHQEKANEGHSRRSKRAI
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```
                        1810      1820      1830      1840      1850
             ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1   kgrkhecskeapaepggeaqehwvheacavwtggvylvagklfglqeamk
zebrafish rai1  RELSRTRPSSRMRFKRLLILQSRLSGASPPAGVEGIETSLQRLQMEAEEK


                        1860      1870      1880      1890      1900
             ....|....|....|....|....|....|....|....|....|....|
HUMAN RAI1   vavdmmcsscqeagatigcchkgclhtyhypcasdagcifieenfslkcp
zebrafish rai1  EHWAHEACAVWTTGIVLVADKLFGLTEAVQKAAHAKCSGCQGEGASVCCG


                        1910      1920      1930
             ....|....|....|....|....|....|....|....|
HUMAN RAI1   khkrlp
zebrafish rai1  WKSCTQSYHYICAKESGCTFEEETFSIRCPKHKVS
```

Fig. 16. Alignment of human and zebrafish RAI1/rai1 amino acids. Like
human RAI1, zebrafish rai1 contains nuclear localization signals, a PHD domain,
and poly serine domains, and shows similarity to TCF20. Zebrafish rai1 is
composed of many amino-acid domains that are conserved in human and share
100% similarity. These amino-acids sequences can be acting as regulatory factor
for normal RAI1 functioning and their conserved nature helps in aligning the
protein. The conservation of the domains between human and zebrafish strongly
suggest that this clone is an ortholog of human RAI1.

List of Domains (% identity to human)
Similarity to TCF20 – MQSFRER (100%)
Glutamine region – QQ (Human RAI1 typically carries 13 Q's, while mouse carries 4)
Nuclear localization signal (3) - KKRKKGRQDEVKAQDSPLDIVHIPKGKRK
                                  KKRRK
                                  KKKQEELSKSQKEDENAIEKKK
Polyserine domain – SPSSSHSPARSVGRSPSYNSTPSPLM (100%)
PHD domain – KCSGCQGEGASVCCGWKSCTQSYHYICAKESGCTFEEETFSIRCPKHK (24%)
Conserved domain - HFPQHSQ (100%)
Conserved domain - PEQYYQT (100%)
Conserved domain – ALTSQVENIS (100%)
Conserved domain – VQQLLLS (100%)
Conserved domain – VSTCS (100%)
Conserved domain – LCGPYYPE (100%)

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CHAPTER 4: Spatial and temporal *rai1* expression in zebrafish embryos

Introduction

In order to understand the dosage-sensitive role of *rai1* gene and its effect on complex phenotypes, it is important to evaluate the zebrafish model that is overexpressing or deficient in *rai1* and compare the result with wild-type zebrafish with normal levels of *rai1*. In humans, *RAI1* mRNA is generally expressed in all tissues but present at high levels in heart and neuronal structures (40, 46). Similarly in mouse, immunohistochemistry (IHC) with a *Rai1* antibody detected expression mainly in cerebral cortex, cerebral hemisphere, ganglion, thymus, and midbrain (Fig. 17). Preliminary data from zebrafish studies show *rai1* expression in the lens, midbrain, and diencephalon. A complete spatial and temporal profile of *rai1* expression will facilitate the background for evaluating the changes in gene expression and morphology in the presence of altered levels of *rai1*.
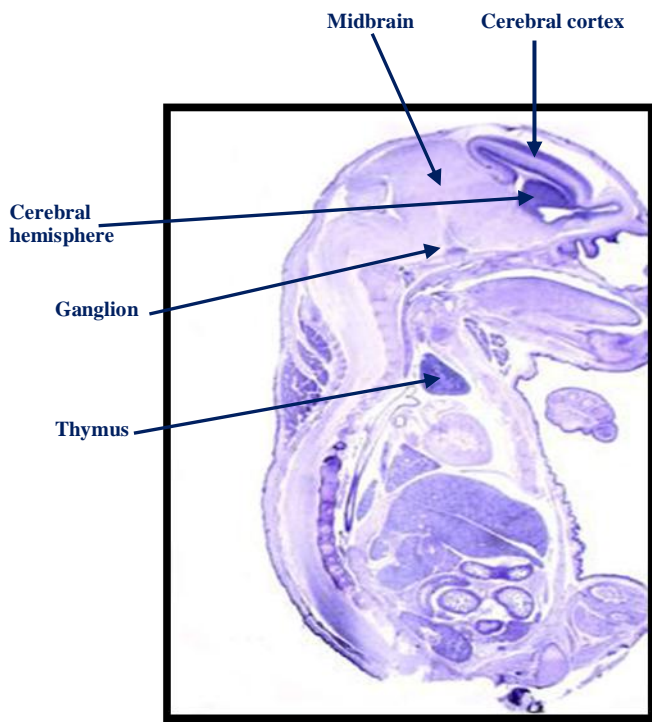
18 dpc
IHC with Rai1 ab
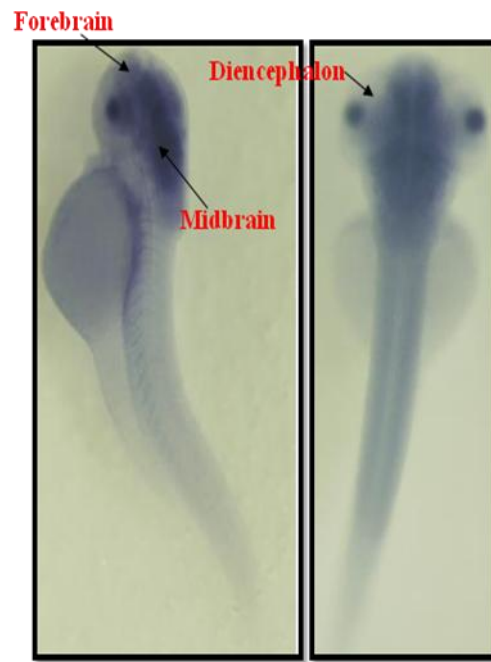
72 hpf
ISH with *rai1* probe

Fig. 17. *rai1* expression in mouse vs. zebrafish. As in mouse, zebrafish show widespread *rai1* expression during embryonic development. Expression in both species becomes more specific in certain areas, especially in the brain. The expression in mouse is detected in the cerebral cortex, ganglion, thymus, and midbrain (Girirajan Dissertation, 2008). The expression in zebrafish is detected in similar regions including midbrain, forebrain and diencephalon.

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Materials and methods

Zebrafish culture and maintenance

<u>Zebrafish lines</u>

Wild-type adult fish of AB strain were used for the experiments.

<u>Aquarium system</u>

Zebrafish were maintained in recirculating system water with the 500 µS as the parameter of conductivity, 27-28°C as the temperature, optimum chemical and biological pressure, UV sterilization, and pH of 7. For breeding, approximately 8 adult fish were placed in 3.0 liter tanks.

Early stages - (<2.5 hpf, 9 hpf, 15hpf and 18hpf)

Collect cultivated embryos

Embryos were harvested and collected as described in Chapter 3.

TRizol treatment

Collected embryos were incubated at 28.5°C until they reached the required stage. At the various stages such as <2.5 hpf, 9 hpf, 15 hpf and 18 hpf, a total of 30 embryos were homogenized in 1 ml of TRizol reagent and stored at -20°C until ready to isolate RNA.

RNA extraction

Total RNA was extracted from the trizol treated embryos using the RNA preparation kit (Invitrogen), according to the manufacturer's instructions.

Reverse-transcriptase PCR (RT-PCR)

First strand cDNA synthesis was performed as described in Chapter 3.

Semi-quantitative PCR

cDNA obtained from <2.5, 9, 15 and 18 hpf embryos was PCR amplified with internal control (*TCOF1*- like gene) primers [F (gcgatctctaccaacatgtg) and R (cttggctgatgggccattga)]. *rai1* was then PCR amplified with primers in the exon 3 region [*rai1*F (agggcactgtctgaagagga) and *rai1*R (ttggcagtgtgagtctgagg)], followed by PCR amplification using primers spanning the exonic boundary of *rai1* exon 2 and 3 [SHE646 (gcgcccagatctaagtgacg) and SHE650 (tcacacacttaccttgtgtttcgg). PCR reaction mix and conditions were followed as described in Chapter 2 (Polymerase chain reaction). PCR products were evaluated by gel electrophoresis.

<u>Later stages</u> - (24, 30, 31, 35, 37, 48 and 72 hpf)

Collect cultivated embryos

Embryos were harvested as described in Chapter 3. There were a few exceptions to the previous protocol, including the addition of 2 µl of methylene blue for fungus prevention and 500 µl of 0.12% of 1-phenyl-2-thiourea (PTU) to inhibit pigmentation, in each petri dish with ~ 80 embryos and then incubated at 28.5°C.

Probe preparation

A glycerol stock of the plasmid containing the 5' end (exon 1) of zebrafish *rai1* gene (Fig. 18) was used to grow colonies for DNA isolation. DNA was extracted using the mini kit by Qiagen (Valencia, California, USA), according to the manufacturer's

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protocol. The isolated DNA was quantified by a spectrophotometer. The DNA template was linearized by digesting 5 µg, overnight at 37° C with *BamH*I enzyme (anti-sense probe) and with *Not*I enzyme (sense probe). After the enzyme digest, the DNA/probe was purified from the solution using the ultraclean kit by Molecular Biology labs (Carlsbad, CA). DNA was then quantified by a spectrophotometer. This was followed by a transcription reaction containing 8 µl of nuclease-free water, 4 µl of transcription buffer (5X), 2 µl of DIG-NTP mix (10X), 1 µl of RNA polymerase (T7 - antisense probe and SP6 – sense probe), 1 µl of RNasin and 1 µg of linearized DNA templates, all assembled in order and incubated at 37°C (antisense probe) and 40°C (sense probe) for 2 hours. This was followed by the addition of 1 µl of RNase-free DNase I after the transcription reaction and incubation at 37°C for 15 mins. Finally, the digoxigenin-labeled probe was purified by using Probe Quant G-50 micro column purification kit by Illustra^{TM} (Buckinghamshire, UK) and 80 µl of hybridization buffer [50% formamide, 5X sodium citrate buffer (SSC), 50 µg/ml heparin, 0.5 mg/ml yeast tRNA, 0.1% tween-20, 9.2 mM of citric acid, and nuclease-free water] was added to the purified probe and stored at -20°C. The probe sequence was aligned with the putative *rai1* gene and it was found to be identical to the 5' end (beginning of exon 1) of the *rai1* gene (Fig. 18 and 19).

In-situ hybridization

As mentioned earlier, embryos were incubated at 28.5°C until they reach the required stage. At these stages, ~ 30 embryos were dechorionated and anesthetized by addition of 20 µl of MESAB before they were fixed in 4% of paraformaldehyde (PFA)

for overnight on the shaker. Next day, PFA solution was replaced with methanol and the embryos were stored at -20°C for overnight. The embryos were then rehydrated through methanol/PBT series washes (100%: 0%, 66%: 33%, 33%: 66% and 0% : 100%) for 5 mins on the shaker. These washes were followed with Proteinase K (PK) treatment: 1 µl of PK in 1 ml of PBT for 2.4 mins for 24 hpf embryos and 4.8 mins for 48 hpf embryos and so on. This was followed by aspiration of PK solution and post-fixation of the embryos with 4% PFA on the shaker for 20 mins. After post-fixation, the embryos were washed several times with PBT and then incubated in Hyb (+) for > 1 hour in water bath set at 65°C. The solution was replaced with diluted probe (1:200) in Hyb (+) and incubated overnight at 65°C. Next day, the diluted probe solution was aspirated and saved for re-use. It was replaced by (Hyb -) buffer [hybridization buffer without tRNA and heparin] for a quick wash followed by series of Hyb (-)/ 2 X SSC series (66% :33%, 33%: 66%, 0%: 100%) for 10 mins at 65°C, and two 20 mins washes of 0.2 X SSC at 65°C. This was followed by three serial washes of 0.2XSSC/PBT (66%: 33%, 33%:66%, 0%:100%) for 5 mins on the shaker. Embryos were then treated with blocking solution [1X PBT, 2 mg/ml bovine serum albumen (BSA), and 2% goat serum (GS)] for 1 hour on the shaker. This blocking solution was replaced with alkaline-phosphatase-coupled anti-digoxigenin FAB fragments diluted (1:10,000) in blocking solution and incubated overnight at 4°C. On day 5, embryos were washed five times with PBT for 15 mins on the shaker. This was followed with three washes of coloration buffer [0.1 M Tris hydrochloride (pH 9.5), 50 mM $MgCl_2$, 0.1 M NaCl and dH_2O] with 0.1% Tween 20 for 5 mins on the shaker. After these washes, the solution was replaced with nitro blue tetrazolium [NBT] [225 µg/ml] and

bromo-4-chloro-3-indolyl phosphate [BCIP] added in coloration buffer with Tween, and the embryos were kept in the dark box on the shaker during the day and incubated at 4°C during the night for two consecutive days. When the embryos were stained sufficiently, the coloration buffer was aspirated and the embryos were given a quick PBT wash. The embryos were then dehydrated with methanol for ~ 20 mins on the shaker to clear out the background. Before performing the imaging, the embryos were rehydrated through a methanol/PBT series and equilibrated in 1X PBS with 50% glycerol. All pictures were taken with an Olympus SZX12 microscope and Olympus DP70 camera using an 80X objective lens.
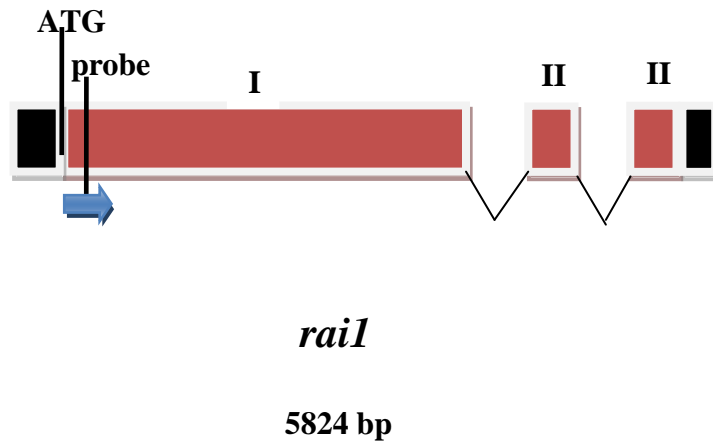
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Fig. 18. Schematic representation of the ISH probe. Probe maps to the 5'end of the exon 1 region of the putative *rai1* gene.

```
                         60        70        80        90       100
                ....|....|....|....|....|....|....|....|....|....|
SP6             ctagtaacggccgccagtgtgctggaattcgccct---tgaattccacca
putative rai1   ----------------------------------cctatctggagtc---a

                        110       120       130       140       150
                ....|....|....|....|....|....|....|....|....|....|
SP6             tgcagtccttcagagagcggagtggtttccatagcaaccagcactgctac
putative rai1   tgcagtccttcagagagcggagtggtttccatagcaaccagcactgctac

                        160       170       180       190       200
                ....|....|....|....|....|....|....|....|....|....|
SP6             cagcaggaaccccaagaattaccgcgcctcgagagctacagacatcatta
putative rai1   cagcaggaaccccaagaattaccgcgcctcgagagctacagacatcatta

                        210       220       230       240       250
                ....|....|....|....|....|....|....|....|....|....|
SP6             cagccagtccagacagggctacgagccacacgctttagcttctgccggga
putative rai1   cagccagtccagacagggctacgagccacacgctttagcttctgccggga

                        260       270       280       290       300
                ....|....|....|....|....|....|....|....|....|....|
SP6             tgtcaacagcgggatctaaagactgttacggacagcagaactatcctgtc
putative rai1   tgtcaacagcgggatctaaagactgttacggacagcagaactatcctgtc

                        310       320       330       340       350
                ....|....|....|....|....|....|....|....|....|....|
SP6             tacaccagcgccacacagactaagaaaccatacagaggaggcaaaactcc
putative rai1   tacaccagcgccacacagactaagaaaccatacagaggaggcaaaactcc

                        360       370       380       390       400
                ....|....|....|....|....|....|....|....|....|....|
SP6             aaaccagcatctgcaggcgggctacagtaaccacatgggcaccggataca
putative rai1   aaaccagcatctgcaggcgggctacagtaaccacatgggcaccggataca

                        410       420       430       440       450
                ....|....|....|....|....|....|....|....|....|....|
SP6             cggcccagtacatgagcgaaggtcacctgcagcagaaatgggatgagtcg
putative rai1   cggcccagtacatgagcgaaggtcacctgcagcagaaatgggatgagtcg

                        460       470       480       490       500
                ....|....|....|....|....|....|....|....|....|....|
SP6             gctcagatgacccagttcgagcaggacatggtgggtcgtctggagtctgg
putative rai1   gctcagatgacccagttcgagcaggacatggtgggtcgtctggagtctgg
```
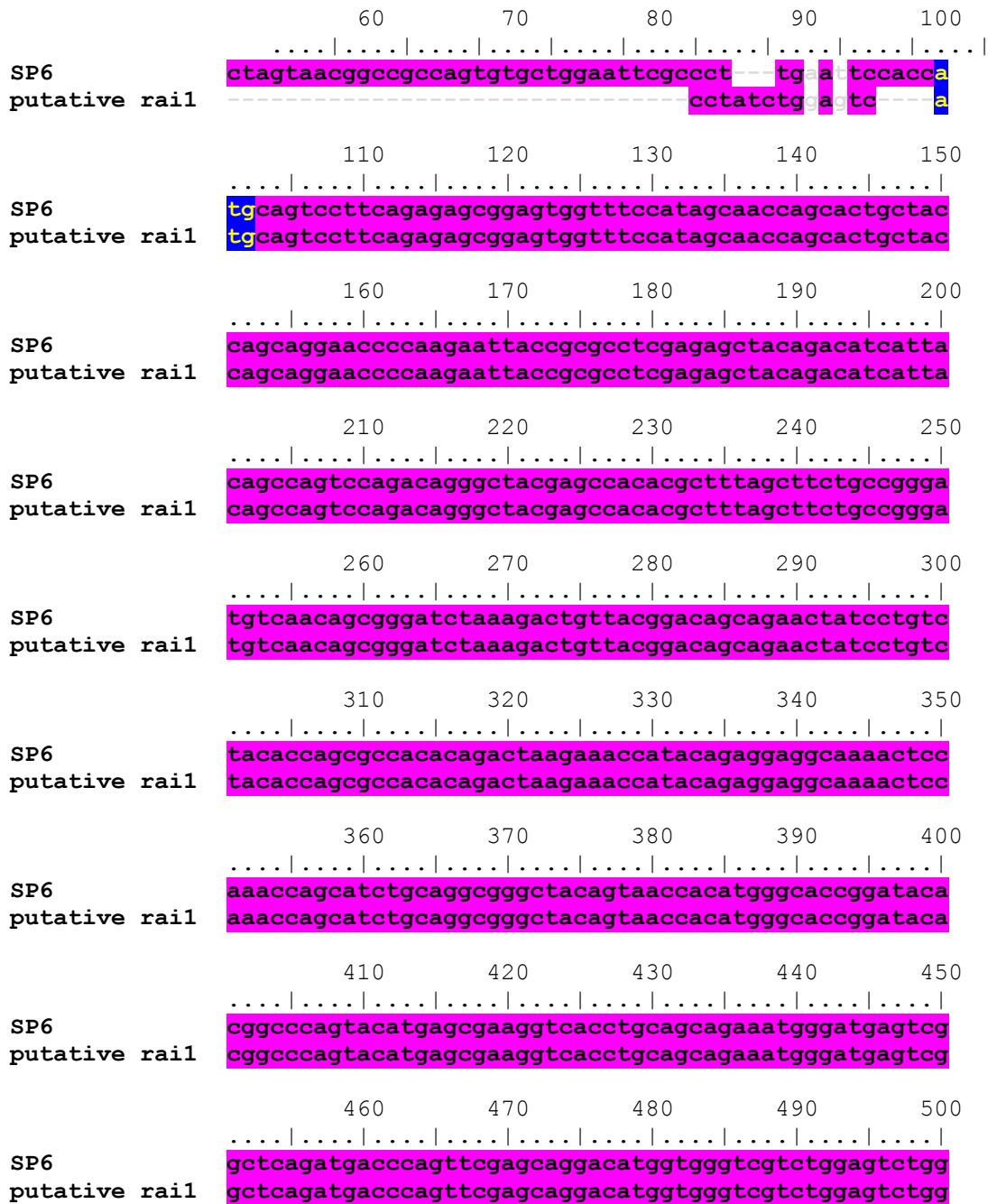
Fig. 19. Alignment of the ISH probe with the *rai1* gene sequence. The anti-sense probe was sequenced with the SP6 primer. This sequence when aligned with the putative *rai1* gene, is identical to the 5' end (beginning of transcription in exon 1-- ATG) of the *rai1* gene.

Results

rai1 expression detected at early stages

Gel electrophoresis results from *rai1* RT-PCR from <2.5 hpf, 9 hpf, 15 hpf, and 18 hpf zebrafish embryos is shown in Fig. 19. Internal control (*TCOF1*- like gene) spanning the exonic region showed expression at all stages verifying the good quality of the cDNA. RT-PCR analysis detected *rai1* gene expression during early zebrafish embryogenesis at 9, 15 and 18 hpf. A maternal *rai1* transcript (< 2.5 hpf) was not detectable/present in early embryonic development.
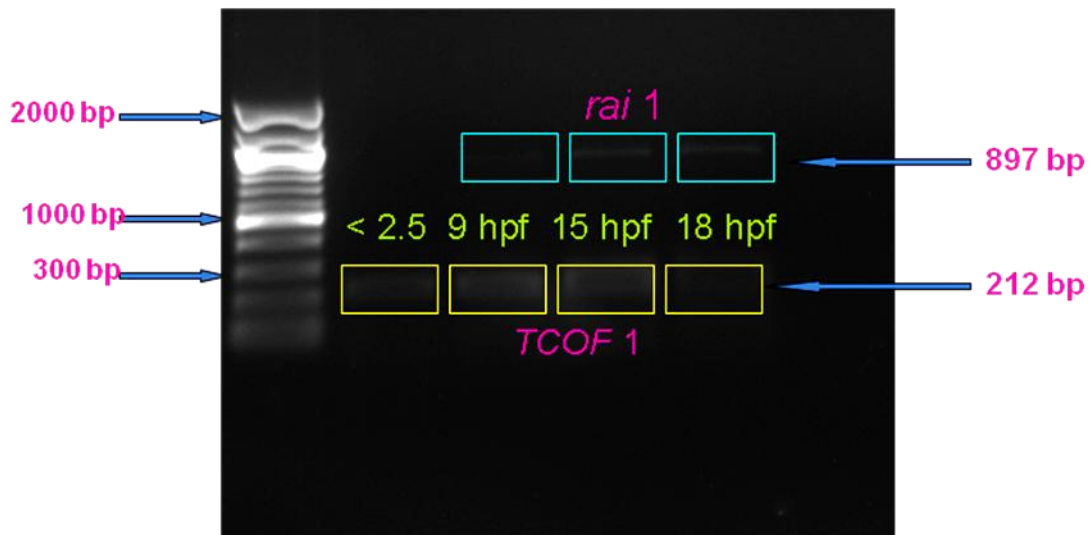

Fig. 20. RT-PCR showing *rai1* expression at early stages. Internal control (*TCOF1*-like gene) shows expression at all stages (yellow). *rai1* expression is detected at 9 hpf, 15 hpf and 18 hpf but not at < 2.5 hpf, using specific primers spanning *rai1* exonic region (blue).

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rai1 expression patterns at later stages

The *rai1* expression patterns observed in zebrafish embryos was similar to the known *Rai1*expression patterns seen in mouse at corresponding developmental stages (Fig. 17). As in mouse, zebrafish embryos showed widespread *rai1* expression during embryonic development, especially in the brain and neuronal areas as shown by ISH. The spatial and temporal profile of zebrafish *rai1* expression is shown in Fig. 21. At 24-30 hpf, *rai1* expression was detected everywhere in the embryos, particularly in the forebrain and diencephalon. As the developmental stage progressed, the expression became more specific in the forebrain, midbrain and diencephalon. The *rai1* expression observed in the lens of the embryos was an artifact as indicated by the similar expression detected in the control embryos.

Fig. 21. Spatial and temporal profile of r*ai1* expression. (a,c) *rai1* expression was detected throughout the embryo at 24-30 hpf. Expression became more specific to the brain regions including midbrain, forebrain, and diencephalon from 31 hpf onwards and was observed in the brain through 72 hpf. **(b,d)** No expression detected in control embryos. Expression in the lens was an artifact, as similar expression was also detected in the control embryo.

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rai1 expression

(c)

35 hpf 37 hpf 48 hpf 72 hpf

(d)

Control
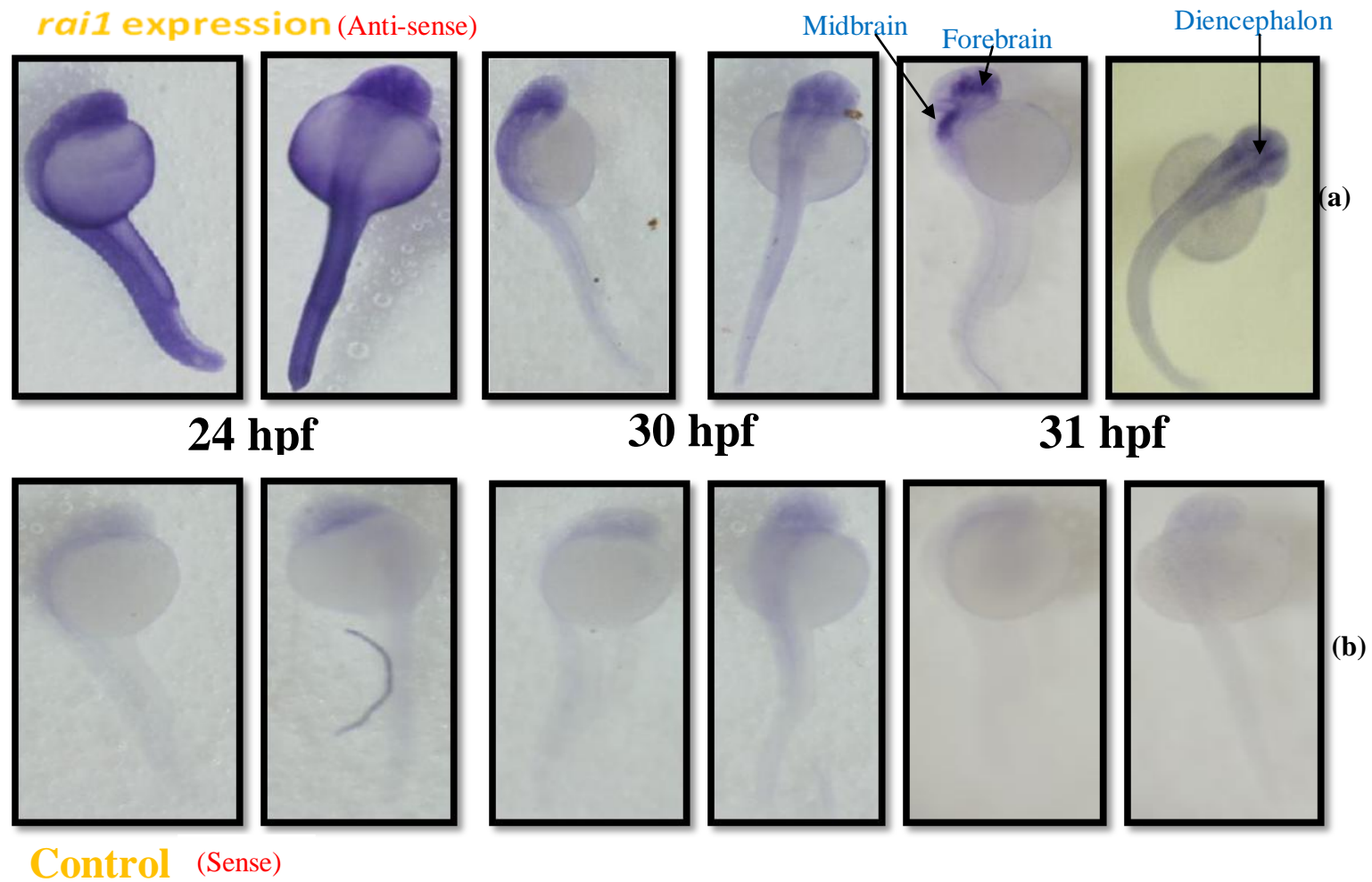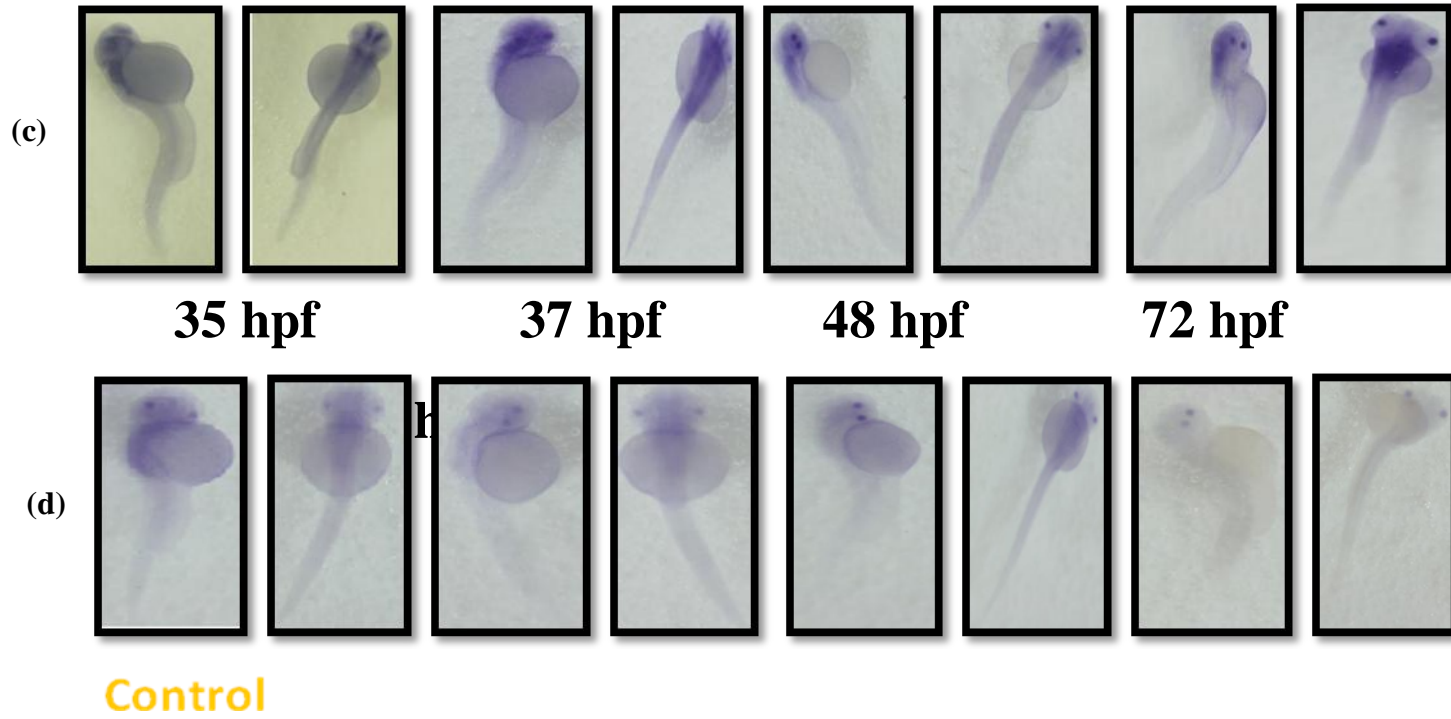
Discussion

Spatial and temporal profile of *rai1* expression

rai1 expression was detected by reverse transcriptase polymerase chain reaction (RT-PCR) and whole-mount *in-situ* hybridization (ISH) in developing zebrafish embryos in tissue-dependent and time-dependent fashion. RT-PCR detected *rai1* expression at 9 hpf, 15 hpf and 18 hpf (Fig. 20). Maternal expression (< 2.5 hpf) was not detected/present in early embryonic development. ISH detected *rai1* expression in zebrafish embryos at different developmental stages (24-72 hpf). Although the expression was present throughout the embryo at 24 hpf; it became more restricted with the progressing developmental stage (~ 35 hpf). The expression was specific in neural tube and brain regions such as diencephalon, midbrain and forebrain as shown in the Fig. 21. But the expression became localized specifically to the brain regions in later stages (~ 48 hpf). The expression observed in the lens was an artifact as seen by similar expression in the control embryos. The completed spatial and temporal profile of normal *rai1* expression established the background necessary to monitor changes in morphology and gene expression associated with *rai1* response to RA treatment.

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CHAPTER 5: *rai1* expression in response to retinoic acid

Introduction

Previous studies have shown *RAI1* as the primary gene underlying the SMS phenotype, and its haploinsufficiency causes SMS (10). Patients with SMS exhibit craniofacial abnormalities, behavioral abnormalities, sleep problems, developmental delay and other manifestations. This suggests that *RAI1* gene dosage is critical for normal growth, development and behavior. Hence, it was important to investigate the factors playing a role in regulating *RAI1* gene dosage and expression.

Retinoic acid

In 1995, Imai et al had shown the upregulation of the *Rai1* gene in mouse P19 cells in response to the induction by RA and RA had led to differentiation of the neurons (26). This suggested an indirect role played by RA in causing SMS phenotype. In addition to this, many other studies (14, 25) have shown the dosage effects of exogenous RA resulting in craniofacial anomalies in both mouse (28) and zebrafish embryos (9) and human. To further investigate the effects of RA concentrations on *rai1* expression and zebrafish development, zebrafish embryos were treated with varying concentrations of RA, and the *rai1* expression and morphology was compared to the wild-type embryos with normal *rai1* expression.

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4-Diethylaminobenzaldehyde

Diethyaminobenzaldehyde (DEAB) is an inhibitor of retinaldehyde dehydrogenases 2 (41, 42) and prevents the production of RA. Assessing the *rai1* expression in the absence of RA helped in determining the overall impact of RA on *rai1* gene expression.

Materials and methods

Preparation of RA and DEAB

All-trans RA was obtained in powder form (Sigma) and RA stocks were made in ethanol at 200 μM concentration. The stock was diluted to 4 μM/ 20 uM in DMSO and then 10 µl of this working stock (4 μM /20 μM/) + 10 µl of DMSO was added to 20 ml of embryo medium to make the final concentration of RA to 2 nM/10 nM. Similarly, DEAB (Sigma) stocks were made in ethanol at 100 mM concentration and diluted to 10 mM in DMSO. Then, 10 µl of this working stock (10 mM) + 10 µl of DMSO was added to 20 ml of embryo medium to make the final concentration of DEAB at 5µM. For the combined treatment of RA + DEAB, 10 µl of RA (4 μM/20 μM) + 10 µl of DEAB (10 mM) was added to 20 ml of embryo medium. Compounds were added at various timepoints to determine the critical period of responsiveness.

Collect cultivated embryos

Embryos were harvested and collected as described in Chapter 3. There were a few exceptions to the previous protocol, including the addition of RA (2 nM/10 nM) only, DEAB (5 μM) only , RA (2 nM /10 nM) + DEAB (5 uM) and DMSO (control) in

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20 ml of embryos medium (1X) instead of water in each petri dish with ~ 80 embryos. This was followed by 2 μl of methylene blue to prevent fungus growth and 500 μl of 0.12% of 1-phenyl-2-thiourea (PTU) to inhibit pigmentation, in each petri dish and then incubated at 28.5°C.

Experimental set-ups

Three different experimental set-ups were arranged to investigate the effect of half-life of RA and DEAB on the developing embryos. Eight petri dishes were set-up with ~ 80 embryos in each of them, and four of them contained embryo medium with RA (only)/DEAB (only)/RA+DEAB (both) and DMSO (control) along with methylene and PTU. In the first set-up, embryos were treated with RA and DEAB only once at 8 hpf. The second experimental set-up contained embryos treated with higher concentration of RA at 10 nM instead of RA at 2 nM on first day (8 hpf), while the third set-up had two different set of groups. The first group of embryos were treated once at 8 hpf while the other group of embryos has the treatment changed on first (8 hpf) as well as second day (27 hpf).

In-situ **hybridization**

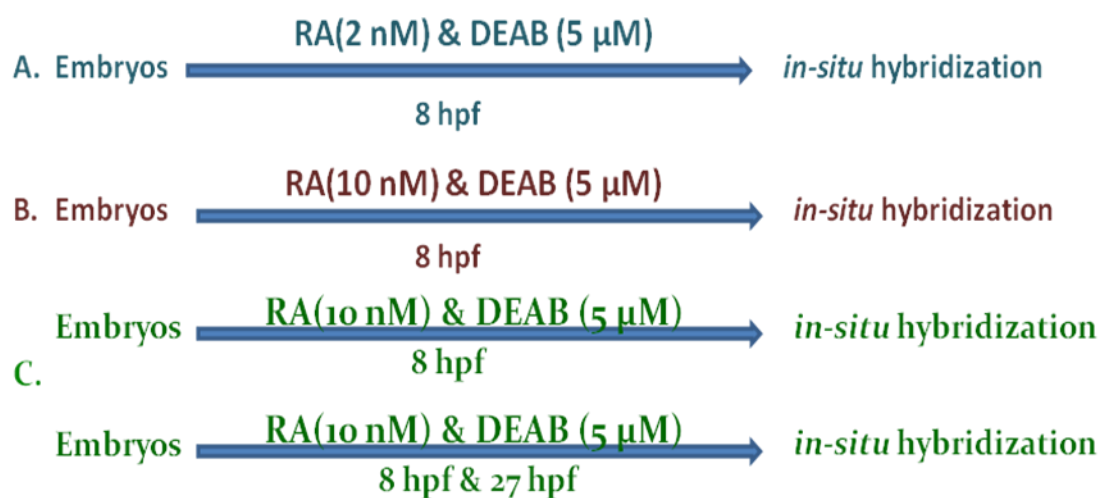Refer to the protocol described in Chapter 4.

Fig 22. Experimental set-ups to investigate the effect of RA and DEAB on embryos. Embryos were treated with RA/DEAB/RA+DEAB treatment at different time points.

Results

Effect of RA on *rai1* expression

Treatment of zebrafish embryos at 24 hpf with 2 nM RA expanded expression of *rai1* throughout the nervous system (Fig. 23). Treatment of 48 hpf embryos with the same concentration of RA (2 nM) induced expression similar to previous stages, but was more specific to the forebrain, midbrain, and diencephalon. Treatment at the same stage (48 hpf) as well as 30 hpf and 72 hpf, with 5-fold higher concentration of RA (10 nM) demonstrated the variable response of the *rai1* expression (Fig. 24). For example, the expression was much higher in specific regions including diencephalon, midbrain, and forebrain. Similar results were observed in embryos treated twice with RA (2 nM/10 nM) at different timepoints (Fig. 25). Hence, in the presence of exogenous RA, *rai1* expression is induced in a non-specific manner throughout the embryo at 24 hpf but

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becomes higher and specific by 48 hpf. Hence, *rai1* is regulated by RA in a tissue and time specific manner.

Effect of DEAB on *rai1* expression

Compared to untreated zebrafish embryo (DMSO), DEAB (5 µM) treated embryos at 24 hpf, 30 hpf , 48 hpf and 72 hpf had a significant reduction of the *rai1* expression in the trunk (spinal cord), but the expression remained high in brain regions (Fig. 23 and 24). Similar results were observed in embryos treated twice with DEAB at different time points (Fig 25). These embryos were deformed with shortened hindbrains, slightly rounded somites, shortened and curved body axes, inflated yolk sac and mild cardiac edema.

RA + DEAB counteract each other

The zebrafish embryos were also treated with the combination of RA (2 nM/ 10 nM) + DEAB (5 µM). The *rai1* expression in these embryos at 24 hpf, 30 hpf, 48 hpf and 72 hpf was similar to *rai1* expression observed in the DMSO treated (control) embryos (Fig. 23 and 24). Similar results were observed in embryos treated twice with RA + DEAB at different timepoints (Fig. 25).

Fig. 23. Effect of RA (2 nM) and DEAB (5 μM) on zebrafish embryos. RA induces *rai1* expression throughout the embryos at 24 hpf and becomes specific to the brain regions by 48 hpf. DEAB reduces expression in the neural tube but it remains high in the brain regions. RA + DEAB counteracts each other and expression is similar to DMSO.

24 hpf

48 hpf

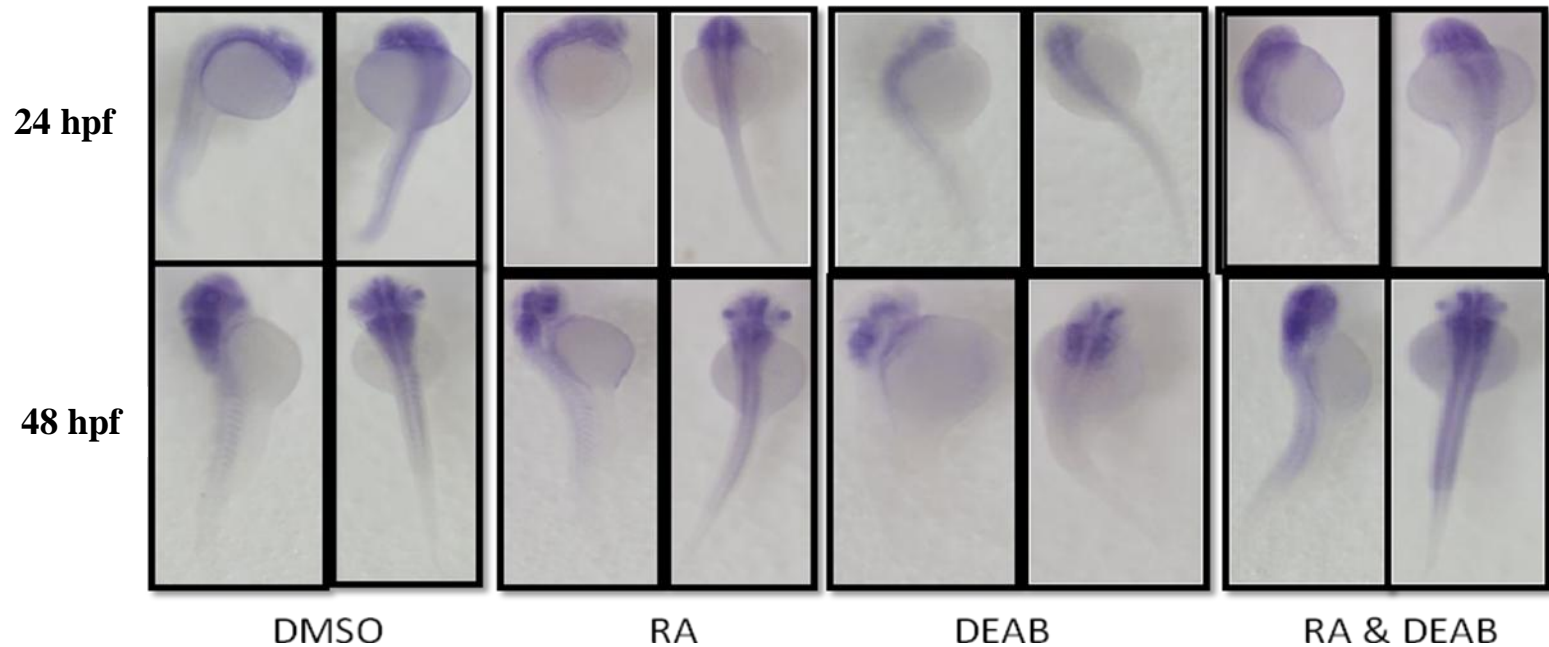DMSO   RA   DEAB   RA & DEAB

Fig. 24. Effect of RA (10 nM) and DEAB (5 µM) on zebrafish embryos. *rai1* expression is similar to Fig. 23.
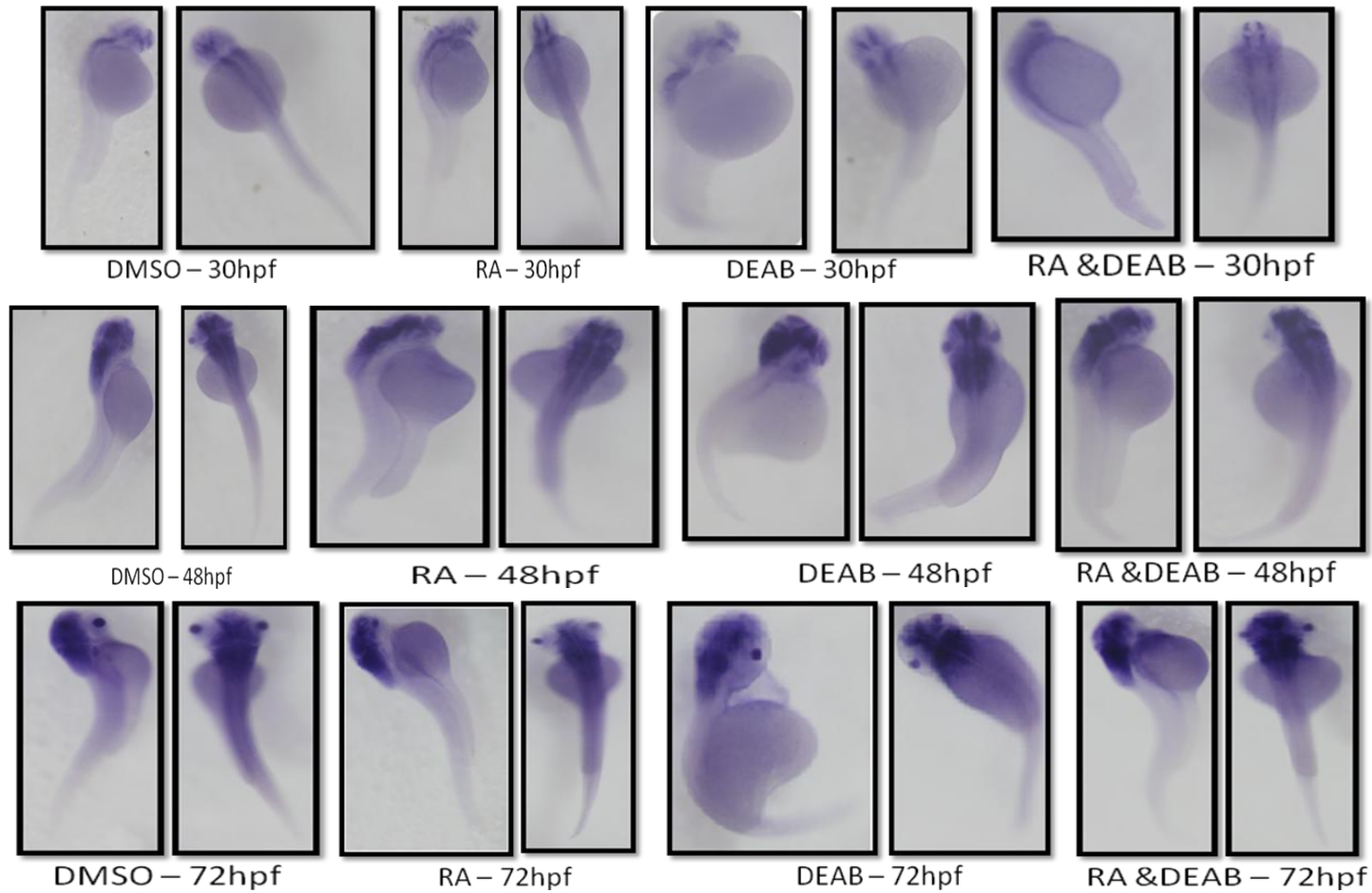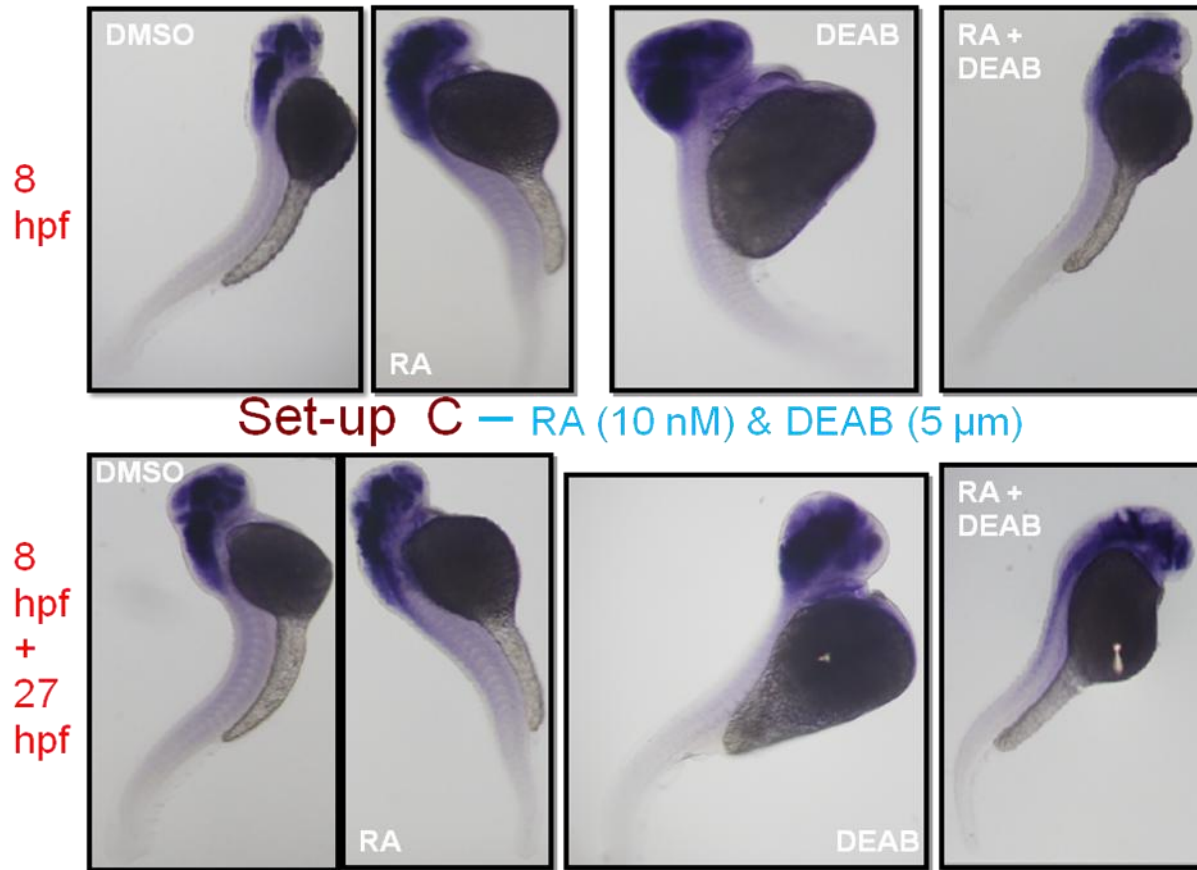
Fig. 25. Effect of RA (10 nM) and DEAB (5 μM) on zebrafish embryos. *rai1* expression is similar to Fig. 23.

Discussion

RA is not solely responsible in inducing *rai1* expression

Expression of *rai1* in response to the presence or absence of retinoic acid at two different concentrations was assessed in the developing zebrafish embryos (Fig. 23 and Fig. 24). Retinoic acid (RA) is a derivative of vitamin A and has been shown to induce the upregulation of the *rai1* gene in cultured cells. On the other hand, diethylaminobenz-aldehyde (DEAB) is an inhibitor of retinaldehyde dehydrogenases and stops the production of RA. Deprivation of RA leads to craniofacial anomalies as shown in previous studies (9, 14, 25, 28). In our study, DMSO treated embryos served as the control for normal *rai1* expression. ISH results from 24 hpf and 48 hpf indicated that RA treatment at 2 nM concentration induced *rai1* expression throughout the embryo as compared to the DMSO treated embryos (Fig. 23). Similar results were also observed in embryo at 30 hpf, 48 hpf and 72 hpf stages when treated with higher concentration of RA (10 nM) (Fig. 24). DEAB treatment at 5 µM concentration reduced the *rai1* expression in the trunk (spinal cord) but the expression remained high in the brain region (Fig. 23 and 24). However, these embryos were greatly deformed with shortened hindbrains and faces, slightly rounded somites, shortened and curved body axes, inflated yolk sac and also displayed mild cardiac edema and were difficult to study. The combination of RA and DEAB treatments counteracted each other, and the *rai1* expression was similar to the DMSO treated embryos. Similar results were observed in embryos treated twice with RA/DEAB or RA + DEAB at different timepoints (Fig 25). Due to the fact that DEAB treatment inhibited RA but did not take away the *rai1* expression throughout the embryo,

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it suggests that RA is not solely responsible for *rai1* expression at the developmental stages studied by ISH. Further studies assessing *rai1* expression at the cellular level should be performed to investigate if *rai1* expression in response to RA is cell-type specific.

CHAPTER 6: Discussion

RAI1/rai1 gene dosage effects in humans and zebrafish

Genetic syndromes such as Prader-Willi, Williams, Down, and Fragile X are complex disorders involving dysregulation of several developmental process that maintain normal growth and behavior. Smith-Magenis syndrome is a complex neurobehavioral disorder caused by a 17p11.2 deletion or a mutation in the _RAI1_ gene. Cases with 17p11.2 deletions always have a deletion of _RAI1_ gene. However, the mutation cases have a single base change or microdeletion in the _RAI1_ gene that causes SMS. Phenotypic comparison between the 17p11.2 deletion and mutation cases indicates that haploinsufficiency of the _RAI1_ gene is responsible for causing SMS features (15). Previous studies in mouse P19 cells have shown that RA induces the _Rai1_ gene (26). Taken together, these data suggest that _RAI1_ gene dosage is vital for normal development and behavior. Zebrafish is an excellent model system to study dosage-sensitivity of the _rai1_ gene. Using zebrafish as a model, we cloned zebrafish _rai1_ gene, evaluated the spatial and temporal profile of _rai1_ expression and investigated the role of RA on _rai1_ expression. Results from this study show the _rai1_ gene was expressed in a time-dependent and tissue-dependent manner and RA was not solely responsible for inducing

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rai1 gene expression. It seems like there are several factors/pathways regulating the expression and function of *rai1* gene.   Future experiments exploring the factors regulating the *rai1* gene and the required *rai1* dosage threshold for normal growth, development and behavior are necessary to increase our understanding of this gene. The results from these studies will facilitate insight about treatment options that can alleviate some of the phenotypic SMS features and prevent the morbidity associated with this syndrome.

Future studies

Future research on SMS and the *rai1* gene using the zebrafish model could include creating a *rai1* antisense morpholino to perform knockdown, or overexpression studies. This will be achieved if the cells are prevented from making rai1 protein or make the protein in excess. If this knockdown or overexpression causes developmental problems, we can learn about the function of rai1 protein and its role in the developmental process. We can use the *rai1* full length clone to perform rescue experiments to show that the phenotype of any knockdown is specific to *rai1*. This will determine the contribution of the *rai1* gene to any phenotypic features observed with knockdown of *rai1* in zebrafish. Additionally, we can investigate upstream and downstream effects of *rai1* RNAi knockdown on other candidate genes. Microarray analysis data obtained from *RAI1*-RNAi knockdown cell lines and cells overexpressing *RAI1* revealed a defined group of genes that had their expression increased or decreased in response to *RAI1* suppression (Girirajan et al., 2008). First, we need to determine if these genes are present in the zebrafish genome and then evaluate how these genes are regulated in the absence or

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presence of *rai1* in zebrafish. In short, the zebrafish model is an ideal system for investigating pertinent questions regarding the molecular and biochemical pathology of SMS and *RAI1* gene.

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Literature Cited

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Refined protocol for *RAI1* mutation screening

1. Run PCRs with *RAI1* specific primers. (Refer to Table 1)

PCR Reaction Mix:

- 2.5 μl 10x PCR buffer
- 0.5 μl 10 mM dNTPs
- 0.5 μl 20 μM forward primer
- 0.5 μl 20 μM reverse primer
- 0.25 μl Taq (5U/μl)
- 4 μl BSA (1 mg/ml) – {except SHE345/664}
- 15.75 μl dH$_2$O
- 1.0 μl genomic DNA (~ 50 ng/μl) → DNA <u>must</u> be added at the <u>END</u>

Total Volume: **25** μl (per reaction)

Cycling Conditions:

94 °C	5 min	
94 °C	1 min	
T$_a$	1 min	35 cycles (T$_a$ – Refer to Table 1)
72 °C	1 min (~ 1 Kb product) 2 min (~ 2 Kb product)	
72 °C	10 min	
4 °C	indefinite	

2. Gel Electrophoresis

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- Make a 1% agarose gel.
  - Weigh out 0.30 g agarose.
  - Combine agarose and 30 mL TBE Buffer in an Erlenmeyer flask.
  - Heat mixture in the microwave in 10 sec increments to completely dissolve the agarose.
  - Cool solution by running flask under cold tap water.
  - Add 3 μL ethidium bromide to the flask. Swirl to mix.
  - Pour agarose solution into gel molds. Let gel set; ~ 15 minutes.
- Run Products
  - Fill electrophoresis chamber with TBE Buffer and place gel in center.
  - Add 3 μL of DNA Ladder (Hyperladder II) to well on far left.
  - On a piece of parafilm place 1 μL drops of 5x loading dye. 1 drop for each PCR product.
  - Add 4 μL of PCR product to one drop of dye and mix with pipet. Load sample in gel.
  - Run gel at 100 V for 30 minutes.
  - Examine gel under UV lamp (11th floor, Sanger Hall) and take a picture.
  - If a single clear band is present with the correct length follow-up with Step 3: Clean PCR Products.
  - If multiple bands are present perform a gel extraction using the Qiagen Gel Extraction Kit and Protocol. Send purified product directly for sequencing—these products do not need treatment with ExoSAP-IT.

3. Clean PCR Products

- Use a 96-well PCR plate for several reactions. Otherwise, use individual PCR tubes.
- Mix 5 μl PCR product with 2 μl ExoSAP-IT (USB 78200) for a combined 7 μl reaction volume.
- Place in thermocycler under these conditions:

  SAP (PCR Machine – User: Santhosh)
  37 °C   30 min
  80 °C   15 min   To inactivate ExoSAP-IT
  4 °C   indefinite

- The PCR product is now ready for use in DNA sequencing. Treated PCR product may be stored at -20 °C until required.

4. Send Treated PCR Products to Massey Core Lab for Sequencing

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- Use either 1.5 ml individual microcentrifuge tubes or 96-well plate for samples.
- Tube 1: Add 3 μl dH$_2$O to 7 μl of treated PCR product for a total volume of 10 μl to be sent for sequencing.
- Tube 2: Add 10 μl diluted primer: 8 μl dH$_2$O + 2 μl of 20 μM primer (Refer to page 3 for details regarding use of forward and reverse primers)

5. **Analyze Sequence Data**

- Analyze by comparing patient sequencing results with the normal *RAI1* gene sequence. Look at sequence quality and for heterozygosity.
- Blast using ClustalX/Bioedit
- Provide sequencing data with a summary sheet of results for each amplimer and sequencing primer to Dr. Elsea

6. **Documentation of Data**

- Draft a Report. (Windows in Lab Office – click on Documents to Elsea lab 2009 to Sequencing Reports, and then use the most updated one as a template, 2008)
- Update Database on Mac (Mutation Summary)
- Mail report to referring geneticist once Dr. Elsea signs and approves final report.
- Attach a copy of report along with the sequences to the patient SMS file and file it in the cabinet.

Sequencing Plan for PCR Products

1st Round of Sequencing

Process the following PCR amplicons with the suggested forward (F) and reverse (R) primers

- SHE323/508
  - Primers – SHE666 (R), SHE506 (R), SHE507 (F)
- SHE327/330
  - Primers – SHE328 (R), SHE329 (F)
- SHE329/332
  - Primers – SHE331 (F)
- SHE513/514
  - Primers – SHE514 (R)
- SHE515/516
  - Primers – SHE515 (F)

- SHE335/669
  - Primers – SHE336 (R), SHE669 (R)
- SHE527/528
  - Primers – SHE527 (F)
- RA46/45
  - Primers – RA46 (F)
- SHE345/664
  - Primers – SHE664 (R)

**If the 1st round of sequencing does NOT cover the entire region, then follow-up with the 2nd round of sequencing.

*You may need to repeat a sequence if the sequence is not clear and readable.

2nd Round of Sequencing
Send the following PCR products with **highlighted** primers only
- SHE**505**/506
- SHE507/**508**
- SHE**325**/326
- SHE**509**/510
- SHE511**/512**

TABLE 1. *RAI1* PRIMERS			
Exon	**Primers**	**Product Size**	**Annealing Temp. (T_a)**
Start E 3	SHE323: TCTGAGGCAAAAGGAAGTGG	1816 bp	62 °C
	SHE508: CATGTCGTCAGGAGAGGTCA		
E 3	SHE327: TGTCAAGAACCTCGTGTCCA	1434 bp	60 °C
	SHE330: AACCAGCTCTGGACCTTTGA		
E 3	SHE329: TCTCGCTGGAGAACCACAG	1765 bp	60 °C
	SHE332: ATGAAGGCCGACTTCCTTTT		
E 3	SHE513: GCTCCAAGACCAAGGAGACA	910 bp	62 °C
	SHE514: GCCTCGGTTTTGAAACAGTCA		
E 3	SHE515: GGCCTCTCCTGGTAATCCTC	1010 bp	64 °C
	SHE516: GCAGCAAACAAGGCAAGAGGT		
End E 3	SHE335:AAACCGAGGCCTTCACATC	1490 bp	60 °C
	SHE669: GTGTGACCTGCCTTAGCCAGAT		
E 4	SHE527: CCAGCCTGTAAAGCTTGAGG	362 bp	62 °C
	SHE528: GGAATGCCTCATTTCCATGT		
E 5	RA46: GGAGTGGAGTGGAGTGTGGAGG	310 bp	64 °C
	RA45: GGACTGTGAAGGAGGTGCGAGG		
E 6	SHE345: ACTGTGAAGTCCGAGGTCGT	313 bp	60 °C + Enhancer
	SHE664: TGGGCAAGAACGCCCT		

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TABLE 2. *RAI1* PRIMERS			
Exon	**Primers**	**Product Size**	**Annealing Temp. (T_a)**
E 3	SHE323: TCTGAGGCAAAAGGAAGTGG SHE324: GACTGGGAATGCTGAGGAAA	770 bp	62 °C
E 3	SHE325: AAAGGCAGAAGCTGCAGAAC SHE326: CAGTGCTGGCTTTTATGCTG	875 bp	62/55 °C
E 3	SHE327: TGTCAAGAACCTCGTGTCCA SHE328: GGGAAACAGTCAAAAGCTGC	777 bp	60 °C
E 3	SHE329: TCTCGCTGGAGAACCACAG SHE330: AACCAGCTCTGGACCTTTGA	904 bp	59 °C
E 3	SHE331: CACTCATCTGCACCAAGGAG SHE332: ATGAAGGCCGACTTCCTTTT	1000 bp	60 °C
E 3	SHE333: CCAAGAAGCTCCTCGACAAC SHE334: TTCCGGCTCCTCTTCTTAGG	763 bp	59 °C
E 3	SHE335:AAACCGAGGCCTTCACATC SHE336: CACACTCGGGACCTTTGAGT	964 bp	60 °C
E 3	SHE339: CTACCCTGAACACTGCCTCC SHE340: AGAGGCAATGGAGACAGGAA	887 bp	60 °C
E 6	SHE345: ACTGTGAAGTCCGAGGTCGT SHE346: GACTGGAAAGGGGACAAACA	766 bp	60 °C + PCR Enhancer (no BSA)
E 3	SHE505: CCCGAGTCATGCAGTCTTTT SHE506: TGGTAATGGAGGGTTTCCTG	925 bp	60 °C
E 3	SHE507: GGTCCAGAATCTTCATGCCTA SHE508: CATGTCGTCAGGAGAGGTCA	947 bp	62 °C
E 3	SHE509: CTGCCAGGGTCAACAGCAAC SHE510: CTCCTCCTCAGCTTCCAGGT	995 bp	64 °C
E 3	SHE511: AGGAGGACCTGGAAGCTGAG SHE512: GTGACCGAAGGATCATGGAGC	861 bp	66 °C
E 6	SHE663: ACCCCAGCCGCGCTCT SHE664: TGGGCAAGAACGCCCT	387 bp	52 °C
E 3	SHE666: TGCAGGCCTTGCTGTGT	Internal Primer	N/A
E 3	SHE669: GTGTGACCTGCCTTAGCCAGAT	Internal Primer	N/A

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Revised genomic sequence of the *RAI1* gene

Genomic sequence surrounding *RAI1*

Exon 3

```
gcgggggagc aggcagggtg gggaggtggg tgggcgggcg ctttctgagg caaaaggaag SHE323
tggcccgtct gaatcgctca tcctctgccc cctccctgcc catcctcccc tccctccttc
cctccctccc tcccttcctt tttcttttca cagataacca gcccgagtca tgcagtcttt start E3/SHE505
tcgagaaagg tgtggtttcc atggcaaaca acagaactac cagcagacct cgcaggaaac
atcacgccta gagaattaca ggcagccgag tcaggccggg ctaagctgcg accggcagcg
gctgctcgcc aaggactatt ataacccgca gccttacccg agctatgagg gtggcgctgg
cacgccctct ggcactgcag ccgcggtggc cgccgacaag taccaccgag gcagcaaggc
cctgcccaca cagcaagggc tgcaggggag gccggctttc cctggctacg gcgtccagga SNP G269C/SHE666
cagcagcccc tacccaggcc gctatgctgg tgaggagagc cttcaggctt gggggggcccc  253del119
acagccacca cccccacagc cgcagccact acctgcaggg gtggccaagt atgatgagaa
cttgatgaaa aagacagcag tgccccccag caggcagtat gcagagcagg gcgcccaggt
gccctttcgg actcactccc tgcacgtcca gcagccaccg ccgcccccagc agcccctggc SNP C493A
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ataccccaag ctccaaaggc agaagctgca gaacgacatt gcctcccctc tgcccttccc    SHE325
ccagggtacc cactttcctc agcattccca gtccttcccc acctcctcca cctactcctc    SHE324
ctctgtccag ggtggtgggc agggggcccac tcctataaga gttgcacagc accgactgcc
cagccccatg acaggccgct gactgccagc tccagcctgg ccccgggggca gcgggtccag    SHE507F
aatcttcatg cctaccagtc gggccgcctc agctatgacc agcagcagca gcagcagcag    SNP G837A
cagcagcagc agcagcagca agcccttcag agccggcacc atgcccagga aaccctccat    SHE506R/Poly#
taccaaaacc tcgccaagta tcagcactac gggcagcaag gccagggcta ctgccagccg
gacgcagccg tccggacccc agagcagtac taccagacct tcagccccag ctccagccac
tcacccgccc gctccgtggg ccgctcacct tcctacagtt ccacaccgtc gccgctgatg
ccaaacctgg agaactttcc ctacagccag cagccgctca gcaccggggc cttccccgca    1119delC
gggatcactg accacagcca cttcatgccc ctgctcaatc cctccccaac ggatgccacc
agctctgtgg acacccaggc tggcaactgc aagccccttc agaaggacaa gctccctgag
aacctgctgt cggatctcag cctgcagagcc tcacggcgct gacctcacag gtggagaaca
tctccaacac cgtccagcag ctgctgctct ccaaggctgc tgtgccgcag aagaaaggtg    SHE327
tcaagaacct cgtgtccagg accccagagc agcataaaag ccagcactgc agccccgaag    SHE326/1449delC
ggagcggcta ctcagccgag cccgcaggca caccgctgtc agagccgccg agcagcacgc
cacagtccac gcatgcggag ccgcaggagg ccgactacct gagcggctcc gaggacccac
tggagcgcag cttcctctac tgcaaccagg cccgtggcag ccctgccagg gtcaacagca    SHE509F
actcgaaggc caagcccgag tccgtgtcca cctgttctgt gacctctcct gacgacatgt    SHE508R
ccaccaaatc tgacgactcc ttccagagcc tacacggcag tctgccgctc gacagcttct
ccaagttcgt ggcgggtgag cgggactgtc cgcggctgct gctcagcgcc ctggcacagg
aggacctggc ctccgagatc ctggggctgc aggaagccat cggtgagaag gccgacaaag
cttgggctga agcacccagc ctggtcaagg acagcagcaa gccccttc tcgctggaga    SHE329
accacagcgc ctgcctggac tctgtggcca agagtgcgtg gccccggcct ggggagccgg    SNP G1992A
aggccctgcc cgactccttg cagctggaca agggcggcaa tgccaaggac ttcagcccag
ggctgtttga agacccttcc gtggccttcg ctacgcctga ccccaaaaag acaactggtc
ctctctcctt tggtaccaag cccacccttg gggttcctgc tccagacccc actacagcag    SHE328
cttttgactg tttcccggac acaaccgctg ccagctcagc ggacagcgcc aacccctttg
cctggccaga ggaaaacctg ggggatgctt gtcccaggtg gggattgcac cctggcgagc
ttaccaaggg cctggagcag ggtgggaagg cctcagatgg catcagcaaa ggggacaccc
atgaggcttc ggcctgcctg ggcttccagg aggaggaccc ccctggggag aaggtggcct
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cgttgcccgg ggacttcaag caggaggagg tgggtggggt gaaggaggag gcaggtgggc
tgctgcagtg ccccgaggtg gccaaggctg accggtggct ggaggacagc cggcactgct
gttccaccgc cgacttcggg gacctcccac tgctgccacc caccagcagg aaggaggacc  SHE511F
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104

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105

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Exon 4

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Exon 5
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Exon 6 and some 3'UTR

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APPENDIX C

cDNA sequence of zebrafish *rai1* gene

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IRB protocol used

Institutional review board (IRB) protocol for the SMS study

2004-present – Protocol number **3784**, "Molecular analysis of Smith-Magenis syndrome."

<u>VITA</u>

Bijal Akshay Vyas was born on November 18, 1986 in Mumbai, India and holds Indian citizenship. She graduated with a Bachelors of Science with a major in Biology and minor in Genetics and Psychology from North Carolina State University in 2007. She joined the Molecular Biology and Genetics Program at Virginia Commonwealth University in 2007 and joined the laboratory of Dr. Sarah H. Elsea to pursue her goal of receiving further knowledge in the field of genetics.