Exploring the Methylation Status of RAI1 and the RAI1 Consensus Binding Sequence

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Exploring the Methylation Status of RAI1
and the RAI1 Consensus Binding Sequence

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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Abstract

EXPLORING THE METHYLATION STATUS OF RAI1 AND THE RAI1 CONSENSUS BINDING SEQUENCE

By Eri Kamura, 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

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Smith Magenis Syndrome (SMS) is a multiple congenital anomalies/mental retardation disorder caused by deletion or mutation of the RAI1 gene on chromosome 17p11.2. The majority of patients with SMS phenotypes have a deletion or mutation of RAI1. However, some patients have been observed with SMS-like phenotypes and yet have no deletions or mutations in the RAI1 gene. One possible explanation could be aberrant methylation of RAI1 since RAI1 is present and yet may be silenced. In order to
study this possibility, patient cell lines were treated with 5-Aza-2’-deoxycytidine. RNA was extracted and real-time PCR was used to check the RAII expression status on the cells. RAII is thought to be a transcription factor, but the DNA binding sequence is still unknown. Sequences from ChIP-chip data were compared to identify a consensus sequence. One gene which contained this consensus sequence was the chemokine-like receptor-1 gene (CMKLR1), which was investigated by luciferase assay. CMKLR1 showed upregulation when co-transfected with RAII.
Chapter 1: Literature Review of Smith-Magenis Syndrome and RAI1

Introduction

Smith-Magenis syndrome (SMS) [OMIM, #182290] is a multiple congenital anomalies and mental retardation disorder. SMS is characterized by craniofacial and skeletal anomalies, such as brachycephaly and midface hypoplasia, mental retardation, obesity, distinctive behavioral phenotypes, including self-injurious behaviors, speech delay, and sleep disturbance due to an inverted pattern of melatonin secretion. The incidence of SMS is estimated to be around 1:15,000 – 25,000 but is thought to be under diagnosed (8,9). SMS is commonly associated with an interstitial deletion involving chromosome 17p11.2 which spans ~3.7 Mb and includes the retinoic acid induced 1 (RAI1) gene. Although the majority of patients with SMS share a common sized deletion, there have been reports of patients with both smaller and larger deletions, along with patients that have a mutation of RAI1 (8,21).
Physical Features

The phenotype of SMS becomes more pronounced and recognizable as individuals age (12). Significantly decreased fetal movement in ~50% of pregnancies was noted in prenatal histories for infants born at term. Craniofacial features that have been reported include brachycephaly, a broad face, frontal bossing, synophrys, hypertelorism, upslanting eyes, midface hypoplasia with a depressed nasal bridge, a tented upper lip (cupid’s bow upper lip), prognathism, and low-set or abnormally shaped ears. Short stature (<5th percentile) is commonly observed in young SMS patients (~67%), but most resolve over time with individuals reaching the 10-25th percentile by adulthood (7,20). Obesity is also common in teens and adult SMS patients. Other skeletal anomalies include brachydactyly, scoliosis, fifth-finger clinodactyly, 2/3 toe syndactyly, elbow limitations, forearm abnormalities, and vertebral anomalies (7,8,10). Otolaryngological abnormalities such as chronic ear infections often associated with hearing loss, and a hoarse voice are also common in these patients (8). Ophthalmologic anomalies are also present in >60% of SMS patients, including myopia, strabismus, nystagmus, microcornea, cataracts, iris anomalies, and retinal detachment (often resulting from violent behaviors) (8).
Neurological and Behavioral Features

Most SMS patients have mild-to-moderate mental retardation with developmental delay observed during childhood. Infantile hypotonia is common in these patients. Patient IQs range between 20-78 with IQ decreasing as the child ages. Many of these children have significant speech delay and motor delay, having better receptive language skills than expressive language skills. Some neurological features include peripheral neuropathy, pes cavus or pes planus, an abnormal gait, hyperactivity, balance problems, and most characteristic of this syndrome is self-injurious behavior. Self-injurious behaviors include head-banging, self-biting, polyembolokoilamania (insertion of objects into bodily orifices), and onychotillomania (pulling out fingernails and toenails) (10). Other behavioral characteristics include attention seeking behavior, impulsivity, an upper body squeeze or “self-hugging”, temper tantrums, aggression, distraction, and disobedience (8).

Sleep Disturbance

The most characteristic finding of SMS is sleep disturbance and is reported in 75% - 100% of patients (7). Sleep disturbances in individuals with SMS include difficulty falling asleep, diminished REM sleep, reduced 24-hour and night sleep, fragmented and shortened sleep cycles with frequent nocturnal and early-morning awakenings, and
excessive daytime sleepiness (8). These patients also have “sleep attacks” where they suddenly fall asleep during evening meal time (6). These abnormal sleep patterns are due to an inverted circadian rhythm of melatonin.

**Melatonin and circadian rhythm**

Melatonin is a naturally occurring hormone secreted by the pineal gland under the influence of the suprachiasmatic nuclei (SCN) of the hypothalamus. The circadian rhythm of melatonin secretion is endogenous; however, it is entrained by the light/dark pattern of light received from the retina to the SCN (17). Melatonin plays an important role in the bioregulation of circadian rhythms, sleep, mood, reproduction, and aging. Concentrations of melatonin in the serum gradually increase from the onset of darkness and peaks around 2 am to 4 am, and then decreases as the night ends. Potocki et al. (2000) conducted a study measuring urinary excretion of 6-sulphatoxymelatonin (aMT6s), the major metabolite of melatonin, showing SMS patients had abnormalities in the circadian rhythm of aMT6s (17). This suggested that the disturbed sleep in these patients could be caused by aberrations in the production, secretion, distribution or metabolism of melatonin (17). β1-adrenergic antagonist therapy has been reported to help reduce the sleep disturbances and improve behaviors (7). De Leersnyder et al. (2001) also conducted a study to determine the circadian rhythm of melatonin in SMS patients. The study found a phase shift in the circadian rhythm of melatonin in these patients in which melatonin onset was around 6 AM, peak time was around 12 PM, and offset of melatonin
was around 8 PM. Normal melatonin onset is around 9 PM, peak time is around 3:30 PM, and offset is around 6 AM. The study showed how the melatonin secretion in SMS patients is inverted compared to normal melatonin secretion patterns and suggests this could be correlated to behavior problems SMS patients have (6). Tantrums, naps, and sleep attacks all could correlate to the patients being tired because of their peak in melatonin secretion during the day/evening time. Also, hyperactivity and attention deficit could also be a result of the patients trying to fight the sleepiness induced by the high melatonin (6).

RAI1

Approximately 90% of reported SMS cases have a deletion of the 17p11.2 region, while 10% have a mutation in the RAI1 gene. Deletions of this region are caused by abnormal chromosomal recombination, which occurs in susceptible repeat-rich regions of the genome, including chromosome 17p11.2. Chen et al. (1997) identified three low-copy number repeats flanking the SMS common deletion region, called SMS-REPs (4). Unequal meiotic crossovers mediated through nonallelic homologous recombination occur between the proximal and distal SMS REPs that were identified resulting in ~70% of the SMS deletion cases resulting in a common deletion of ~3.7 Mb (8,18). Slager et al. (2003) conducted a study where they looked at three individuals with strong clinical suspicions of SMS but were negative for the 17p11.2 deletion. They sequenced three genes that localized to the SMS critical region; developmentally regulated GTP binding
protein 2 (DRG2), RAS dexamethasone-induced 1 (RASD1), and RAI1 and found mutations only in RAI1. This study supports the idea that haploinsufficiency of RAI1 leads to SMS (18,19,22).

The primary RAI1 transcript (GenBank AY172136, AJ271790; NM_030665.3; NP_109590.3; OMIM*607642) consists of six exons which generate a ~8.5kb mRNA and a 1906 amino acid protein (8). The protein contains a bipartite nuclear localization signal, polyglutamine and polyserine tracts, and a C-terminal plant homeodomain (PhD)/zinc-finger domain which is similar to that in the trithorax family of nuclear proteins (Fig. 1). Amino-acid sequence motifs representing these four domains in RAI1 are similar to the transcription factor stromelysin-1 platelet-derived growth factor-responsive element-binding protein, TCF20. This transcription factor is involved in growth and neurobehavioral regulation and therefore suggests RAI1 might also be a transcription factor(1,2,8).
**Fig. 1: RAI1 structure.** a) The genomic structure of RAI1 with six exons. The light color in exon III-VI show the coding exons. b) Protein structure of RAI1 with a bipartite nuclear localization signal, polyglutamine and polyserine tracts, and a C-terminal plant homeodomain (PhD)/ zinc-finger domain.

*Source: Elsea and Girirajan 2008*
Chapter 2: Exploring the methylation status of RAI1

Introduction

Genomic DNA Methylation

There are several epigenetic modifications of DNA in the mammalian genome that affect the transcription of genes. One of the most important, and the focus in this study, is DNA methylation. DNA methylation is a mechanism that does not alter the gene product but affects when and where the gene is expressed (5). DNA methylation in the promoter region of a gene is associated with silencing of that gene (13). DNA methylation is now considered the main contributor to the stability of gene expression status (15). There are two kinds of methylation, which are maintenance DNA methylation, and de novo methylation. DNA methylation involves the addition of a methyl group to the number five carbon of the cytosine in a CpG dinucleotide. In mammals, methylation is essential for normal development and also plays a key role in imprinting and X-chromosome inactivation. The most common place for DNA methylation to occur in vertebrates is at 5’-CpG-3’ dinucleotides or CpG islands. A CpG island is defined as a region with at least 200 bp with a GC percentage of over 50% and an observed/expected CpG ratio greater than 60%. However, most CpG island promoter regions are protected from
methylation which leads to an expressed gene, whereas CpG sites in non-coding or coding regions are often methylated which would lead to a silenced gene (5). DNA methylation patterns in somatic cells are stably inherited due to the function of DNA methyltransferases. These enzymes perform maintenance DNA methylation at hemimethylated CpG sites after DNA replication (11). Genetic disorders such as Prader-Willi syndrome and Angelman syndrome are results of altered genomic imprinting, in which DNA methylation plays an important role (11). This suggests that it may be possible that altered DNA methylation also plays a role in Smith-Magenis syndrome.

**SMS-like patients**

In the Elsea lab, there is a cohort of patients (~75% of patients referred to the lab), referred to as “SMS-like”, that are clinically suspected to have Smith-Magenis syndrome but do not have a molecular diagnosis of SMS. These patients have the classical clinical phenotypes of SMS, including craniofacial and neurological abnormalities, specifically self-injurious behaviors, hypotonia, and sleep disturbance but do not have a deletion of 17p11.2 or a mutation of *RAI1*. Previously, members of the Elsea lab have worked to provide evidence for the occurrence of *RAI1* haploinsufficiency in patients with Smith-Magenis syndrome. *RAI1* mRNA expression levels were measured using qRT-PCR using SMS patient lymphoblast cell lines and control lymphoblasts. In the study, 7 out of 17 “SMS-like” patients were found to have a lower expression of *RAI1*, even though they do not have a mutation or deletion of this gene (Fig. 2). (Lily Troung, Dissertation 2008)
Fig. 2  RAI1 expression in SMS and SMS-like patient cell line. Mean results for each sample measured by real-time qRT-PCR and calibrated against SMS282 to obtain relative RAI1 mRNA expression levels ($\Delta\Delta C_t$ ratio). The relative mRNA expression level is numerically denoted above each sample. For each sample, real-time qRT-PCR measurements were performed in triplicate and repeated at least twice.

Source: Lily Troung Dissertation 2008
The methylation status of the *RAI1* gene has never been investigated, and aberrant methylation could be a likely explanation for the reduced *RAI1* expression in the “SMS-like” patients. In order to investigate this issue, SMS patient cell lines were grown along with control cell lines and treated with 5-Aza-2’-deoxycytidine, a demethylating agent, and *RAI1* expression was compared between the cell lines to identify any differences.

Demethylating agents like 5-Aza-2’-deoxycytidine inhibit methylation by being incorporated into the nucleic acids of dividing cells where they act as inhibitors of DNA methyltransferases (DNMT), which catalyze the methylation reaction. This results in the expression of previously silenced genes (14). Treatment with 5-Aza-2’-deoxycytidine that results in an increase in expression via real-time PCR suggests that DNA methylation at that locus could be responsible for downregulation of that gene.
Methods

Cell Culture

SMS patient cell lines (lymphoblasts) were taken from the frozen stock and thawed in a 37°C for ~20 min. RPMI with 10% FBS and 1% Pen/strep was also warmed in the 37°C water bath for ~20 min. The cells in the cryovials were moved into a 25 cm² cell culture flask along with 10 ml of warmed RPMI. The cells were then maintained at 37°C in a CO₂ incubator.

Counting cells (lymphoblasts)

Cells were grown for about 3 weeks depending on the growth rate of all the cells in the batch that was grown together. Cells were harvested when it was determined that the slowest cell line had enough cells to seed them to a density of 500,000 cells per ml. Cells were put in a 15 ml tube and centrifuged for 3 min at 12,000 rpm in a tabletop centrifuge. The supernatant was discarded and the cell pellet was resuspended in 5 ml of new RPMI with 10% FBS and 1% P/S. This was then pipetted up and down in order to break up any cell clumps. Cells were dyed with tryphan blue and counted using a hemocytometer. Cells were counted on the gridlines of the hemocytometer and the numbers were averaged to get a final cell count.
**Splitting cells**

The cells were split to a density of 500,000 cells/ml. They were split into three 25 cm² per cell line and labeled 0 M, 5 µM, 10 µM. The cells were suspended in 5 ml of RPMI with 10% FBS and 1% P/S. Cells were incubated at 37°C for 48 hrs. Cells were grown in batches of about 3-4 cell lines which included control, *RAI1* deletion, and “SMS-like” cell lines. They were grown for approximately 3 weeks depending on how fast the *RAI1* deletion and “SMS-like” cell lines grew.

**5-Aza-2’-deoxycytidine treatment of cells**

After the initial 48 hr incubation after the cells were split, 2 ml of the old media were removed and replaced by 2 ml of fresh RPMI. The cells were then treated with 5-Aza-2’-deoxycytidine. 25 µl of 5-Aza was put in the flasks labeled 5 µM and 50 µl of 5-Aza was put in the flasks labeled 10 µM. Cells were then incubated for another 48 hrs at 37°C. After the 48 hr incubation time, cells were put in a 10 ml tube and centrifuged for 3,min at 12000 rpm. The supernatant was discarded, and cells were resuspended in 3 ml of PBS in order to get rid of any remaining residue of 5-Aza-2’-deoxycytidine. The cells were then centrifuged again for 3 min at 12,000 rpm. The supernatant was discarded, and the cells were resuspended in RPMI with FBS and P/S and put in a 25 cm² cell culture flask and incubated at 37°C for 48 hrs.
**RNA extraction**

After the 48 hr incubation period, RNA was extracted using the RNeasy mini kit (Qiagen) according to the manufacturer’s protocol. An optional on-column DNase digestion was performed using the RNase-Free DNase set (Qiagen) according to the manufacturer’s protocol during the RNA purification process.

**Spectrophotometer**

RNA concentration was measured using spectrophotometry at 260 nm. This was done in duplicates for each sample and the values were averaged to calculate the RNA concentration.

**Reverse transcriptase**

First-strand cDNA synthesis was performed using SuperScript™ II RT (Invitrogen) according to the manufacturer’s protocol. The maximum amount of RNA was used by normalizing the RNA concentrations for each tube to the lowest concentration RNA. The max amount of RNA from the lowest concentration RNA was added and the others were adjusted in order to add the same amount of RNA to the reaction. Oligo(dT)_{12-18} (500 µg/ml) was used and the optional RNase OUT (40 units/µl) was added. RNaseH (2 units) was then added after cDNA synthesis, and the reaction was incubated at 37°C for 20 min.
Real-time PCR

Real-time PCR was performed on the AB7500 FAST system. The master mix included Universal PCR Master Mix, No Amp Erase® UNG (Applied Biosystems), Taqman Gene expression Assay probes (ABI), and ddH$_2$O. The cDNAs prepared from the SMS patient cell lines were diluted 1:4 with ddH$_2$O in order to minimize pipetting error. A total volume of 6 µl of master mix and 4 µl of the diluted cDNA was added to each well on a 96 well-plate. Samples were done in triplicates and normalized to an endogenous GAPDH. The results are shown with Relative Quantification values (RQ) which take into account the endogenous GAPDH control and the calibrator. The RQ values are automated calculations using the 7500 Fast PCR system (ABI). The mean and SD were calculated for each study.
Results

The methylation status of \textit{RAI1} was assessed by treatment of lymphoblast cell lines with 5-Aza-2’-deoxycytidine. Real-time PCR was used in this study to compare the \textit{RAI1} expression levels for control lymphoblast cells and control cells treated with the demethylating agent. The methylation status of the “SMS-like” cohort of patients was also investigated by comparing the expression levels of \textit{RAI1} between the patient cell lines and the 5-Aza-2’-deoxycytidine treated cell lines. The lymphoblast cell lines were grown in batches to minimize the variability and included cases with 17p11.2 deletions, one or two “SMS-like”, and control controls, usually 3-4 cell lines total per batch. All samples were normalized with a \textit{GAPDH} endogenous control probe in order to control for RNA sample variability and also normalized to a control cell line grown with each sample batch of cells in order to obtain relative expression levels (\(\Delta\Delta C_t\) ratio). One major problem encountered while running these experiments is that the deletion cell lines and some “SMS-like” cell lines were very slow to grow and sometimes did not grow at all. These were then discarded from the batch leaving the batch with one less cell line for evaluation and reducing the ability to compare expression levels.

The data include all usable data from a total of 35 cell lines that were grown in batches. Cell lines were not included in the data if they did not grow, if the Ct values were higher than 30, or if the Ct std error was >0.2, since these results were too variable to analyze. All of the raw relative quantification (RQ) values shown are relative to one control line,
from the batch the individual cell lines were grown in, as a calibrator. The RQ values were then combined into one graph as shown in the figures.

The results for *RAI1* expression of control lymphoblast cell lines treated with 5-Aza-2′-deoxycytidine are shown in Fig. 3. The cell lines were not grown together, but were all calibrated to their own normal untreated cell line. The trends are different between cell lines but for all the cell lines there is no difference between untreated cells and drug treated cell *RAI1* expression. No increase in RAI1 expression is observed comparing the 5-Aza-2′-deoxycytidine treated cells to the untreated cells suggesting that *RAI1* is not normally methylated. Fig. 4 shows expression of *RAI1* in *RAI1* deletion patients relative to a control set as 1. The cell lines are calibrated to the untreated control cell line in the batch they were grown in. The results did not show lower expression in the *RAI1* deletion cell lines as was expected since deletion patients are haploinsufficient for *RAI1*. Instead *RAI1* expression was as high as the controls. Fig. 5 shows 5-Aza-2′-deoxycytidine treated *RAI1* deletion cell lines but they do not show a consistent change in expression. The cell lines are all calibrated to the untreated control cell line in the batch they were grown in. Fig. 6 shows untreated “SMS-like” cell lines compared to the control. In contrast to the preliminary data (performed by a previous student in the Elsea lab), they do not display a decreased expression compared to the controls. All cell lines were calibrated to the untreated control cell line from the batch they were grown in. Fig. 7 shows 5-Aza-2′-deoxycytidine treated “SMS-like” cell lines compared to the control. The results of expression change from the drug treatment differ from cell line to cell line and they are not consistent amongst themselves. There is no *RAI1* expression increase
that can be observed comparing the demethylating agent treated cell lines to the untreated cell lines suggesting that methylation seems to not play a part in these “SMS-like” cell lines. Fig. 8 shows the RAI1 expression of untreated control cell lines. The study was repeated twice and the RQ values are calibrated to cell line 288. The results show large variation in values even for the same cell lines. Control cell lines are inconsistent and therefore suggests some other factor playing a role in RAI1 expression, like the timing of when RNA is extracted.
Fig. 3  *RAII* expression of 5-Aza-2’-deoxycytidine treated control cell lines. Cell lines shown are all control cell lines which have been calibrated to the untreated line of each individual cell line. All untreated control cell lines are shown as a raw RQ value of 1. Study was performed twice and error bars represent the SD of the two experiments. Cell line 211 does not have an error bar due to the fact it failed to grow during the repeat experiment. Experiment was done twice and the qPCR was run in triplicate. RQ values are calculated by normalizing to GAPDH and calibrating to the control.
Fig. 4 *RAII* expression of untreated *RAII* deletion cell lines. Cell lines shown are *RAII* deletion cell lines with the raw RQ value determined by calibrating against the control cell line from the same batch. There are no error bars due to the fact that these cell lines are extremely difficult to grow since they are slow in growth and often die before reaching the preferred density. RQ values shown are an average of triplicate wells from real-time PCR from a single study. RQ values are calculated by normalizing to GAPDH and calibrating to the control.
Fig. 5 RAI1 expression of 5-Aza-2’-deoxycytidine treated RAI1 deletion cell lines. Cell lines shown are RAI1 deletion cell lines treated with 5-Aza-2’-deoxycytidine. RQ values are determined by calibrating against the control cell line from the same batches. Error bars on the control treated lines are the SD of the 6 control cell lines. The deletion cell lines have no error bars since they were only done once with qPCR done in triplicate. RQ values are calculated by normalizing to GAPDH and calibrating to the control.
Fig. 6 *RAI1* expression of untreated “SMS-like” cell lines. Cell lines shown are all “SMS-like” patient cell lines which were calibrated against a control grown with the individual cell lines. There are no error bars due to the fact the study was done once in triplicate. RQ values are calculated by normalizing to GAPDH and calibrating to the control.
Fig. 7  RAILI expression of 5-Aza-2’-deoxycytidine treated “SMS-like” cell lines.  Cell lines shown are all “SMS-like” patient cell lines calibrated to their individual control controls in the same batches. The error bars on the treated control cell lines represent the SD of the 6 control cell lines. There are no error bars due to the fact they were done once and qPCR was done in triplicate. RQ values are calculated by normalizing to GAPDH and calibrating to the control.
**Fig. 8** *RAII* expression of untreated control cell lines. All cell lines shown are control cell lines calibrated to cell line 288. Samples were evaluated twice in triplicate and the error bars show the SD of the mean of those two triplicate values. Cell line 211 does not have an error bar due to the fact that it failed to grow during the repeat study. Experiment was done twice and qPCR was done in triplicates each time. RQ values are calculated by normalizing to GAPDH and calibrating to the control.
Discussion

The methylation status of RAI1 has not been studied before. In this study, the RAI1 expression levels in control lymphoblast cells were compared to the expression of control lymphoblast cells that were treated with 5-Aza-2’-deoxycytidine. If RAI1 was normally methylated, the gene expression after treatment with the demethylating agent should increase. However, the results from this study showed no increase in RAI1 expression in these cell lines compared to the ones treated with 5-Aza. This suggests that RAI1 is not normally methylated as was expected, as RAI1 is expressed in the population.

The other part to this study was to investigate whether the reason behind the cohort of patients known as “SMS-like” having similar phenotypes as SMS patients was due to aberrant promoter methylation of RAI1. The preliminary data did not have control cell lines grown with each RAI1 deletion and “SMS-like” cell line and therefore needed to be repeated. RAI1 mRNA expression levels were measured by quantitative real-time PCR and compared with the same cell lines which were treated with 5-Aza-2’-deoxycytidine. The results of this study were not consistent with the q-PCR preliminary data (performed by a previous student in the Elsea lab). The range of the control cell lines were different and also the “SMS-like” cell lines and RAI1 deletion cell lines did not exhibit reduced RAI1 expression as previously shown. The deletion cases and “SMS-like” cases did not have a decreased expression of RAI1. RAI1 deletion patients are haploinsufficient and therefore should have a 50% reduced expression. Differences in results compared to previous data may be due to the fact that RAI1 expression is sensitive to the growth
period and can differ depending on when the RNA is extracted. The previous data may have lacked accuracy since this was not considered. This problem was addressed by growing cells in batches so as to minimize environmental differences and length of growth. A control cell line was always grown with a deletion cell line and “SMS-like” cell lines. However, the deletion cell lines and some “SMS-like” cell lines have a slower doubling time, so the length of time they are grown is hard to control. These cells were all started at the same time, harvested at the same time as soon as the slowest cell line grew enough to be seeded at the required density, RNA extraction was done at the same time, and reverse transcriptase was also done together. However, cell densities were often not the same due to these differences in growth rates. Since the data was inconsistent, the control cell lines were repeated to observe whether the \( RAI1 \) expression would be similar every time. The results show that this is not the case and there is great variability in \( RAI1 \) expression even in biological repeats. Since the control data was also inconsistent, the method used for reverse transcriptase was investigated. A comparison of RTs done with oligo dT, random primers, and both oligo dT and random primers revealed no differences (Fig. 9). The conclusion from this experiment was that as long as the same method was used, i.e. just oligo dT or just random primers, the outcome would not be different between cell lines. Oligo dT was used for all real-time PCRs in the study as was in the preliminary study performed by a previous student. Next, to check for technique errors in real-time PCR, cDNA prepared by the same individual was taken and a real-time PCR was run along with another individual using the same cDNA. The
results again showed no difference between the two real-time PCR expression levels, and therefore real-time PCR technique error was ruled out (data not shown).
Fig. 9 RAI1 expression RT comparison. Data shown are four different control lymphoblast cell lines. There are no error bars due to the fact that the study was done once in triplicate. RQ values are calculated by normalizing to GAPDH and calibrating to the control.
Chapter 3: Identification of the RAI1 consensus binding sequence: RAI1 regulates chemokine-like receptor 1

Introduction

Transcription factors are proteins that bind to specific DNA sequences and regulate transcription of other genes. RAI1 is thought to be a transcription factor (8); however, it is still unknown which genes RAI1 regulates or to what DNA sequence it binds. In order to identify genes that RAI1 regulates, other members in the Elsea lab conducted a ChIP-chip experiment, which is a combination of chromatin immunoprecipitation and microarray technology on a genome wide scale. This technique will identify regions of the genome where the RAI1 protein binds. The chip contains sequences that represent promoters and regulatory regions of known human genes. An antibody to a Flag-tagged RAI1 was used to pull down any chromatin sequence that bound RAI1 (or a protein bound to RAI1). The acquired data were processed through the Signal Map program in order to narrow the putative RAI1 binding regions to the top ~200 candidates. Data from this study identified a large number of promoter sequences to which RAI1 was potentially
bound and thus, identified a cohort of genes that RAI1 may control. Consensus sequences were identified by comparing the two shortest sequences from the top candidate genes obtained from the ChIP-chip study. Potential target sequences were identified and functionality studies using luciferase assays were initiated.
Methods

Chip-chip

The Nimblegen standarad ChIP protocol was followed using the HG18 RefSeq promoter array. The array has a 51 Mb coverage with 24,659 transcripts represented. Signal Map software was used to identify most likely hits. (done by Stephen Williams, data not shown)

Identifying 5-7bp binding sequence

In order to identify the RAI1 consensus binding region, the two shortest sequences from the top ChIP-chip candidate genes were chosen. Microsoft Office Word was used to compare these two sequences by using the “ctrl find” function and taking the first 5 bases from the 1st sequence and trying to find the consensus sequence in the 2nd regulatory region sequence. One base was shifted every time for a new search (Fig. 9). About 25 sequences of 5 ~7 bp in length were identified, and those sequences were then checked to determine if they were known transcription factor binding sequences using the program TESS and if they were present in the other gene promoter sequences in the ChIP-chip. The top 2 unique candidate binding sequences were chosen.
Amplifying putative binding region by PCR

Primers were designed to amplify approximately 200 bp around the 5–7 bp sequence from genomic DNA. PCR was conducted with the program set to 4 min initial denaturing at 94°C, denature for 1 min at 94°C, and 35 cycles of 1 min annealing at temperature according to different primers, and extension for 30 sec at 72°C, followed by a final extension for 10 min at 72°C.

Primer Sequences for *CMKLR1*: (334 bp)

Forward – TAGTCATGTAGGATGCCA

Reverse - ATGCTGTGCTCTAGAGACA

Taq treatment

10 μl of gel extracted product was combined with 0.25 μl Taq (5 U/μl) and incubated at 72°C for 10 min. This was done to ensure the As were still on the end of the PCR products for optimal cloning into the TA vector.

Cloning

The amplified PCR products were TOPO cloned into the TOPO2.1 vector according to the manufacturer’s protocol (Invitrogen). PCR product that was Taq treated was used. The TOPO cloned product was then transformed into *E.Coli* cells by adding 2 μl of the
ligated product to 30 µl of One-shot chemically competent cells (Invitrogen) and incubated on ice for 30 min. Then the cells were heat shocked at 42°C for 30 sec. They were then immediately transferred to ice and 250 µl of S.O.C. medium was added. The tube was then shaken horizontally at 37°C for 1 hr. After the incubation, 40 µl was spread onto pre-warmed 50 µg/ml kanamycin plates. Colonies were grown and DNA was prepared by standard methods (Fermentas). Once the insert was confirmed, the purified DNA was cut with \(KpnI\) and \(XhoI\) for ligation into the pGL3 promoter vector.

**Ligation into pGL3**

The concentration of \(CMKLR1\) sequence in the TOPO plasmid was evaluated by the spectrophotometer. The following equation was used to determine the amount of insert and vector to add to the reaction. Ligation and transformation were carried out according to manufacturer’s protocol (Promega).

\[
\left( \frac{ng_{vector} \times kb_{insert}}{kb_{vector}} \right) \times 4 = ng_{insert}
\]

**Maxi-prep**

The pGL3 promoter vector with the \(CMKLR1\) insert was maxi-prep’d for transfection. The Qiagen plasmid purification protocol was followed, starting with inoculating 100 ml medium. Cells were harvested, and the protocol for high-copy plasmids was used. The re-
centrifugation step to remove the supernatant containing plasmid DNA was substituted by filtering the supernatant from the step before through a Whatman filter 3 by gravity flow. Subsequent steps were carried out as written in the protocol (Qiagen).

Growing cells (HEK293 cells)

Cells were grown in DMEM with 10% FBS and 1% Pen/Strep and maintained at 37°C in a CO₂ incubator.

Count cells (fibroblasts)

Cells were washed with 5 ml of PBS then treated with trypsin for 5 min at 37°C. The sides of the flask were tapped to ensure all cells come off bottom of the flask. 5 ml of DMEM with 10% FBS was added to inactivate the trypsin. Cells were centrifuged at 12,000 rpm for 2 min to form a pellet. The cell pellet was resuspended in DMEM with FBS w/o P/S and counted using a hemocytometer. Cells (HEK293) were seeded at a density of 1.6x10⁵ cells in 2 ml of growth medium (DMEM w/FBS –P/S) in a 6-well plate.

Transfection

The Invitrogen lipofectamine™ reagent protocol was used. The transfection procedures were followed after scaling up for a 6-well plate. The cells were transfected with 1)
CMKLR1, RAI1, and β-galactosidase, 2) CMKLR1 and β-gal, 3) RAI1 and β-gal, and 4) with just β-gal by lipofection using Lipofectamine™ (Invitrogen) according to manufacturer’s protocol. DNA (2 µg) was put in 100 ml of OptiMEM (Invitrogen) and mixed with 25 µl lipofectamine reagent diluted in 100 µl OptiMEM (Invitrogen). The mixture was incubated at room temperature for 40 min to allow complexes to form. The complexes were then added to the HEK293 cells and incubated for 24 hrs at 37°C in a CO2 incubator. Following transfection, cells were washed twice with PBS, then treated with lysis buffer and cells were scraped off into a micro-centrifuge tube. They were then centrifuged and the supernatant was collected.

**Luminometer**

Cell lysate (50µl) was placed into a 96-well plate and the lipofectamine reagent protocol (Invitrogen) was followed along with the Galacto-Light System protocol (Applied Biosystems).

**Data analysis**

The data were analyzed by taking the value from the luciferase and dividing that by the value from the β-galactosidase. The CMKLR1 + RAI1 was set as 1 and the other values were divided by the CMKLR1 + RAI1 value. The mean and SD were calculated for each study.
Results

Putative RAI1 consensus DNA binding sequence

The ChIP-chip data revealed ~200 candidate gene promoters that bound RAI1 (or a protein bound to RAI1). From the top ~200 genes, a 5~7 bp sequence was chosen via the method listed above (Fig.10). The sequences that were most prevalent among the top~200 candidate genes were chosen with the sequences TGAATTT and AGAATAT. Variations of these two sequences; TGAAT, TGAATT, TGAATTT, AGAAT, AGAATA, and AGAATAT were checked in the top 100 candidate genes for their prevalence. The results revealed that the three variations of the sequence TGAATTT appeared in 74% of the top 100 genes, and the three variations of the sequence AGAATAT appeared in 77% of the top 100 genes.

From these sequences, the top candidate genes that were deemed to be relevant to Smith-Magenis syndrome were selected to try and identify genes that RAI1 had a direct effect on. The genes that were chosen for cloning were bone morphagenic protein 5 (BMP-5), minichromosome maintenance protein 10 (MCM10), zinc finger protein 545 (ZNF545), and beta-defensin 112 precursor (DEFB-12). While amplifying these potential RAI1 binding regions in the top candidate genes, DNA sequencing results of one of the genes revealed a completely different gene, than the sequence that was expected originally. Although this gene was not the gene the primers were designed for, it turned out to be the
Chemokine-like receptor-1 gene (CMKLR1). The gene was then checked on the array data to see if it was included in the top 200 candidate genes, and it was. This sequence was then checked to see if the 5~7 bp potential binding sequence was included and was found to have the AGAATT sequence in three places (Fig. 11). Since the sequence amplified was in intron 2 of CMKLR1 and could be in an enhancer regulatory region, the CMKLR1 sequence was further studied to analyze whether or not RAI1 had an effect on its expression. The other candidate gene sequences were not able to be cloned into pGL3 due to wrong sequences, failure to PCR amplify, and unable to transfer into pGL3 from the TOPO vector.

A luciferase assay was conducted by co-transfection of RAI1 and CMKLR1 regulatory region sequence. The CMKLR1 sequence (Fig. 11) was put into a pGL3 vector that contained the luciferase gene and an SV40 promoter, and β-galactosidase was used as a control. The relative light units were measured and compared with HEK293 cells that were transfected with 1) CMKLR1 regulatory region and β-galactosidase, 2) CMKLR1 regulatory region + β-galactosidase + RAI1, 3) RAI1 and β-galactosidase, and 4) β-galactosidase alone. This experiment was done twice, each done in duplicate (Fig. 12). The results show an increase in relative light units when CMKLR1 regulatory region is co-transfected with RAI1. However, when a confirmation was done to confirm the presence of this gene on the array it was found that the gene that existed on the array was the chemokine receptor like-1 gene (CCRL1) and not chemokine-like receptor-1 (CMKLR1). Also checking the array that was used in the ChIP-chip revealed that the
The \textit{CMKLR1} gene was not present on the array and therefore would not have appeared in the ChIP-chip data. Thus, studies were continued on the \textit{CMKLR1} gene.

An additional transfection was done with RAI1 isoform-C to observe any fold change differences due to the fact that this isoform lacks a NLS (nuclear localization signal) and a PHD domain, which is thought to be the main binding domain for RAI1. The luciferase activity was expected to stay the same as the controls and not increase since it lacks the NLS and the PHD domain. The results show that the RAI1 isoform-C transfection with \textit{CMKLR1} regulatory region sequence reveals increased luciferase activity compared to the control but has decreased activity when compared to the full length RAI1 co-transfection.
Fig. 10  Identifying the 5~7 bp RAI1 binding sequences. The two sequences shown are the two shortest sequences from the top candidate genes given by the ChIP-chip results. The highlighted/underlined/italicized sequences are the matching sequences in both regulatory region.
chr12:107,223,494-107,223,656

GCCGCCAGTGTGATGGATATCTGCAGAATTGCCCTTTATGCTGCTCTAGAGACACCTCCAGATCCCTGTATCC
CCAGGCTCTGACAGACTCGGGAACCTGGAAACCACACAGTGTCTGCAGATGTCCAGCCACAGGCCTGGCTGCC
AGCCCCAGGACAGAATTAGCTCCGTGTCAGGAGAGAATTTCCTCCCTGGTCCACCAGCC

**Fig. 11** Amplified *CMKLRI* putative binding region sequence. The highlighted sequences are the putative RAI1 binding sequences.
Fig. 12 RAI1 co-transfection with CMKLR1 regulatory region. Results shown are from two experiments done in duplicate. Error bars indicate SD (N=4).
Fig. 13 RAI1 isoform-C co-transfection with CMKLR1 regulatory region. Results shown are from one experiment done in duplicate. Error bars indicate SD (N=2).
Discussion

Based on these studies, a gene potentially regulated by RAI1 is the chemokine-like receptor-1 gene (CMKLR1) [OMIM, #602351], also known as ChemR23, DEZ, MGC126105, or MGC126106 (Fig. 13). CMKLR1 is a G-protein-coupled receptor which functions in skeletal system development, chemotaxis, immune response, and the G-protein coupled receptor protein signaling pathway, and may also be associated with obesity and the metabolic syndrome (3). Chemerin (TIG2), an adipokine which, upon proteolytic cleavage is able to activate CMKLR1, is the endogenous ligand for CMKLR1 (16). Bozagolu et al. (2007) conducted a study in which they found both chemerin and CMKLR1 expression to be elevated in adipose tissue (3,16). When the chemerin gene expression was compared in normal and type 2 diabetic P. obesus, it showed that chemerin was elevated in the obese animals and associated with the characteristics of metabolic syndrome (3). In another study conducted by Martensson et al. (2005), it was shown that the expression of chemerin and CMKLR1 is induced by all trans retinoic acid (ATRA) from a luciferase reporter gene assay using ATRA stimulated cells; however, the study failed to identify any RA-responsive element (16). Since chemerin and CMKLR1 are both induced by RA, we hypothesized that RAI1 may be playing a role in regulating CMKLR1. This would be a reasonable hypothesis since one of the classic characteristics of Smith-Magenis syndrome is obesity.

Although this was not the gene we expected to amplify, when we conducted a search on the CMKLR1 sequence we amplified, we found the 6 bp consensus sequence (AGAATT)
in 3 different places within the sequences and decided to further investigate the potential
effect of RAI1 on its expression (Fig. 12). In order to investigate whether RAI1 was
directly playing a role in the expression of CMKLR1, we conducted a co-transfection of
RAI1 and a fragment of the CMKLR1 gene containing the 6 bp potential binding
sequence cloned into the pGL3-promoter vector and conducted a luciferase assay to
evaluate the luciferase activity. The luciferase assay was done by co-transfecting RAI1 in
pDest26 (Invitrogen) and CMKLR1 in pGL3 (Invitrogen), which contains an SV40
promoter upstream of the luciferase gene. We expected to see an increase in expression
of CMKLR1 compared to a control transfection if RAI1 was binding to the CMKLR1
sequence and had a direct effect on CMKLR1 expression. The results from the luciferase
assay show an increased activity of luciferase when it was co-transfected with RAI1,
compared to the control transfections (Fig. 12). This suggests that RAI1 is directly
binding to the CMKLR1 regulatory region and is enhancing the expression of CMKLR1
or acting as a coactivator not directly bound.

Additionaly, a co-transfection was done with the CMKLR1 sequence in pGL3 and RAI1
isoform C in GFP. The RAI1 isoform C has 964 amino acids and lacks a NLS and PHD
domain. The results from this transfection show that the RAI isoform-C transfection still
shows increased luciferase activity. This suggests that the RAI1 isoform-C is still getting
into the nucleus probably due to effects of transfection and also that the PHD domain,
which was thought to be the main DNA binding domain for RAI1, is not the only DNA
binding domain. The RAI1 isoform-C may be binding via the Poly-Q domain, Poly-S
domain or another domain that is still unknown. RAI1 could also be acting non-directly.
Fig. 14 *CMKLRI* gene structure. There are 3 exons with the last exon being a coding exon. The red box in intron 2 represents the location of the potential regulatory region sequence that was amplified and inserted into pGL3.
Chapter 4: Discussion

Exploring the Methylation Status of RAI1

The goal of this study was to investigate the methylation status of normal RAI1 and also whether “SMS-like” patients had aberrant promoter methylation that was causing RAI1 expression to be reduced. From the study in Chapter 2, by treating the cells with a demethylating agent, 5-Aza-2’-deoxycytidine, no increase in expression was observed in control cell lines compared with non-drug treated cells. However, when “SMS-like” patient cell lines were investigated for RAI1 expression, the study revealed no decrease in RAI1 expression. To further investigate the reason for the inconsistent data, a technique check was done on both reverse transcriptase and real-time PCR. Both revealed no problem in the techniques. The fact that the expression of RAI1 looks different in the data obtained from this study and the previous study may be due to the previous study combining all cell line data from different experiments; cells were grown and RNA and cDNA prepared at different times. All possible trouble shooting was done, but this project may need to be repeated in different cell types since the lymphoblastoid cells did
not give consistent expression results. Future studies for this project include repeating the RAI1 deletion cell line treatment and “SMS-like” cell line treatments since these were only performed once. Also, conditions of the 5-Aza-2’-deoxycytidine treatment can be tested by also looking at a gene known to be methylated. Another project would be to synchronize the cells by serum starvation to observe any differences in RAI1 expression during different times in the cell cycle. Since lymphoblastoid cells are transformed cells, it is still unknown and studies still have not shown as to whether these are the optimal cells to investigate methylation on. At this point a conclusion can not be made accurately since some cell lines were only performed once, however, preliminarily the results seem to suggest that aberrant RAI1 methylation does not play a role in contributing to SMS-like patients. In the future, if the RAI1 promoter region is sequenced and cloned, bisulfate sequencing or methylation specific PCR is another way to detect promoter methylation in this gene.

**RAI1 Consensus Binding Sequence**

RAI1 is thought to be a transcription factor but nothing about its binding or function is known. The project in Chapter 3 aimed to find a consensus binding sequence by comparing the data sequences obtained from another lab member’s ChIP-chip study. The comparison of the sequences revealed 2 distinct sequences (and their variations) in ~75% of the top 100 gene candidates given by the ChIP-chip study. A gene accidently found from this experiment is the *chemokine-like receptor-1 (CMKLRI)*. This gene was
accidently amplified but still contained the consensus sequence and also showed an increase in expression when co-transfected with RAI1. The results from this study reveal an interesting relation of CMKLR1 and RAI1, thus further studies should be done to investigate its properties and interactions with RAI1.

Future studies for this project include repeating the RAI1 isoform-C luciferase assay, and also conduct a RAI1 dosage response luciferase assay by varying the amount of RAI1 added. Future studies to investigate the exact RAI1 binding sequence should use site directed mutagenesis. The 6 bp sequence in the CMKLR1 binding sequence could be mutated and transfection would be repeated to compare results with the transfection with the correct 6 bp binding sequence. If the expression decreases, this might be an indication that the 6 bp potential binding sequence is correct. If the expression still remains elevated, deletion constructs could be made by shortening either side of the CMKLR1 sequence and repeating transfections with the deletion constructs until a decrease in expression is found. An experiment that can be done to find direct evidence of RAI1 binding could be to perform a electrophoretic mobility shift assay (EMSA). This would show a band shift if RAI1 binds to the CMKLR1 sequence compared to a band that contains only DNA and would support the direct binding of RAI1 and CMKLR1.

For some future studies regarding the CMKLR1 gene, real-time PCR experiments can be done to assess CMKLR1 expression in human adipose tissue obtained from liposuction. The expression of CMKLR1 will be compared to the expression level of CMKLR1 in
adipose tissue of subjects that are not overweight or obese. This study can also be done in mice by assessing Cmklr1 expression in mouse adipose tissue from RAI1 transgenic, RAI1 knockout, and non-transgenic mice. This study would show whether Cmklr1 expression is really elevated in the adipose tissue and whether there is a difference in Cmklr1 expression in obese mice versus lean mice.

Some other studies are using the co-transfected cells with RAI1 and the CMKLRI sequence and looking at CMKLRI expression via real-time qPCR. This study would show whether the expression if CMKLRI is increased in the cells co-transfected with RAI1 versus cells that have just CMKLRI. Increased expression levels of CMKLRI would be expected in obese individuals in humans, and increase expression of Cmklr1 would be expected in RAI1 knockout mice since they are also obese.


Vita

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