SEQUENCING OF ESTROGEN RELATED RECEPTOR BETA (*ESRRB*) AND ITS ROLE IN DENTAL CARIES EXPERIENCE

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Dental caries is a significant public health problem and is estimated to affect 60 to 90 percent of school children as well as a large number of adults. It is a chronic, infectious, multifactorial disease in which the host's diet, microbiota, and genetic background play a role. Initial linkage studies suggested the estrogen related receptor beta (*ESRRB*) locus is linked to high caries experience in humans. Our hypothesis is that rare genetic variation in the coding region of *ESRRB* influences caries. Ninety-three whole saliva samples from a clinically well-characterized cohort were collected and extracted (62 caries samples and 31 caries free controls). We sequenced the exons and exon-intron boundaries of *ESRRB* and compared our results with the reference sequence transcript ENST00000505752 from the Ensembl genome browser. Eight SNPs were found in our samples with no evidence indicating these are disease-causing variants. Individuals with dental caries have an over-representation of the T allele of rs55835922 (74% versus 54%; $p = 0.01$). The SNP rs61742642 is a missense mutation (P386S), but its frequency was just slightly elevated in cases with dental caries (13% versus 9.5%). SNP rs35544003 is a synonymous change. Through bioinformatics analysis, we determined the SNP rs61742642 missense mutation is a benign change. Our results indicate that ESRRB may contribute to caries, but coding mutations causing the disease are not commonly found.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

I would like to thank all of those who made my master's thesis project possible. First and foremost, I would like to thank Dr. Alexandre Vieira, my advisor and mentor. Through his guidance I have been able to work toward my goal of becoming a successful independent researcher.

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I am very grateful for the opportunity to complete my master's degree here at the University of Pittsburgh.

1.0 INTRODUCTION

1.1 DENTAL CARIES

Dental caries is a chronic, slow progressing infectious disease. It is characterized by the gradual demineralization of enamel, dentin, and cementum. The localized area of destruction of these tissues is called the carious lesion. Caries ranges anywhere from initial small amounts of mineral loss to total destruction of the tooth. The start of a lesion is usually characterized by a white spot, which is the result of an increase in internal enamel porosity causing the enamel to appear opaque and white (Fejerskov and Kidd, 2008). "Active" caries are lesions that are currently progressing while "arrested" or "inactive" lesions are not. Caries that are on an unrestored surface of the tooth are known as "primary" caries, and caries lesions next to a previous filling are known as "secondary" caries. While caries are diagnosed based on the mineral loss seen by the clinician, their formation requires oral bacteria from dental plaque on the tooth which will be discussed later (Fejerskov and Kidd, 2008).

W.D. Miller and G.V. Black helped establish dentistry as a science in the early 1900s and were some of the first scientists to describe dental caries (Fejerskov and Kidd, 2008). G.V. Black described the dissolution of the minerals and soft tissue of the teeth while touching on the possibility of microbiological aspects behind caries (Black, 1908).

Years later, other factors were found that play a role in dental caries. In Sweden, the well-known Vipeholm studies supported the idea that sugar plays a role in the disease (Gustafsson et al., 1954; Krasse, 2001). Studies also confirmed the role of microorganisms in dental caries through observations of mice who were germ free but engaged in high sugar diets (Braunschneider et al., 1948; Stewart et al., 1953). One scientist, Dr. Paul H. Keyes, a dentist and former member of the National Institute of Dental Research, studied dental caries extensively in the 1940s-1980s. He was one of the pioneers in determining that caries is a multifactorial disease with factors playing a role such as the patient's diet, immune response, genetics, and microbiota present in the oral cavity (Keyes, 1960; Keyes, 1962). A simple representation was created from this and is shown in Figure 1. This diagram has been adapted and used throughout the years by many scientists studying dental caries. The three main categories of causes of dental caries discussed below include the diet, the host, and the oral microbiota.

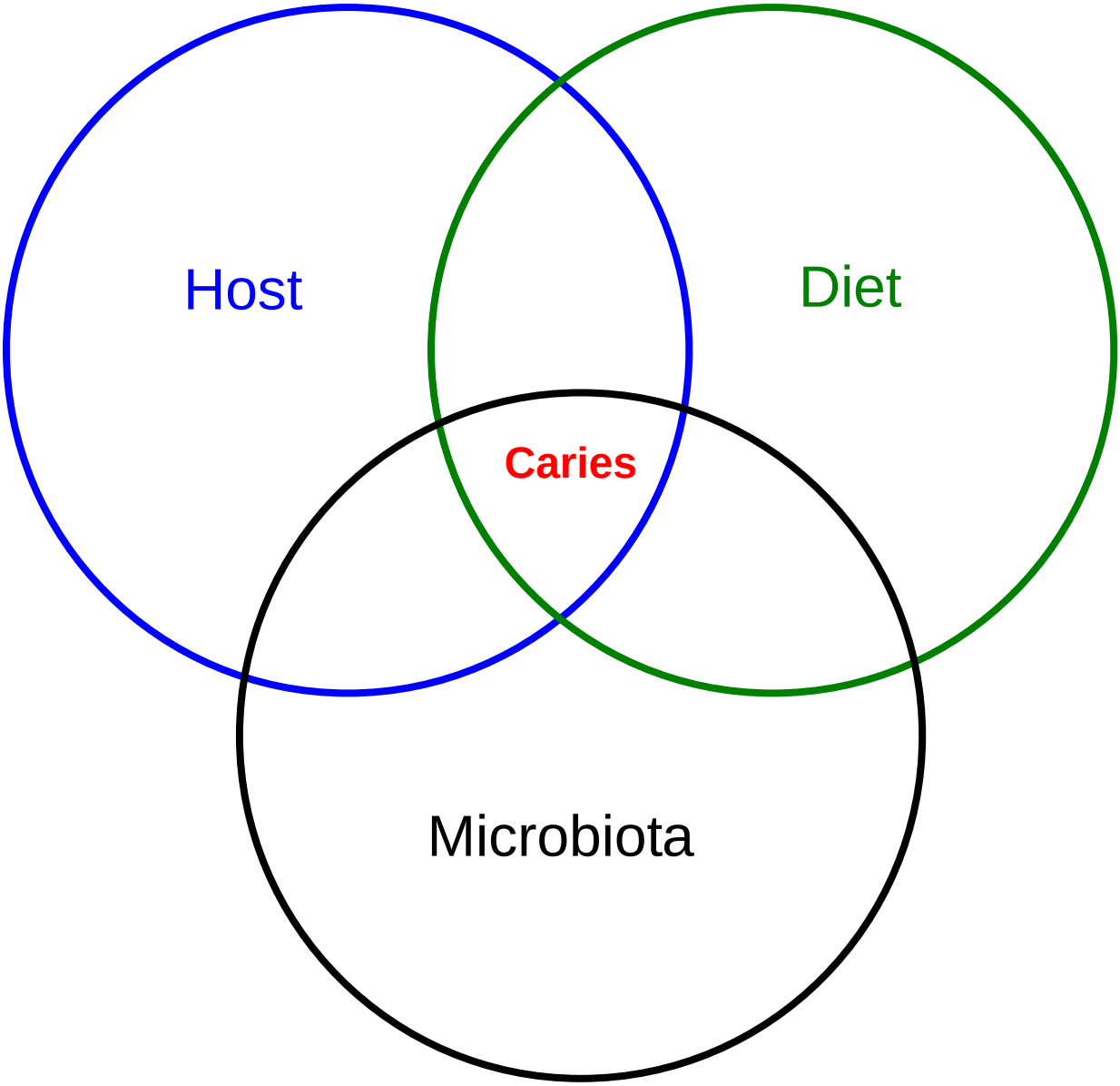

Figure 1. Representation of contributing factors of dental caries. When host factors such as saliva composition and immunology, pathogenic oral microbiota, and a diet heavy in sugars and carbohydrates come together, dental caries can develop in susceptible individuals.

The diet is a well-known contributor to caries experience. As mentioned before, the Vipeholm studies played a large role in laying the foundation studying the role of diet in dental caries. These studies were conducted in Sweden from 1945-1954 at Vipeholm Hospital, a medical institution for patients with mental handicaps (Gustafsson et al., 1954; Krasse, 2001). Patients' stays in the hospital were relatively permanent, which allowed for long-term studies to be conducted in a controlled environment (Gustafsson et al., 1954; Krasse, 2001). The Vipeholm studies concluded that patients who had increased intake of sugars through sweet breads and toffees between meals also had an increase in caries experience (Gustafsson et al., 1954; Krasse, 2001). However, there were also groups of patients who either developed no new dental caries despite increased sugar intake, and patients who developed new carious lesions despite restrictions of sugar in their diet (Gustafsson et al., 1954; Krasse, 2001). These last two conclusions showed that dental caries is reliant on other factors, not only sugar intake. The Vipeholm studies led to further study of the role of diet in dental caries while increasing the desire for sugar substitutes and increased preventative practices (Krasse, 2001). Additional studies have supported the correlation between dental caries and sugar intake (Sreebny, 1982). Some researchers have tried to start at the beginning and research older populations to look for a difference in caries experiences throughout the ages. In a study by Grimoud et al, it was found that older populations from the Chalcolithic period had less dental caries than populations from the Middle ages. A reason for this could be that agriculture and consumption of cereals, and thus carbohydrates, were significantly less common in the Chalcolithic period than the Middle ages (Grimoud et al., 2011).

In addition to the diet, oral microbiota play a part in dental caries. Humans have resident microflora throughout their body. These microflora have protective properties for the host against pathogenic microbiota such as being competitors for resources and saturating colonization sites (Fejerskov and Kidd, 2008). Some resident microbiota can over colonize, though, and cause problems, such as those involved in caries.

When babies are born, their mouths are usually sterile. They begin acquiring resident oral microbiota soon after birth from their mothers' saliva and from nutritious fluids and food (Li and Caufield, 1995; Li et al., 2005). Tooth eruption provides a habitat for even more species of microbiota, as the teeth are a non-shedding surface that allows the formation of biofilms in the form of dental plaque (Fejerskov and Kidd, 2008). The bacterium usually associated with dental caries is Streptococcus mutans (Loesche, 1986; Ge et al., 2008). Lactobacilli are also strongly associated with areas on the teeth that are in need of restorations due to carious lesions (Loesche et al., 1984). S. mutans and lactobacilli are found in most patients' mouths, even those without caries. However, their greatest concentration is usually in and around carious lesions (Loesche, 1986). In general, the bacteria thrive when the host consumes a high sugar or high carbohydrate diet and their metabolic processes produce acid, which contributes to enamel erosion. Other factors contribute to the formation and progression of dental caries, but it is because of the presence of oral bacteria and their contribution that we can call dental caries an infectious disease (Keyes, 1960; Loesche et al., 1984; Fejerskov and Kidd, 2008).

Aside from diet and oral microbiota, the host itself plays a role in dental caries. For instance, the immune system of the host plays a part in the disease since caries triggers inflammation and is considered infectious. Arachidonate 15-lipoxygenase (ALOX15) and Beta-defensin 1 (DEFB1) are examples of immune response genes that may play a role in the

inflammatory response triggered by carious lesions (Nandula et al., 2007). Aquaporins have also been associated with dental caries. Aquaporins are water channel proteins that may be involved in formation of saliva or other salivary contributions to caries experience (Matsuki-Fukushima et al., 2008). Host behavior may also contribute to the disease since the patient's dietary preferences may contribute. For example, taste preferences may influence dietary behavior, which in turn may influence whether or not a patient develops caries. Studies have shown some taste genes to be associated with caries (Wendell et al., 2010). There are also a range of enamel formation genes such as amelogenin, tuftelin, and ameloblastin, which exhibit variation and association to dental caries (Patir et al., 2008; Ergoz et al., 2014).

Certain groups of people are also at a higher risk for dental caries and poor oral health than others due to a large variety of reasons. Studies have shown that caries incidence increases with age and that women have more dental caries than men (Warren et al., 2000; The World Health Organization, 2003; Fejerskov and Kidd, 2008; Saunders and Meyerowitz, 2005; Lukacs, 2007; Mungia et al., 2008; Jindal et al., 2011; Ferraro and Vieira, 2010; Gati and Vieira, 2011; Lukacs, 2011). Tobacco use causes poor oral health and poor response to oral infections, so users are more prone to dental caries than non-users (The World Health Organization, 2003). Socioeconomic status can also play a role. For instance lower income patients all over the world tend to have poorer oral health due to limited or no access to dental care and inability to practice good oral hygiene, as well as potential for limited access to fluoridated water (The World Health Organization, 2003).

There are a huge number of other factors that can play a part in poor oral health such as having additional diseases like diabetes, HIV/AIDS, epilepsy, and asthma, as well as factors such as ethnicity and education (The World Health Organization, 2003; Fejerskov and Kidd, 2008;

Anjomshoaa et al., 2009; Nedwick-Castro and Vieira, 2012; Ergoz et al., 2014; Johnston and Vieira, 2014). Because of this, it is obvious that dental caries is multifactorial and the disease cannot be narrowed down to one single contributor or cause.

1.2 PREVIOUS RESEARCH FROM OUR GROUP

The overall goal for studying dental caries is to determine the mode of the disease. This overarching goal can be divided in to smaller goals, one of which is identifying genes associated with caries to better understand the host's role in the disease. In 2008 a genome wide linkage study was performed to look for possible genomic regions that play a part in caries experience. This linked five regions to caries experience (Vieira et al., 2008). Three regions, 5q13.3, 14q11.2, and Xq27, were associated with low caries experience while two regions, 13q31.1 and 14q24.3, were associated with high caries experience (Briseño-Ruiz et al., 2013; Shimizu et al., 2013; Küchler et al., 2013; Küchler et al., 2014). Each of these regions was studied extensively to determine how they play a part in caries experience. The region included in the specific aims of this study was 14q24.3.

Before the sequencing described in this paper occurred, other studies were performed to better understand 14q24.3. The region 14q24.3 spans an area of chromosome 14 that includes the gene for estrogen-related receptor beta (*ESRRB*). Single nucleotide polymorphisms (SNPs) were selected using data from the international HapMap project on Caucasians and Chinese subjects (Gibbs et al., 2003), which was viewed using Haploview (Barrett et al., 2005). Based on pairwise linkage disequilibrium and haplotype data, 25 SNPs were selected in this region for fine mapping. Of those 25 SNPs used for fine mapping the region 14q24.3, eight markers within or

flanking *ESRRB* were over-transmitted. Four of the eight SNPs were located within *ESRRB* while the other four were flanking it. These results are shown in Figure 2. Replication studies using Turkish and Brazilian populations confirmed association of some SNPs within and flanking *ESRRB* and dental caries (Weber et al., 2014).

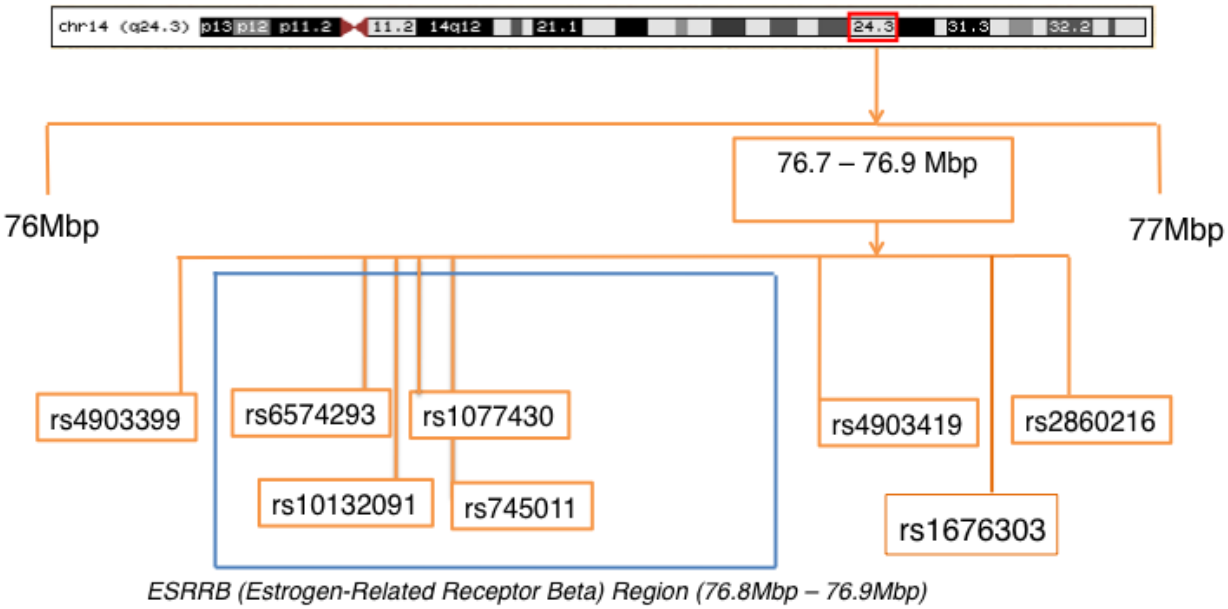

Figure 2. Summary of fine mapping results. Fine mapping studies found 8 SNPs to be associated with caries. This figure shows the four SNPs found within ESRRB and the four SNPs found to be flanking ESRRB.

1.3 ESTROGEN RELATED RECEPTOR BETA (ESRRB)

Estrogen related receptor alpha (ESRRA) and estrogen related receptor beta (ESRRB) are orphan nuclear receptors. Nuclear receptors are transcription factors that depend on a ligand to function in gene expression (Gronemeyer et al., 2004; Yuan et al., 2009). These receptors have a central DNA binding domain, allowing the receptor to be targeted by hormone response elements (Mangelsdorf et al., 1995). ESRRA and ESRRB are similar to estrogen receptors and bind to similar types of small molecule targets. However, they do not bind estrogen and do not activate gene transcription in response to estrogen (Sladek et al., 1997).

Esrrb has been mapped to mouse chromosome 12 while *ESRRB* has been mapped to 14q24.3 (Sladek et al., 1997). During embryogenesis, *Esrrb* is expressed in extra-embryonic ectoderm, which eventually forms the chorion, suggesting that *Esrrb* is involved in early placental development (Luo et al., 1997). Mutants made by targeted disruption of *Esrrb* have severely impaired placental tissue and usually die within 11 days post-coitum. Upon further examination, it has been shown that they have too many trophoblast giant cells and not enough diploid trophoblasts (Luo et al., 1997). A study by Chen and Nathans in 2007 determined that Esrrb is expressed in endolymph-producing cells of the inner ear. Several week old mice with mutations in *Esrrb* exhibited head bobbing and spun or ran in circles as adults, suggesting deformities in the inner ear (Chen and Nathans, 2007). A study by Mitsunaga et al determined Esrrb is expressed in primordial germ cells, and mutations cause the number of germ cells in male and female mouse gonads to be significantly reduced (Mitsunaga et al., 2004).

Studies in humans have shown that *ESRRB* mutations play a role in congenital nonsyndromic hearing impairment. A study by Collin et al revealed a seven base pair duplication in the coding region of *ESRRB*, which causes a premature stop codon to form. All affected individuals had this mutation (Collin et al., 2008). In situ hybridization experiments in mice showed Esrrb expression during inner ear development, suggesting that ESRRB plays a similar role in humans. Another study found a missense mutation in *ESRRB* that alters the ligand-binding site and causes hearing impairment in affected individuals (Ben Saïd et al., 2011). In two additional studies, a deletion in *ESRRB* was found to cause autosomal recessive nonsyndromic hearing loss in a family in Pakistan, and a point mutation found in a family from the Czech Republic caused hearing impairment as well (Lee et al., 2011; Šafka Brožková et al., 2012).

1.4 HYPOTHESIS AND CURRENT OBJECTIVES

Because the previous genome-wide linkage scan found that the estrogen related receptor beta (*ESRRB*) locus is linked to high caries experience, and subsequent association studies suggested that variants in the gene may cause higher caries experience, our hypothesis is that rare genetic variation in the coding region of *ESRRB* influences caries. Our objective was to sequence the exons and exon-intron boundaries of *ESRRB* to look for these variants.

2.0 MATERIALS AND METHODS

2.1 SUBJECTS

The original cohort of samples included 172 patients from Istanbul, Turkey. Istanbul University and the University of Pittsburgh Institutional Review Boards approved the study of these samples, and appropriate written informed consent was obtained from the parents of all participating children. There were 79 males and 93 females who had a mean age of 5.4 years and an age range of three to six years. Ninety-two of these children had a decayed-missing filled teeth (dmft) score of four or more contributing to high caries experience, while 80 of the children were caries free. Caries free children had no evidence of prior caries or white spot lesions. There were no children with low caries experience included in the cohort (Patir et al., 2008). From the 172 patients, we chose 93 females for sequencing.

2.2 CARIES DIAGNOSIS

Dental caries was diagnosed using a modified World Health Organization protocol recommended for oral health surveys (The World Health Organization, 2003). Teeth lost to trauma or primary teeth lost to exfoliation were not included in the final dmft scores. When records indicated that teeth were extracted for orthodontic reasons or periodontal disease, or

treatments were performed in sound teeth, these situations were not included in the final dmft scores. Carious lesions were recorded as present when a break in enamel was apparent upon visual inspection and included white spot lesions as evidence of caries. All examiners carried out the clinical examination after being calibrated by an experienced specialist. A k of 10 was found when intra-examiner agreement was assessed in ten percent of the sample two weeks after initial examination (Patir et al., 2008). In this study, the groups were classified as either 'no caries experience' or 'caries experience', based on dmft scores. Children with a dmft 4 or more were placed in the "caries experience" group. Children with no caries were used as controls.

2.3 SEQUENCING

Ninety-three samples from the Turkish cohort were used (62 caries samples and 31 caries free control samples). Subjects were asked to spit and provide unstimulated saliva samples, which were collected and stored in Oragene DNA Self-Collection kits (DNA Genotek Inc.) at room temperature. From this saliva, DNA was extracted according to standard protocol, without centrifugation. All of the exons and exon-intron boundaries of *ESRRB* were sequenced and compared with the reference sequence transcript ENST00000505752 obtained from Ensembl Genome Browser (http://useast.ensembl.org/index.html). Primers are listed in Table 1.

Table 1. Primer sets used for sequencing *ESRRB* exons and exon-intron boundaries.

Forward Primer (5'-3')	Reverse Primer (5'-3')	Temperature	Product Size (Base Pairs)
ACTTTCCTGCGTCCATCAGT	CAGAGTCTGGGGAGGAGAAA	56.5	378
ATGTTTCCGCAGCATTTATC	GCCACATGCTCTCTAAATCC	51.3	578
CTCCTCCCACTCTGCGTTC	GGACAGACAGACCGAGAAGC	57.7	214
CCACAAACAGTGTGTCTGCAT	GGTCTGGCCATTCATTCATT	55.9	248
ATGCAATGTGACCCTAGAGC	AAGACAGCATGGTCTGCATC	54.9	337
TAATGCCAGAAACTTGCTCC	CACAGAAGTACCGCTCCAAC	53.0	720
TGCTTTGAGAACACTAGGGG	AAGAAATTCCAATTCCCACC	54.5	448
CCCTGCGTCCTCTGTCTCTA	AGAGCAAGACTCCGTCTCCA	58.0	430
GGATGCGCCATTACTGTTAG	CCCAAGATCCACATTGTCTC	53.6	424
GGAGCTCTTAGGAACCCAAC	TCCTCTCCAATGCTACAAGG	55.1	439
TTACGCTACACAGGGAAAGC	CTTAGGAAATGCTCAGCCAG	54.9	640
ACCTCTTGAGAAATGTCCCC	CATGATACAGGGGTTGAAGG	54.3	577
TGCTAATGCTCGTCCTTGTG	GAGCCATGATACAGGGGTTG	55.2	380
TGTGGGGCTCGACTGTAACT	CACTCCTGAAGGGAGTCAGC	58.3	98
TCCCAGGAAACTCCTCTACC	AGCTGCTCTGCAGTTTGTG	55.6	477
CTCACTGTGCTGTGTCCTTG	ATGGACCCCTTCAGTACCAG	55.9	393
CTGGCATCTGCTGTCTGTCT	GGAGAGGGTCCTCATCTGG	57.1	365
AGTTCAGGGGCCAACTTCTT	TGACAAAGCCAGTCTGGAAA	56.6	336
TCCCCTTGTCCTTATACGTTC	CCAGAAAGACAATGTGTGAAGAA	53.8	600

Primers used for polymerase chain reaction (PCR) amplification were designed using Primer3 software and supplied by Integrated DNA Technologies (Integrated DNA Technologies, Inc.) (Rozen and Skaletsky, 2000). The samples were sent to Functional Biosciences, Inc. for purification and sequencing. The sequences were then verified against the reference sequence transcript and the sequences from two unrelated CEPH (Foundation Jean Dausset-Centre d'Etude du Polymorphisme Humain) DNA samples using Sequencher 5.1 software (Gene Codes Corporation) (Dausset, 1886).

2.4 STATISTICAL ANALYSIS

Samples were separated based on the presence of one or two copies of each allele. Chi-square was used to test for Hardy-Weinberg equilibrium. The number of homozygous and heterozygous samples was divided by the total number of samples to determine the percentage of the genotypes in population. The distribution of genotypes and alleles between caries affected and caries free individuals was compared using chi-square with an alpha of 0.05 to determine statistically significant differences.

2.5 BIOINFORMATICS

For each of the SNPs found in our results, we used the UCSC Genome Browser Gateway to determine if the sequences were conserved across species (Kent et al., 2002). We used the

program RESCUE-ESE to predict if any SNPs found are involved in exonic splicing enhancer activity (ESE) (Yeo et al., 2004).

It was also determined if any of the eight SNPs were involved with transcription factor binding sites using the program Alibaba 2.1 developed by Niels Grabe (http://www.gene-regulation.com/pub/programs/alibaba2/index.html).

The programs SIFT, PolyPhen-2, and the Ensembl genome browser Variant Effect Predictor were used to determine the impact of nonsynonymous substitutions found by sequencing (Kumar et al., 2009; Adzhubei et al., 2010; McLaren et al., 2010). All programs listed in this section are internet-based and free of charge.

3.0 RESULTS AND DISCUSSIONS

3.1 SEQUENCING RESULTS

From the sequencing of *ESRRB* exons and exon-intron boundaries, SNPs rs10132091, rs61742642, rs3813545, rs3829784, rs45533334, rs35544003, rs2361292, and rs55835922 were found in our samples. A representation of the relative positions of these SNPs in ESRRB can be found in Figure 3. Of the eight SNPs, four were found in exons while the other four were found in exon-intron boundaries. We used chi-square to test that each of the SNPs were in Hardy-Weinberg equilibrium, with our results located in Table 2. We also checked Hardy-Weinberg equilibrium for the caries and caries-free groups. The results are located in Table 3. Individuals with dental caries have an over-representation of the T allele of rs55835922 (74% versus 54%; p=0.01). The SNP rs61742642 is a missense mutation (P386S), but its frequency was just slightly elevated in cases with dental caries (13% versus 9.5%). SNP rs35544003 is a synonymous change not thought to have any detrimental effects. Chi-square results and p-values for each SNP are contained in Table 4.

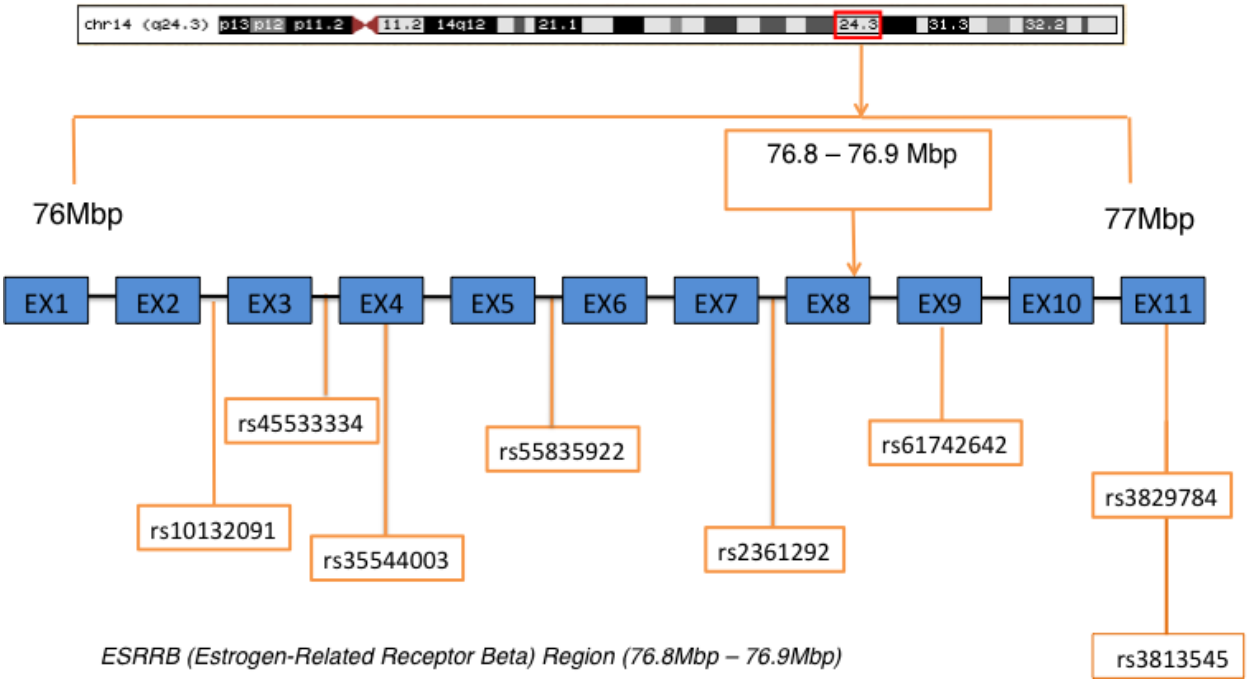

Figure 3. Representation of relative locations of 8 SNPs found via sequencing. SNPs rs61742642 and rs35544003 are located in exons of *ESRRB.* SNPs rs3829784 and rs3813545 are also located in an exon but are in a noncoding region. The other SNPs are located within intron-exon boundaries of ESRRB.

3.2 SEQUENCING DISCUSSION

Patients with dental caries were found to have a significant over-representation of the T allele of rs55835922 (74% versus 54%; p=0.01). This could be due to either the SNP being close to the part of the gene involved in caries experience or due to some other function of the SNP we did not detect in our experiment. We did not find any evidence that any of the SNPs we found are disease-causing mutations. Therefore, this result can be interpreted as a potential risk factor for dental caries, if indeed it is related. Perhaps patients with the T allele could be instructed in more comprehensive caries prevention techniques because they may be more at risk for the disease.

Ninety-three samples from the 172 sample Turkish cohort were used for sequencing (62 caries samples and 31 caries free control samples). These patients were chosen because they are all female patients. We decided to choose females for two reasons. First, studies have shown that females tend to have more dental caries than men (Lukacs, 2007; Jindal et al., 2011; Lukacs, 2011). Second, we wanted to eliminate additional factors from the study that could confuse our results. Dental caries is a multifactorial disease. It is easiest to study one factor at a time to determine the etiology. By choosing only females, we eliminated any factors that may come from sex disparities. By choosing only children for the study, we eliminated additional factors that could come from differences in age, such as differences between adults and children. Studies have shown that caries tends to increase with age (Saunders and Meyerowitz, 2005; Mungia et al., 2008; Gati and Vieira, 2011; Vieira et al., 2014).

We also chose children from the same ethnic background because differences in caries experience has been linked to differences in ethnicity. Additionally, all of the patients for the study came from similar socioeconomic backgrounds, eliminating differences that come with differing class levels, levels of dental care available, and educational differences.

We used samples without caries as our control samples. We also used two unrelated CEPH (Foundation Jean Dausset-Centre d'Etude du Polymorphisme Humain) DNA samples. The foundation these samples came from provides resources for research involving the human genome, including a registry of DNA samples. We used CEPH samples because they have been tested before both by our lab and the foundation from which they came from and could verify that our sequencing methods were sound.

We checked the population using chi-square analysis to see if it was in Hardy-Weinberg equilibrium. Our calculations included one degree of freedom. From the tables, it is shown that some of the SNPs are not in equilibrium. This could be due to our smaller sample sizes, especially when they are separated into caries and caries-free groups. It could also be due to the highly multifactorial nature of dental caries, which could cause a distortion in equilibrium among the caries and caries-free groups. Other factors that may distort it could be genetic, such as genes related to immune response differences or susceptibility to bacterial colonization and enamel dissolution in the mouth (Nandula et al., 2007; Patir et al., 2008). Also, behavior of the patients can affect equilibrium such as dietary decisions and oral hygiene habits because it these are environmental factors that can influence the patients susceptibility to caries despite their genetic makeup (Wendell et al., 2010; Feeney et al., 2011). Additionally, patients who are caries-free may still be in the disease state if they have the infectious bacteria in their mouths. All of these factors may be

working independently or together to cause an equilibrium distortion, especially if there are genes underlying them. Perhaps our testing of these distributions of alleles are serving as a surrogate to mark other untested factors involved in the disease.

When looking at the specific SNPs not in Hardy Weinberg equilibrium (rs45533334 and rs5583992), we can see that these SNPs were associated with dental caries experience in Table 4. This association may be the true reason these SNPs were the ones not in Hardy Weinberg equilibrium, with the factors listed previously being only potential issues.

Table 2. Results of using chi-square to test for Hardy-Weinberg equilibrium in all samples.

Marker	Genotypes	# Observed	X^2	p-value
rs10132091	TT CT CC	22 44 14	0.99	0.32
rs61742642	CC CT TT	71 9 0	0.28	0.59
rs3813545	TT CT CC	53 20 1	0.34	0.56
rs3829784	TT CT CC	10 43 18	3.65	0.055
rs45533334	GG CG CC	52 4 1	5.00	0.02
rs35544003	CC CA AA	69 6 0	0.13	0.71
rs2361292	CC CG GG	11 27 40	3.02	0.08
rs55835992	TT CT CC	40 18 15	14.2	0.000

Table 3. Results of using chi-square to test for Hardy-Weinberg equilibrium in caries and caries-free samples.

Marker	Genotypes	# Caries Samples	X^2	p-value	# Caries-free Samples	X^2	p-value
rs10132091	TT CT CC	16 27 10	0.05	0.81	6 17 4	1.91	0.17
rs61742642	CC CT TT	40 6 0	0.22	0.64	31 3 0	0.07	0.79
rs3813545	TT CT CC	37 12 1	0.000	0.98	16 8 0	0.96	0.33
rs3829784	TT CT CC	7 27 12	1.62	0.20	3 16 6	2.23	0.14
rs45533334	GG CG CC	34 4 1	3.01	0.08	18 0 0	-	-
rs35544003	CC CA AA	48 5 0	0.13	0.71	21 1 0	0.01	0.91
rs2361292	CC CG GG	9 16 29	5.29	0.02	2 11 11	0.12	0.74
rs55835992	TT CT CC	28 14 5	2.21	0.14	12 4 10	12.4	0.00

Table 4. Summary of sequencing results and case-control comparisons.

Marker	Genotypes/ Alleles	Caries Affected	Caries Unaffected	X^2	p-value
rs10132091	TT	16	6	1.05	0.59
	CT	27	17		
	CC	10	4		
	T	59	29	0.06	0.81
	C	47	25		
rs61742642	CC	40	31	0.35	0.55
	CT	6	3		
	TT	0	0		
	C	86	65	0.33	0.57
	T	6	3		
rs3813545	TT	37	16	1.12	0.57
	CT	12	8		
	CC	1	0		
	T	86	40	0.18	0.67
	C	14	8		
rs3829784	TT	7	3	0.22	0.89
	CT	27	16		
	CC	12	6		
	T	41	22	0.00	1
	C	51	28		
rs45533334	GG	34	18	2.53	0.28
	CG	4	0		
	CC	1	0		
	G	72	36	4.81	0.03
	C	6	0		
rs35544003	CC	48	21	0.50	0.48
	CA	5	1		
	AA	0	0		
	C	101	43	0.48	0.48
	A	5	1		
rs2361292	CC	9	2	2.28	0.31
	CG	16	11		
	GG	29	11		
	C	34	15	0.001	1
	G	74	33		
rs55835992	TT	28	12	8.27	0.02
	CT	14	4		
	CC	5	10		
	T	70	28	6.45	0.01
	C	24	24		

3.3 BIOINFORMATICS RESULTS

Sequence conservation for each of the eight SNPs was checked using the UCSC Genome Browser Gateway (Kent et al., 2002). For the four SNPs found in exons (rs61742642, rs35544003, rs3829784, rs3813545), the sites of the amino acid changes were conserved across all species for which there was data (Figure 5). For the other four SNPs, which were intronic, the sites of the nucleotide change were relatively conserved with only rs45533334 not conserved in the mouse, rs55835922 not conserved in the Rhesus monkey, and rs2361292 not conserved in the chicken (Figure 6). You can see that the mutations and surrounding sequences are generally conserved. Full results can be found at http://genome.ucsc.edu/cgi-bin/hgGateway.

Each SNP was entered into the program RESCUE-ESE to predict if any SNPs found are involved in exonic splicing enhancer activity. Sequences with both alleles were entered separately to determine if either polymorphism was involved in exonic splicing enhancer activity. SNP rs3829784 was involved in exonic splicing enhancer activity for both alleles, while rs35544003 and rs3813545 were involved for only one allele, A for each of them. The other five SNPs were not involved in exonic splicing enhancer activity. These results are included in Figure 4.

Figure 4. Output of RESCUE-ESE showing exonic splicing enhancer of SNPs. Column "a" includes the wildtype sequences for each of the SNPs. Column "b" includes sequences with the changed alleles, showing how the exonic splicing activity changes as well.

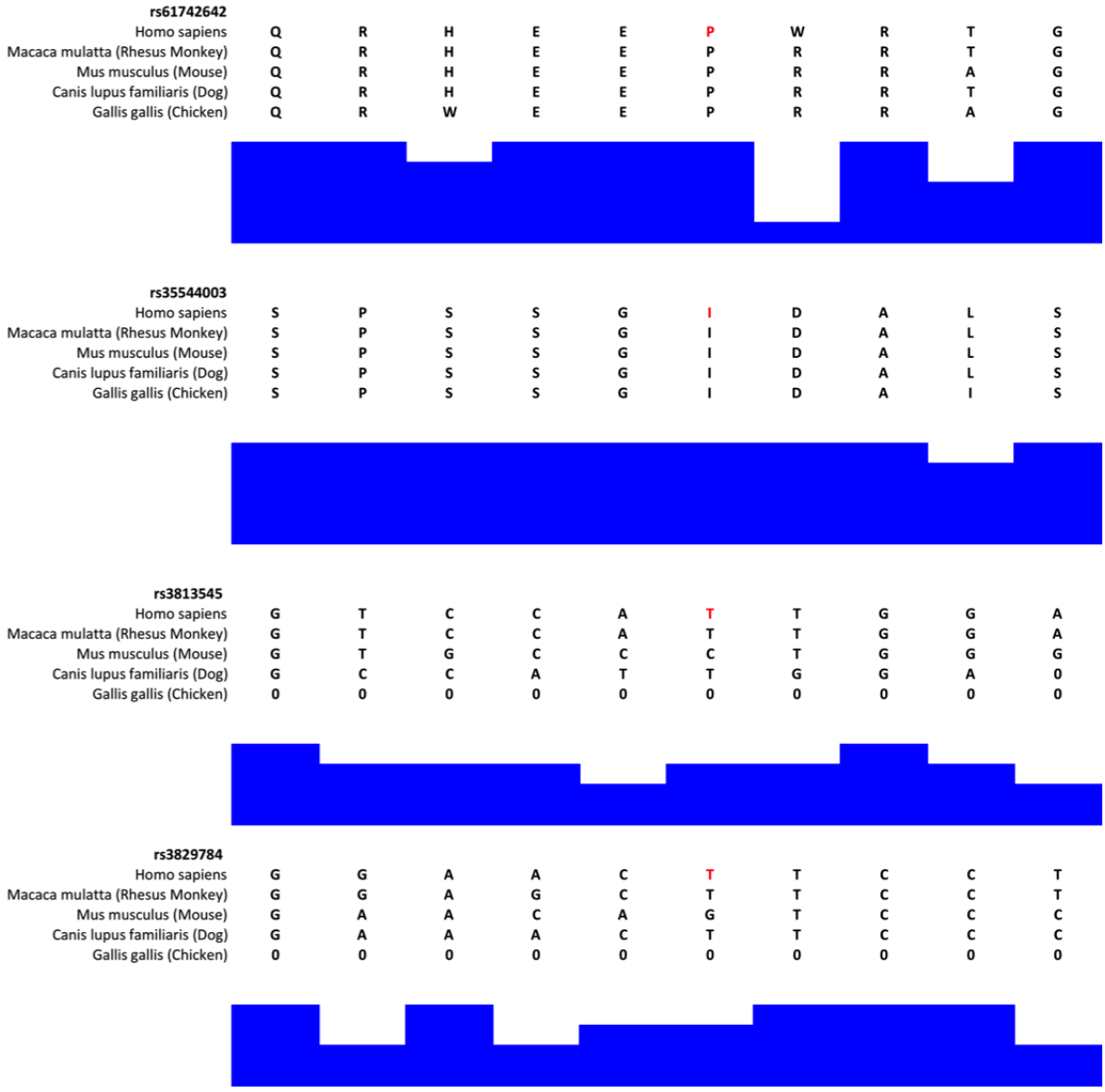

Figure 5. Results of conservation studies for exonic SNPs found in sequencing. The amino acid changes are highlighted in red. Changes for the first two SNPs, which are in coding regions of exons, are conserved across species. In general, the surrounding sequences are conserved as well. Full results can be found via the UCSC Genome Browser Gateway.

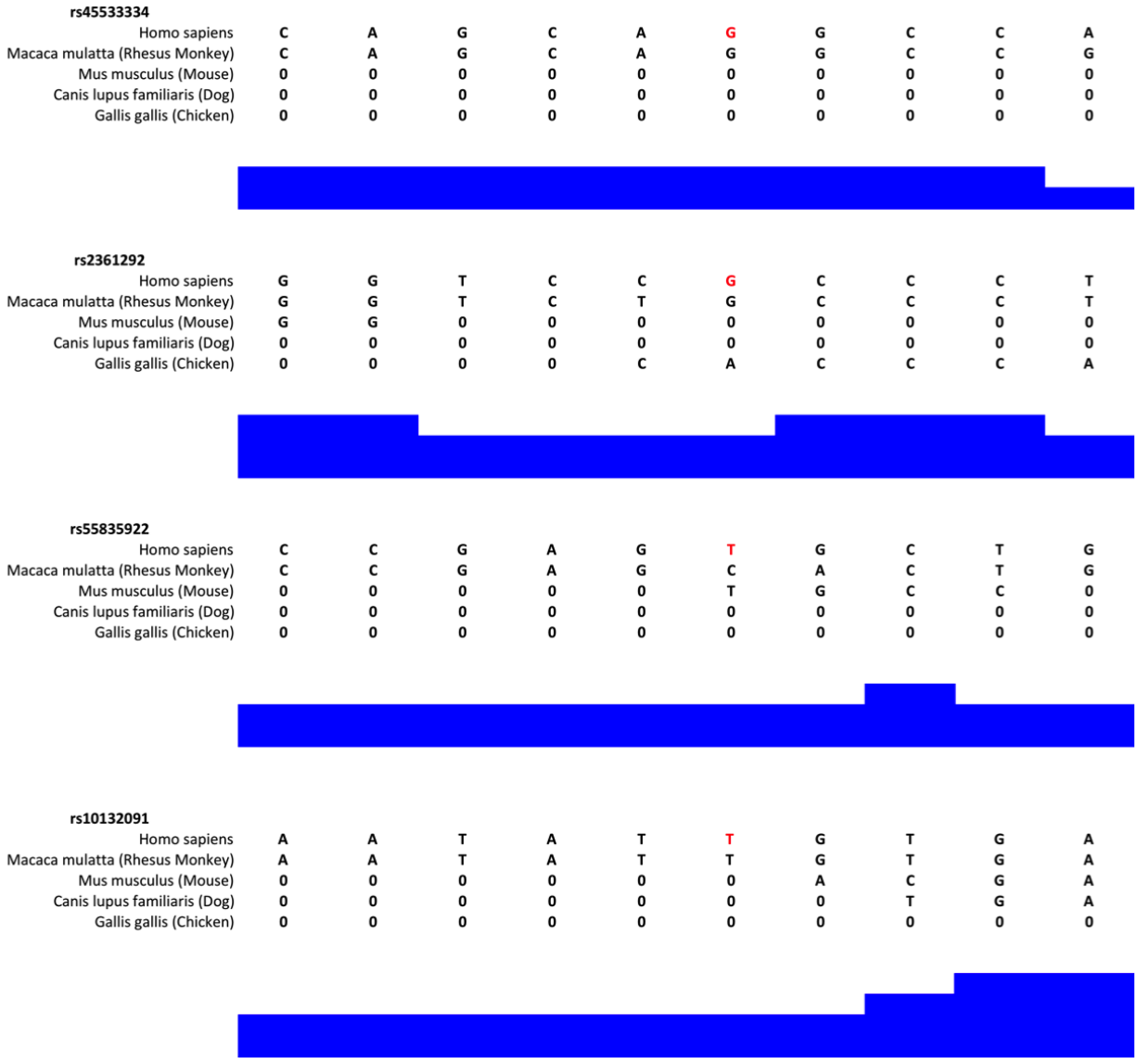

Figure 6. **Results of conservation studies of intronic SNPs found in sequencing.** The nucleotide changes are highlighted in red. The sequences are conserved, especially between humans and monkeys. Full results can be found via the UCSC Genome Browser Gateway.

The SNP rs61742642 was analyzed using SIFT, PolyPhen-2, and the Ensembl genome browser Variant Effect Predictor. The Variant Effect Predictor confirmed the mutation as missense and also corroborated the SIFT and PolyPhen-2 results. SIFT predicted that the mutation would be tolerated, and PolyPhen-2 also agreed that it would be a benign change.

It was also determined if any of the eight SNPs were involved with transcription factor binding sites using the program Alibaba 2.1 developed by Niels Grabe (http://www.gene-regulation.com/pub/programs/alibaba2/index.html). These results are included in Figure 7. Our results show that the missense mutation rs6172642 and synonomous change rs35544003 do not have any transcription binding factor activity. The other SNPs, both wildtype and changed sequences alike, bind to transcription factors such as specificity proteins (SP), CCAAT-enhancer binding proteins (C/EBP), TATA-binding proteins (TBP), nuclear factors (NF), and activating enhancer binding proteins (AP).

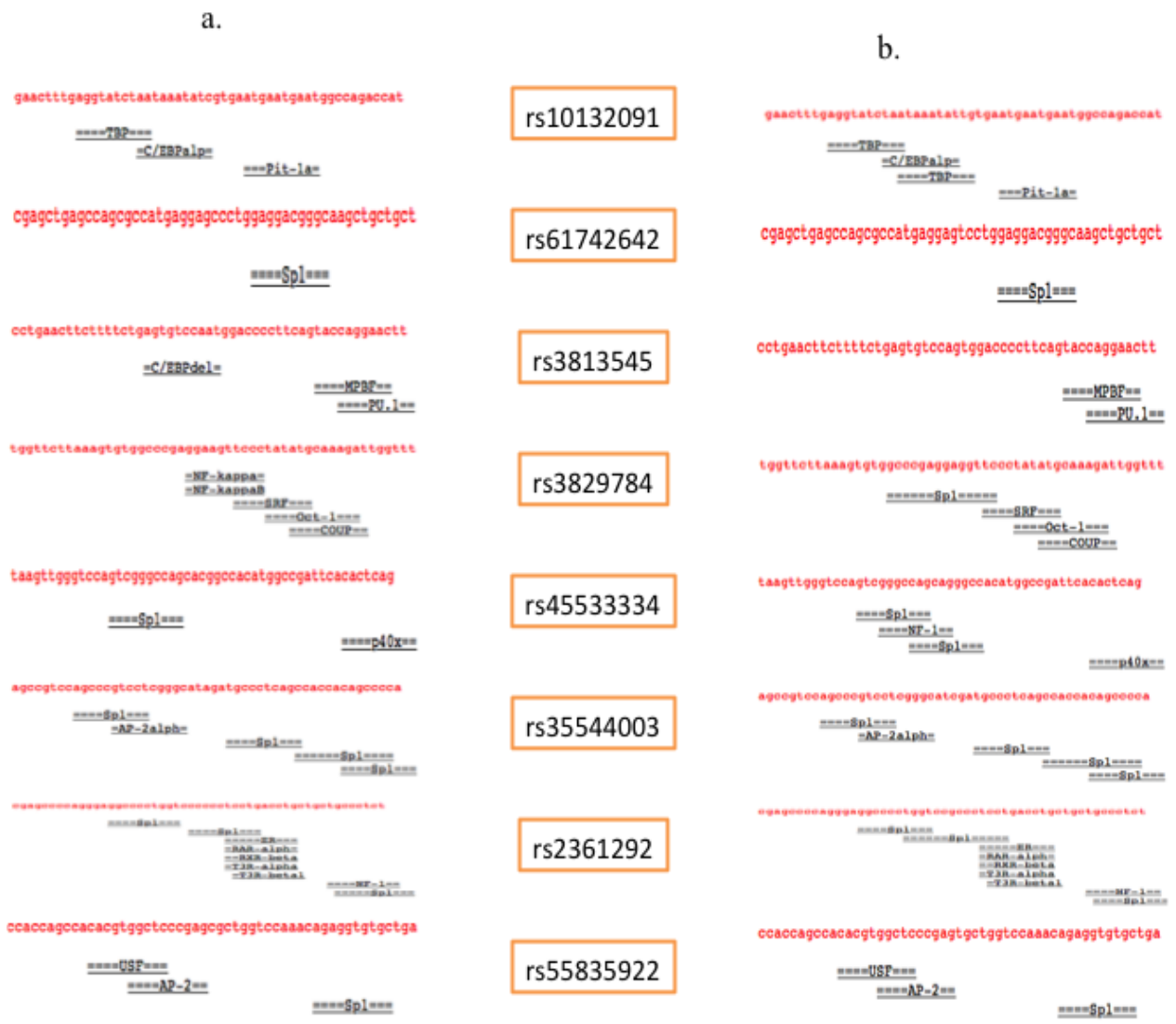

Figure 7. Output of Alibaba 2.1 software showing the transcription factors and their binding sites. Column "a" includes the wildtype sequences and the output of Alibaba showing transcription factor binding sites. Column "b" includes the Alibaba output for changed sequences with transcription factors and binding sites.

3.4 BIOINFORMATICS DISCUSSION

Bioinformatics is, loosely, a term used to describe the combination of biology with computational analysis. Luscombe et al proposed the following definition for bioinformatics: "…conceptualizing biology in terms of macromolecules (in the sense of physical-chemistry) and then applying "informatics" techniques (derived from disciplines such as applied mathematics, computer science, and statistics) to understand and organize the information associated with these molecules, on a large-scale (Luscombe et al., 2001)." Other scientists have come up with similar explanations, emphasizing the requirement of organization of genomic data and a need for visualizing how the genome works in regards to proteins and functionality within the organism (Mackenzie, 2003). We will used bioinformatics tools in this study to help determine if genetic variation in estrogen related receptor beta (ESRRB) has a potential functional effect that influences dental caries.

When looking at mutations or polymorphisms, sequence conservation can provide insight on the impact of genetic variation. Sequences that are conserved across species tend to be more resistant to changes. This is because those that are conserved usually play a functional role in the organism, and changes result in loss of function or deleterious effects (Cooper and Brown, 2008). Sequences that are not as conserved are usually involved in evolutionary change between species (Cooper and Brown, 2008). It was interesting that rs61742642 was heavily conserved, specifically the proline which is changed to a serine via the missense mutation. Usually, heavily conserved regions do not tolerate mutations well. However, through protein analysis with SIFT, PolyPhen-2, and the Ensembl Variant Effect Predictor, it was determined that the missense

mutation would be tolerated and therefore benign, which most likely explains why such a heavily conserved region of the genome is able to withstand the mutation.

SIFT predicts whether substitution of a different amino acid will affect the function of the protein. We chose SIFT because of its application to nonsynonomous mutations. According to the website, predictions by SIFT are due to degree of conservation of amino acid residues in sequence alignments supplied by PSI-BLAST (Kumar et al., 2009). PolyPhen-2 is another tool for predicting the effect of nonsynonomous SNPs on the structure and function of proteins (Adzhubei et al., 2010). We used this along with the Ensemble Variant Effect Predictor, another similar bioinformatics tool, to corroborate the results of the SIFT analysis. The Variant Effect Predictor determines the effect of variants on genes, transcripts, proteins, and regulatory regions (McLaren et al., 2010). All of these tools determined the missense mutation would be benign to the structure and function of the ESRRB protein.

To try to explain why this missense mutation may not be a functional problem for the ESRRB protein and why it can happen within a sequence that is heavily conserved, we can look at the amino acids themselves. Proline is hydrophobic and usually found within a protein (Denise Ferrier, 2014). Serine is a slightly polar amino acid that is often neutral when it comes to mutations in sequence, and because of its neutrality can be found within or outside of a protein (Betts and Russell, 2003). Both serine and proline have hydroxyl functional groups, which may be one of the reasons they can be substituted for one another in mutations without causing too much of a change. Additionally, these hydroxyl groups can both form hydrogen bonds, which means serine can essentially mimic proline in protein function (Betts and Russell, 2003). Therefore, because of their similar structures and functions, a proline to serine substitution may not have a dramatic effect on the protein, helping to explain our bioinformatics results.

From Alibaba 2.1 analysis we determined that some of the SNPs in *ESRRB* are involved in transcription factor binding activity. However, the SNPs rs61742642 and rs35544003, which were a missense mutation and synonomous change respectively, did not bind to transcription factors. Transcription factors help control whether or not genes are turned on or off (Denise Ferrier, 2014). However, other transcription factor binding sites were found flanking the SNPs, which may affect the SNPs that are downstream. Also, transcription factors may work together, explaining some of the overlap seen in the output for the other SNPs (Denise Ferrier, 2014).

3.5 LIMITATIONS AND FUTURE DIRECTIONS

This study is obviously limiting in itself because it is only sequencing, which is a very small part of determining whether a gene may have a role in a disease known for being incredibly multifactorial. However, further studies have concluded similar results that ESRRB is associated with dental caries, as this study was part of a larger series of studies in determining the role of ESRRB in oral health (Vieira et al., 2008; Weber et al., 2014).

For the sequencing, we only used female patients from a cohort of Turkish children. While using female children helps to eliminate extra variables associated with gender and age differences in dental caries, it does prevent us from determining the risk involved in ESRRB and dental caries for males and adults. Ideally, we would hope to find similar results if we sequenced males, adults, and patients who are not Turkish but from other ethnicities. Therefore, future studies could involve testing additional populations to help determine if this is a true risk factor for dental caries for other patients.

While we were able to use bioinformatics to determine the effect of the nonsyonomous mutation at rs61742642, not much is known in general about the protein structure and function of estrogen related receptor beta. Scientists only have a basic understanding of this receptor protein. Hopefully future studies will begin giving us a more complete picture of the role of ESRRB, and we can use this knowledge to help learn more about the etiology of dental caries.

Studying a genetic basis for dental caries experience is a difficult task. Dental caries is a highly multifactorial disease. While there is clear evidence that the host's genetic background plays a role in the disease, it is nearly impossible to study only one factor at a time. Although we

can choose to study homogeneous populations to eliminate confounding variables, it is still difficult to control variables such as diet or immune response. For this study, therefore, we cannot say definitively what the role of ESRRB is in dental caries experience.

However, it is still important to study this gene as well as other potential genetic causes despite environmental factors playing such a big role in the disease. Perhaps one day, polymorphisms or mutations in a gene such as *ESRRB* could be used as markers to determine a patient's risk for the disease. Then, this patient could be advised to partake in more comprehensive oral hygiene and good diet practices. Earlier intervention for those at risk could also stem the progression of the disease. While markers in *ESRRB* may not lead to a direct treatment, knowing which patients are more at risk could help prevent future oral health problems and monetary commitments if prevention methods are employed for the disease.

4.0 CONCLUSIONS

Although we did not find mutations that may affect protein structure and function, we did find that estrogen related receptor beta (ESRRB) might be involved in elevated risk of dental caries. Patients with dental caries were found to have a significant over-representation of the T allele of rs55835922. This could be due to either the SNP being close to the part of the gene involved in caries experience or due to some other function of the SNP we did not detect in our experiment. Perhaps closer study of the etiology of dental caries and ESRRB would provide a plausible model of risk assessment for dental caries in patients, allowing more personalized dental care and stricter preventative measures for those at risk.

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