ORIGINAL INVESTIGATION

Role of *TRAV* locus in low caries experience

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Abstract Caries is the most common chronic, multifactorial disease in the world today; and little is still known about the genetic factors influencing susceptibility. Our previous genome-wide linkage scan has identified five loci related to caries susceptibility: 5q13.3, 13q31.1, 14q11.2, 14q 24.3, and Xq27. In the present study, we fine mapped the 14q11.2 locus to identify genetic contributors to caries susceptibility. Four hundred seventy-seven subjects from 72 pedigrees with similar cultural and behavioral habits and limited access to dental care living in the Philippines were studied. An additional 387 DNA samples from unrelated individuals were used to determine allele frequencies. For replication purposes, a total of 1,446 independent subjects from four different populations were analyzed based on their caries experience (low versus high). Forty-eight markers in 14q11.2 were genotyped using TaqMan chemistry. Transmission disequilibrium test was used to detect over transmission of alleles in the Filipino families, and Chi-square, Fisher's exact and logistic regression were used to test for association between low caries experience and variant alleles in the replication data sets. We finally assessed the mRNA expression of *TRAV4* in the saliva of 143 study subjects. In the Filipino families, statistically significant associations were found between

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low caries experience and markers in *TRAV4*. We were able to replicate these results in the populations studied that were characteristically from underserved areas. Direct sequencing of 22 subjects carrying the associated alleles detects one missense mutation (Y30R) that is predicted to be probably damaging. Finally, we observed higher expression in children and teenagers with low caries experience, correlating with specific alleles in *TRAV4*. Our results suggest that *TRAV4* may have a role in protecting against caries.

Introduction

Caries is still a major problem in most industrialized countries, affecting 60–90 % of schoolchildren and the vast majority of adults (World Health Organisation 2003). The etiology is complex and multifactorial with contributions from external factors, such as the host, oral microbial flora, type of diet, shared behaviors, practices and habits within family, and pressures from society (Fisher-Owens et al. 2007).

Currently, identifying children at risk for caries prior to the occurrence of the disease still depends on classic methods, including application of diet questionnaires, inspection of oral hygiene level, and detection of *Streptococcus mutans* in saliva. However, these attempts have had limited success in determining caries risk at the population level, and the most promising methods reach sensitivity and specificity of around 80 % (Gao et al. 2010). Previous caries experience continues to be the best predictor for future disease (Powell 1998). A child's resistance or susceptibility to caries can occur, regardless of exposure to external risk factors. These facts suggest that there is biological influence on disease susceptibility, likely controlled by genetic factors of the host.

Although caries is a complex disease, studies on twins and families have provided strong evidence for a role of genetic inheritance for caries, with an estimated genetic

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contribution to caries development of 40–60 % (Conry et al. 1993; Bretz et al. 2005, 2006; Wang et al. 2010). The importance of genetic factors in caries is also supported by animal studies that have identified a few chromosomal loci (in chromosomes 1, 2, 7, and 8) for caries susceptibility (Suzuki and Kurihara 1998; Nariyama et al. 2004). Regarding the mode of inheritance, both a mouse crossbreeding study (Nariyama et al. 2004) and a family segregation analysis in humans (Werneck et al. 2011) suggest a major gene dominant effect for caries.

Our previous genome-wide linkage scan for caries in 624 individuals from 46 Filipino families identified three loci for low caries susceptibility (5q13.3, 14q11.2, and Xq27.1) and two loci for high caries susceptibility (13q31.1 and 14q24.3) (Vieira et al. 2008a). Recently, our group showed that the 5q13.3 locus may be related to caries susceptibility through genes related to immune response (Shimizu et al. 2013). The 14q11.2 locus had significant results under a recessive model (Vieira et al. 2008a). Our hypothesis was that genetic factors located in 14q11.2 underlie biological mechanisms that influence low caries experience in humans. Here, we present the fine mapping of the 14q11.2 locus and the identification of a gene that may be relevant for caries susceptibility.

Materials and methods

Study subjects

The study sites, subject recruitment and DMFT/dmft (Decayed, Missing due to caries, Filled Teeth) data collection have been described in our original genome-wide linkage study (Vieira et al. 2008a), but the number of families was increased from 46 to 72 for further fine mapping. DNA samples were extracted from peripheral blood according to standard protocols. In an attempt to reduce the influence of environmental factors as potential confounders in this study, the sample population consisted of 477 subjects (224 females and 253 males) from 72 pedigrees living in the same area in the Philippines. An additional 387 DNA samples from unrelated individuals from the same area collected during preliminary assessments of oral health needs were available for determining allele frequencies. Families included were part of existing studies of other craniofacial defects (Murray et al. 1997). The mean age of the subjects was 25.8 years and ranged from 2 to 72 years of age and the mean DMFT/dmft score was 9.7 and ranged from 0 to 32 (Table 1). This study protocol was approved by the University of Pittsburgh and H.O.P.E. Foundation International Institutional Review Boards, and appropriate written informed consent was obtained from all participants. Age appropriate assent

Table 1 Demographic and caries experience (DMFT/dmft) of study subjects

	Filipinos	Turkish	Brazilian Curitiba	Brazilian Rio de Janeiro	Guatemala	Argentina[a]
Sample size (mean DMFT ± standard deviation)	477 (9.7 ± 7.3)	176 (3.7 ± 3.7)	539 (1.5 ± 1.5)	501 (2.4 ± 2.4)	230 (7.45 ± 7)	143 (7.1 ± 7.1)
High caries experience individuals (mean DMFT ± standard deviation)	298 (13.3 ± 6.7)	90 (7.2 ± 2.3)	129 (4.3 ± 1.4)	174 (5.8 ± 2.6)	90 (8.3 ± 3.3)	66 (13.0 ± 7.9)
Low caries experience group (mean DMFT ± standard deviation)	179 (3.6 ± 2.4)	86 (0)	410 (0.6 ± 0.6)	327 (0.6 ± 0.6)	140 (3.5 ± 3.3)	77 (2.0 ± 2.0)
Females	224	96	261	236	161	83
Males	253	80	278	265	69	60
Age in years (mean ± standard deviation)	25.8 ± 16.3	5.4 ± 0.8	12 ± 0.5	9.1 ± 3.1	31 ± 10.7	21.7 ± 15.6
The number of pedigrees	72	Unrelated	Unrelated	Unrelated	Unrelated	Unrelated
Artificially fluoride water	No	No	Yes	Yes	No	No

High and low caries experience were defined based on Table 2

DMFT Decayed, Missed due to caries, Filled permanent Teeth, *dmft* = decayed, missed due to caries, filled primary teeth

[a] Argentina samples were used for expression analysis only

Table 2 Definition of caries experience (based on Vieira et al. 2008a, b) based on age and DMFT (Decayed, Missing due to caries, Filled Teeth) scores

	Number of individuals	DMFT
Caries experience level	477	
Children (up to 12 years of age)	115	
Low caries experience	26	0–2
High caries experience	89	3 or higher
Teenagers (from 13 to 19 years of age)	104	
Low caries experience	44	0–5
High caries experience	60	6 or higher
Adults (20 years of age and older)	258	
Low caries experience	99	0–8
High caries experience	159	9 or higher

documents were used for children between 7 and 14 years of age with written informed consent obtained from the parents as well. Caries experience data (DMFT/dmft scores; World Health Organisation 1997) were collected by a single examiner who was calibrated by one of the authors (A.R.V.). Intra-examiner agreement was assessed by a second clinical exam in ten families after 2 weeks, with a κ of 1.0. The criteria of caries experience level based on age and DMFT scores, and DMFT cut-offs used for statistical analysis were the same used in the original genome-wide linkage study (Table 2) (Vieira et al. 2008a).

Association analyses

The 14q11.2 locus covers a 1-LOD interval (upstream and downstream) from the point with the maximum LOD score on chromosome 14 from our previous work in the Philippines (Vieira et al. 2008a). We selected single nucleotide polymorphisms (SNPs) to fine map the 14q11.2 region, which covers about 7.3 million base pairs and spans 128 genes using the data from the International HapMap Project on Caucasians and Chinese (http://www.hapmap.org), viewed through the software Haploview (Barrett et al. 2005). We selected 48 SNPs in the 14q11.2 region based on pairwise linkage disequilibrium properties, haplotype blocks, and structure of the genes present in the region. Genotyping was performed using Taqman chemistry on ABI 7900HT real-time PCR equipment. Endpoint analysis was performed using the ABI SDS software (Applied Biosystems, CA, USA). Association between caries experience and the SNPs was tested with the transmission disequilibrium test (TDT) as implemented in family-based association test (FBAT) (Horvath et al. 2001). Correction for multiple comparisons in single marker analyses was performed using the SNP spectral decomposition (SpD) method (Nyholt 2004). Significance was set at $p < 0.00035$ by the SpD method. Calculations of linkage disequilibrium were computed with the Graphical Overview of Linkage Disequilibrium (GOLD) software (Abecasis and Cookson 2000).

Replication dataset

The replication dataset consisted of 1,446 individuals from four populations including Turkish, Guatemalan, and two Brazilian cohorts (Table 1). Caries experience was defined as described in Table 2, taking into consideration age of subjects (Vieira et al. 2008a, b). Details regarding the demographics and caries experience of the four study groups are presented in Table 1. With the exception of Brazil, drinking water in the regions where samples originated is not artificially fluoridated. DNA samples from the

replication dataset were extracted from whole saliva collected in Oragene kits (DNA Genotek, Inc., Ottawa, ON, Canada) according to the manufacturer's instructions. Subjects were asked to spit 2–4 ml of saliva within 5–10 min. Absorbing sponges provided by the manufacturer were used for children younger than 7 years of age.

These replication experiments targeted SNPs showing suggestive association with caries experience in the Filipino dataset. When the allele frequency was low in a specific replication group, we selected additional markers in the same region. Hardy–Weinberg equilibrium tests were performed to test for deviations in the genotype and allele distributions. Distribution of genotypes and allele frequencies in the Turkish cohort was tested by Chi-square test. For the Guatemalan and Brazilian cohorts, we used logistic regression analysis and haplotype analysis [as implemented in PLINK (Purcell et al. 2007)] to investigate main-effect models and predict caries status. Since the definitions of caries experience are based on age, there was no need to adjust for age in the regression analysis. For genetic association analyses, a nominal p value ≤ 0.05 was considered statistically significant.

Turkey

The study sample from Istanbul, Turkey, consisted of 172 unrelated children (93 females and 79 males) from 3 to 6 years of age. All children younger than 6 years of age visiting the Pedodontics Department for treatment during 2007 were invited to participate in the study. Ninety children had a dmft score of more than four and 82 children were caries-free (Patir et al. 2008). Children with dmft scores between 1 and 3 were not included. Both Istanbul University and University of Pittsburgh Institutional Review Boards approved the study of these samples and appropriate written informed consent was obtained from the parents of all participants.

Guatemala

The cohort from Tiquisate, Guatemala consisted of 230 DNA samples from unrelated individuals (161 females; mean DMFT 8.3 ± 7.5, and 69 males; mean DMFT 6.6 ± 6.5, mean age of 31 years) who participated in studies conducted by the University of Pittsburgh Center for Craniofacial and Dental Genetics, specifically a 2006 research trip in collaboration with a Children of the Americas medical mission. As part of the research protocol, the cohort received a dental examination (Deeley et al. 2008). These samples were collected with the approval of both the University of Pittsburgh Institutional Review Board and the Oversight Ethics Committee of the Hospital

Nacional de Tiquisate, and all subjects gave informed written consent.

Brazil

Two sample data sets from Brazil were available for this study. The first consisted of 539 unrelated children living in Curitiba (261 females and 278 males) with a mean age of 12 years (between 10 and 14 years) and mean DMFT of 1.5 ± 1.9. These children were all 5th, 6th, or 7th grades students of 12 public schools during the year 2006 (Brancher et al. 2011) to obtain a representative sample of Curitiba county. These samples were used with the approval of both the Pontifical Catholic University of Paraná and the University of Pittsburgh Institutional Review Boards. Age appropriate assent documents were used for all children and informed written consent was obtained from the parents. The second group consisted of 501 unrelated subjects living in Rio de Janeiro participating in studies of other craniofacial anomalies (236 females and 265 males) with mean age of 9 years (between 2 and 21 years) and mean DMFT of 2.4 ± 3.0, ascertained for studies of craniofacial anomalies (Tannure et al. 2012). These samples were used with the approval of both the Federal University of Rio de Janeiro and the University of Pittsburgh Institutional Review Boards. Age appropriate assent documents were used for children between 7 and 14 years and informed; written consent was obtained from the child as well as from the parents.

Caries assessment

Caries experience data (DMFT/dmft) were collected according to the World Health Organisation protocol of 1997 (World Health Organisation 1997). In brief, visible lesions in dentin as well as visible active lesions in enamel (white spots) were scored as decayed. An explorer was gently used for assessing the smoothness of tooth surfaces. Gauze was used to dry and clean teeth prior to exam. Artificial light and a dental operatory were used for all evaluations, with the exception of the Philippines and Guatemala, where a bed was available for subjects to lay down. Exam calibrations were performed according to the following protocol: First, the calibrator presented to the examiner(s) the criteria for caries detection, showing pictures of several situations to be observed in the exam (sound and decayed tooth surfaces, filled teeth with and without secondary lesions, missing teeth due to caries or due to other reasons) and discussing each of these situations in a session that lasted 1–2 h. Next, the calibrator and examiner(s) examined 10–20 subjects and discussed each case. In the case of data from Turkey, one of the authors (A.P.) carried out the clinical examination after being

calibrated by an experienced specialist (F.S.). The intra-examiner agreement was assessed by a second clinical exam in 10 % of the sample after 2 weeks, with a κ of 1.0. In the cases of Argentina and Guatemala, data were collected by one single experienced specialist examiner (A.R.V.). Subjects in these projects are seen over a period of no longer than 3–5 days and intra-examiner agreement data could not be generated. Samples from Rio de Janeiro, Brazil, were collected by two examiners (E.C.K. and P.N.T.) and calibrated by an experienced specialist (M.C.C.). The intra-examiner agreement was assessed by a second clinical exam in 20 children after 2 weeks, with a κ of 0.99. Cohen's kappa values for agreement between examiners were 0.91. Finally, caries experience data from Curitiba, Brazil, were collected by two examiners (J.A.B. and G.D.P.) and calibrated by an experienced specialist (P.C.T.). Inter- and intra-examiner reproducibility was taken on 10 % of the sample and κ was 0.93 for inter- and 0.99 for intra-examiner reliability.

Mutation analysis

We carried out mutation analysis of *TRAV4* since SNPs flanking the gene showed significant or suggestive association with caries experience in the Filipinos, Turkish, Guatemalans, and Brazilians.

Twenty-two samples from individuals with low caries experience carrying two copies of the associated SNP alleles were selected for sequencing all exons and intron–exon boundaries. Reference sequences were obtained from the Ensembl Genome Browser (http://useast.ensembl.org/index.html). The primers used for PCR amplification were 5′GCTCATTGCAGCTCAGACAC3′ and 5′GAGCTGCCTGGAGAAAAATG3′ for exon 1 and 5′AAGTAGAGCGTGGGGATGGA3′ and 5′GGACTTCTTTCTAGGCTGTGA3′ for exon 2. PCR products were 205 and 438 base pairs long, respectively. Reactions conditions were as follows: exon 1 (one cycle of 94 °C for 10 min, then 40 cycles at 94 °C for 15 s, 58 °C for 20 s, and 72 °C for 40 s, with final extension at 72 °C for 4 min); exon 2 (one cycle of 94 °C for 10 min, then 35 cycles of at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min). PCR products were directly sequenced using an ABI PRISM BigDye[TM] Terminator Cycle Sequencing Ready Reaction Kit in an ABI 3730xl DNA Analyzers (Applied Biosystems, CA, USA). Sequences obtained were compared against the sequences in the Ensembl Genome Browser and two unrelated CEPH (Foundation Jean Dausset-Centre d'Etude du Polymorphisme Humain) controls. We used PolyPhen-2 (Adzhubei et al. 2010) to predict the functional effects of any nonsynonymous single nucleotide changes detected.

Gene expression analyses

Samples

From Argentina, 143 DNA and RNA samples from unrelated individuals living in 12 Patagonia sites (San Carlos de Bariloche, El Bolsón, Esquel, El Maitén, Maquinchao, Ingeniero Jacobacci, Rio Colorado, Choele Choel, Valcheta, Sierra Grande, Santo Antonio Oeste, and General Roca) were studied to determine expression of *TRAV4* in whole saliva as described below. These samples are part of the University of Pittsburgh Center for Craniofacial and Dental Genetics studies. The mean age was 21.7 years (between infants under 1 and 72 years with median of 18 years) and both the Centro de Educación Médica e Investigaciones Clínicas "Norberto Quirno" (CEMIC) and University of Pittsburgh Institutional Review Boards approved the study of these samples and appropriate written informed consent was obtained from all participants (parents provided consent for the participation of individuals 17 years of age and under).

mRNA expression of TRAV4 in whole saliva

Whole saliva RNA samples were used for isolation of total RNA from 143 Argentinean individuals using the RNeasy Micro kit (Qiagen, CA, USA). Total RNA from saliva was successfully isolated from 118 samples. mRNA expression analysis of *TRAV4* was performed in real-time PCR using SYBR Green chemistry in a 7900HT Real-time PCR system (Applied Biosystems, CA, USA). Primer sequences were forward 5′ATGAGGCAGTGGCGAGAGTG3′ and reverse 5′GGCTGGGAAACTGTTGGTAC3′. A PCR product of 175 base pairs was obtained. Products spanned at least one intron, so that cDNA products were distinguishable from potential genomic DNA products. *β-Actin* was used as an endogenous control. Reaction conditions were 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min, then a dissociation stage of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. The quantification of mRNA expression levels relative to *β-Actin* was performed by relative quantification or 2DDCT method (Livak and Schmittgen 2001). Differences between low ($N = 73$) and high ($N = 45$) caries experience samples were analyzed using the Student's t test. $p \leq 0.05$ was considered statistically significant. mRNA levels were also compared based on genotypes of the four SNPs found to be associated with the Filipino families. DNA samples from the same subjects were used and genotypes were generated as described earlier.

Results

Association analyses

From the 48 single nucleotide polymorphisms used for fine mapping the 14q11.2 locus, four markers were over transmitted at nominal significance ($0.01 \leq p \leq 0.05$) depending on caries experience level (Table 3).

Philippines

Nominal association between *TRAV4* markers rs1997532, rs1997533, rs8011979, rs7150049 and caries experience was detected under the recessive model. The T allele of rs1997532 ($p = 0.04$), the G allele of rs1997533 ($p = 0.02$), the T allele of rs8011979 ($p = 0.01$), and the G allele of rs7150049 ($p = 0.02$) were associated with low caries experience (Table 3), indicating that these alleles may have a protective effect for caries. No strong linkage disequilibrium was detected between these markers based on D′ and r^2 values (data not shown). Haplotype analysis did not improve the single marker analysis results.

Turkey

In the Turkish cohort, we also detected significant association for all four *TRAV4* markers initially associated with low caries experience in the Filipinos. The T allele of rs1997532 ($p = 0.0005$), the G allele of rs1997533 ($p = 0.01$), the T allele of rs8011979 ($p = 0.001$), and the G allele of rs7150049 ($p = 0.001$) were associated with low caries experience (Table 4), indicating that these alleles may have a protective effect for caries.

Guatemala and Brazil

In the Guatemala cohort, we detected significant association between the T allele of *TRAV4* rs1997532 ($p = 0.03$) and low caries experience by logistic regression adjusted for sex. No associations were found in the Brazilian cohort from Curitiba, although the sample from Rio de Janeiro showed association between low caries experience and *TRAV4* rs7150049 ($p = 0.05$).

Mutation search

We found four variants in *TRAV4* in six individuals: two silent mutations (S26S and L92L), one intronic variant (C1011-66T), and one missense mutation (Y30R). The missense mutation represents a change from an uncharged polar tyrosine to a basic charged polar arginine (Table 5). PolyPhen-2 predicts this change as probably damaging to the TRAV4 protein.

TRAV4 expression analyses in whole saliva

We observed differential expression of *TRAV4* mRNA levels based on individual genotype (Fig. 1). When *TRAV4* expression was analyzed in subjects with low caries experience carrying at least one copy of the less frequent allele of each marker, statistically significant higher expression of *TRAV4* was seen in children younger than 17 years of age (Fig. 2).

Discussion

It is not uncommon to encounter caries-free individuals, who have never gone to the dentist, or paid special attention to diet or adequate oral hygiene. This evidence suggests that there is an underlying biological mechanism that inhibits one or more factors that increase individual's risk to caries. One plausible mechanism is the influence on bacterial colonization, which likely involves the host immune system.

In this study, 2,453 individuals were studied to assess the association and potential role of 14q11.2 in caries. Our previous genome-wide linkage scan has identified the 14q11.2 locus as linked to low caries experience in Filipino families (Vieira et al. 2008a). We fine mapped the 14q11.2 region in 477 subjects from 72 pedigrees with similar cultural and behavioral habits and limited access to dental care living in the Philippines. For replication purposes, a total of 1,446 independent subjects from four different populations were analyzed. Statistically significant associations were found between low caries experience and markers in the *TRAV4* gene. We were able to replicate

Table 3 Summary of the single marker association results for low caries experience in Filipino families

Position (base pair)	Marker	Gene	Region	Associated allele	Allele frequency	*p* value
22197477	rs1997532	*TRAV4*	3′ Downstream	T	0.499	0.04
22197558	rs1997533	*TRAV4*	3′ Downstream	G	0.499	0.02
22201900	rs8011979	*TRAV4*	5′ Upstream	T	0.458	0.01
22201888	rs7150049	*TRAV4*	5′ Upstream	G	0.482	0.02

Table 4 Summary of the single marker association results for caries experience in Turkish children

Marker	Genotypes			Alleles	
rs7150049	AA	AG	GG	A	G
Children with 4 or more lesions	29	51	22	109	95
Caries-free children	38	40	5	116	50
Summary statistics		$p = 0.003$		Model free	$p = 0.001$
					OR = 0.49 (95 % CI 0.32–0.76)
				Recessive model	$p = 0.01$
					OR = 0.47 (95 % CI 0.26–0.95)
rs8011979	CC	CT	TT	C	T
Children with 4 or more lesions	30	51	21	111	93
Caries-free children	39	37	5	115	47
Summary statistics		$p = 0.004$		Model free	$p = 0.001$
					OR = 0.49 (95 % CI 0.31–0.75)
				Recessive model	$p = 0.009$
					OR = 0.45 (95 % CI 0.24–0.82)
rs1997533	CC	CG	GG	C	G
Children with 4 or more lesions	37	40	27	114	94
Caries-free children	15	32	37	62	106
Summary statistics		$p = 0.007$		Model free	$p = 0.0005$
					OR = 0.48(95 % CI 0.31–0.73)
				Recessive model	$p = 0.009$
					OR = 0.44 (95 % CI 0.24–0.82)
rs1997532	CC	CT	TT	C	T
Children with 4 or more lesions	20	59	26	79	111
Caries-free children	8	41	37	57	115
Summary statistics		$p = 0.01$		Model free	$p = 0.01$
					OR = 0.7(95 % CI 0.45–1.07)
				Recessive model	$p = 0.007$
					OR = 0.44 (95 % CI 0.24–0.81)

Table 5 Summary of the results of the *TRAV4* mutation search

Location	Base pair position	Protein position	Type of mutation	Frequency
Exon 1	A77C	S26S	Silent	4/22
Exon 1	T88A	Y30R	Missense	1/22
Exon 1	A276G	L92L	Silent	2/22
Intron 1	C1011-66T	–	–	3/22

these results in populations with higher caries experience from underserved areas. Direct sequencing of the *TRAV4* gene in 22 individuals with low caries experience revealed one missense mutation (Y30R) that changes an uncharged polar to a basic charged polar and this alteration could impact *TRAV4* function. However, we believe that this finding alone does not explain our relatively modest association results, since the frequency of the mutation in the studied Filipino population is 0.19 %. The frequency is higher in the Filipinos who carry two copies of the

haplotype comprising four SNPs that we found associated with low caries experience (4.5 %), but the frequency of Filipinos homozygous for this haplotype is 4.1 %. Additional variants in *TRAV4* may exist to explain our association results, since the *TRAV4* Y30R mutation alone cannot. We checked the location of the four associated SNPs against the aligned consensus sequences of the TRAV genes (Koop et al. 1994; data not shown) and none of the SNPs was located in these sequences, which suggests that these SNPs are not functional. *TRAV4* appears to be expressed in higher levels in younger individuals with lower caries experience and this expression is a reflection of carrying specific SNP variants. Our results suggest that *TRAV4* may have a role in protecting against caries.

Correction for multiple comparisons in single marker analyses was performed using the SNP spectral decomposition (SpD) method (Nyholt 2004). We chose this method over the popular Bonferroni correction to avoid overcorrecting our results and reducing power since the genetic
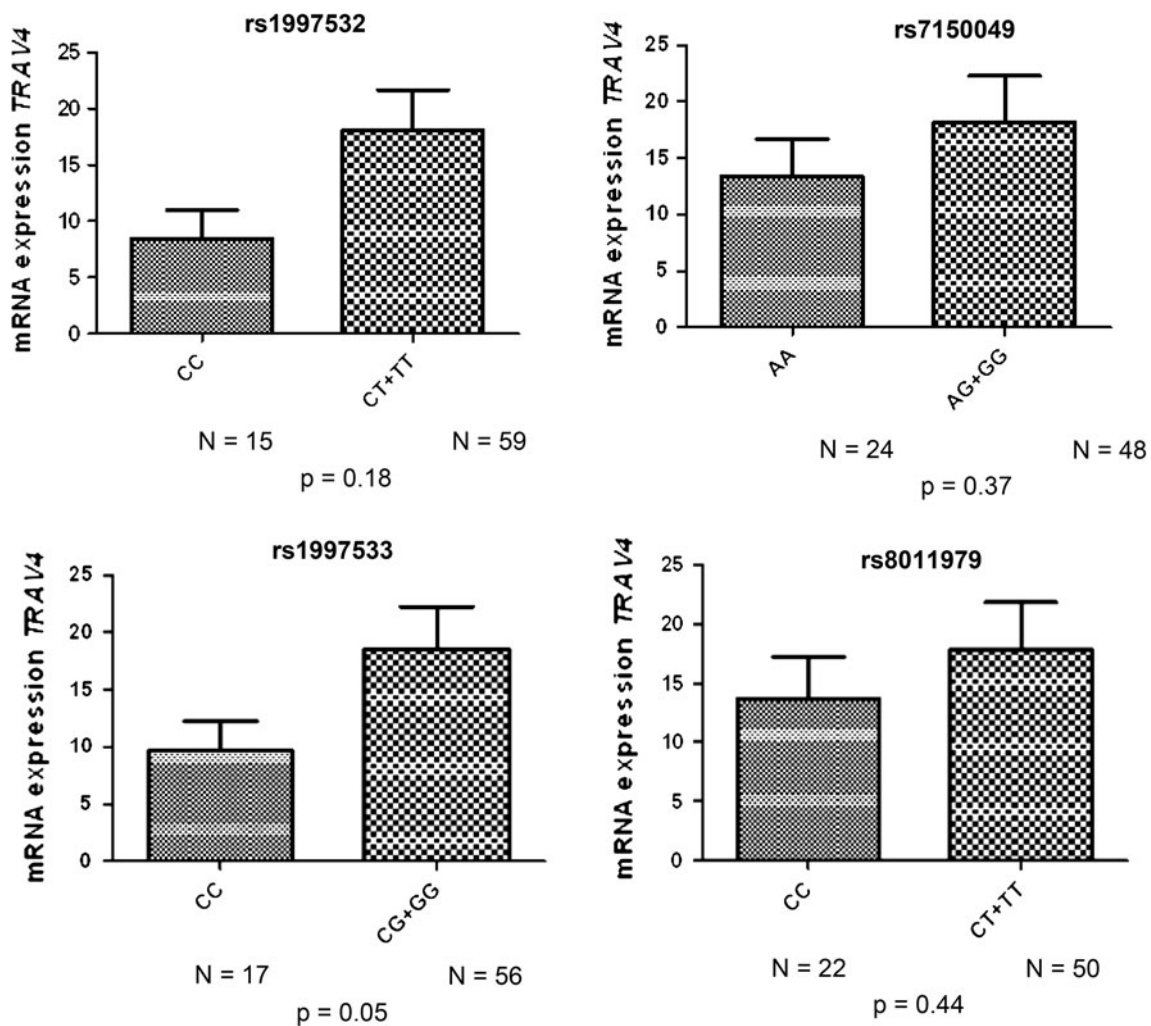
Fig. 1 *TRAV4* mRNA expression levels in whole saliva based on marker genotype and allele frequencies

markers we studied were purposely chosen to avoid complete linkage disequilibrium. While concerned about multiple testing, we still decided to further explore the four nominal significant statistical results in the Filipino families (Table 3), since the original linkage analysis suggested linkage to the locus (Vieira et al. 2008a). If we had ignored those, we would not have found the statistically significant associations in the Turkish and Guatemalans and would not have performed the expression experiments. Our previous work on other craniofacial anomalies has shown that known associations will be missed if we ignore observed trends for association in the data (Vieira et al. 2008b). The lack of association in the Brazilians may be due to their less severe caries experience and better oral health (Table 1). We could still see a trend for association for one of the markers and we may be able to detect an association if sample sizes were larger for this study population. In the future, it may be helpful to compare caries free with low caries and high caries experience individuals, which may

allow us to discriminate possible different influences that younger and older patients may have with regard to genetic factors.

TRAV genes play a role in immune response. This mechanism may relate *TRAV4* genetic variation with caries susceptibility. T-helper and T-killer cells play a critical role in the vertebrate immune response. There are two types of T-cell receptors, αβ and γδ. The α, β, δ, and γ polypeptide chains are made up of variable (antigen recognition) and constant (membrane attachment and activation) regions (Koop et al. 1994). The variable regions are further encoded by multiple variable (V), diversity (D), and joining (J) gene segments for the α and γ chains. These segments undergo somatic DNA rearrangements during lymphocyte differentiation to generate contiguous V/J or V/D/J genes. T-cell receptor alpha variable (TRAV) genes (Fig. 3) have their RNA transcripts spliced and translated to form one complete polypeptide chain. The many functional T-cell receptors and addition and/or replacements of nucleotides
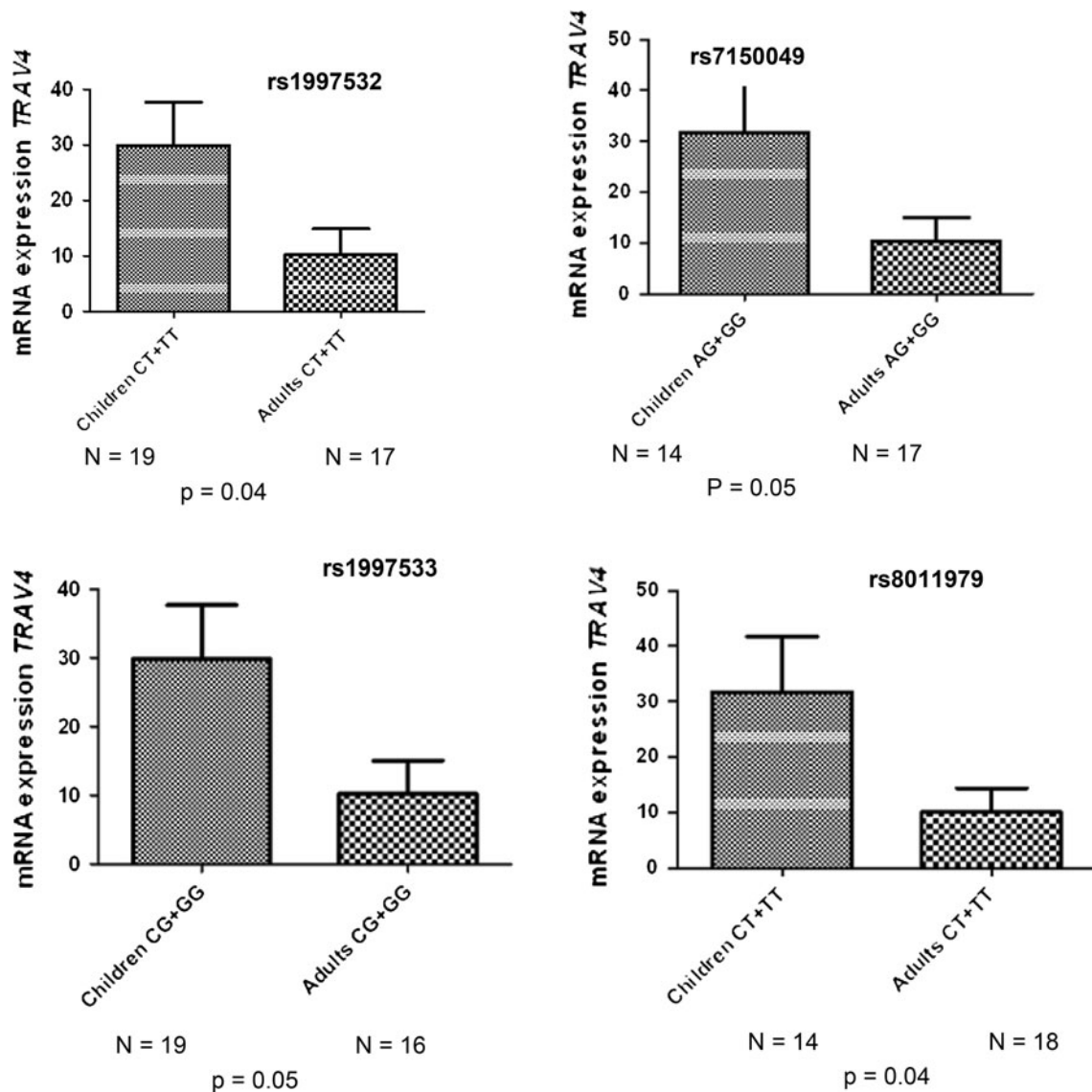
Fig. 2 *TRAV4* mRNA expression levels in whole saliva based on marker genotype (at least one copy of the minor allele), caries experience (low experience), and age (individuals 17 years old or younger versus adults)

at the junctions of these genes enable them to recognize the vast array of potential antigenic peptides (Lafaille et al. 1989; Meier and Lewis 1993). The number of TRAV segments in the human genome is 54, of which 44 are functional (expressed protein), eight are pseudogenes (no expressed protein), and two are not yet characterized. The human TRAV genes are grouped into more than 30 subgroups. The TRAV genes are linked to 61 TCR α-chain J gene segments (TRAJ1 to TRAJ61), of which 11 are functional, but the TRAJ genes cluster in a region that is 3′ to both the TRAV genes and all of the TCR δ-chain segments, including the variable (TRDV), diversity (TRDD), joining (TRDJ), and constant (TRDC) region segments. The TRAJ genes are found 5′ to the single TCR α-chain C segment (Ceri and Mody 2004).

The exact mechanism by which TRAV genes, including *TRAV4*, could influence oral health and caries is not known. Similar to what we see in cancer (He et al. 2012), TRAV genes may be related to the diversity of oral antigens and the likely differential immune responses of the host. Our data showed that *TRAV4* mRNA can be detected in whole saliva and the same genetic variants we found associated with low caries experience showed variable expression depending of the age of individuals that have low caries experience. These are intriguing findings and suggest that caries needs to be considered a lifelong threat that changes overtime. Individuals with primary dentition or with recently erupted permanent dentition are thought to be particularly susceptible to caries attack. Typically the accumulation of disease experience, as reflected by DMFT
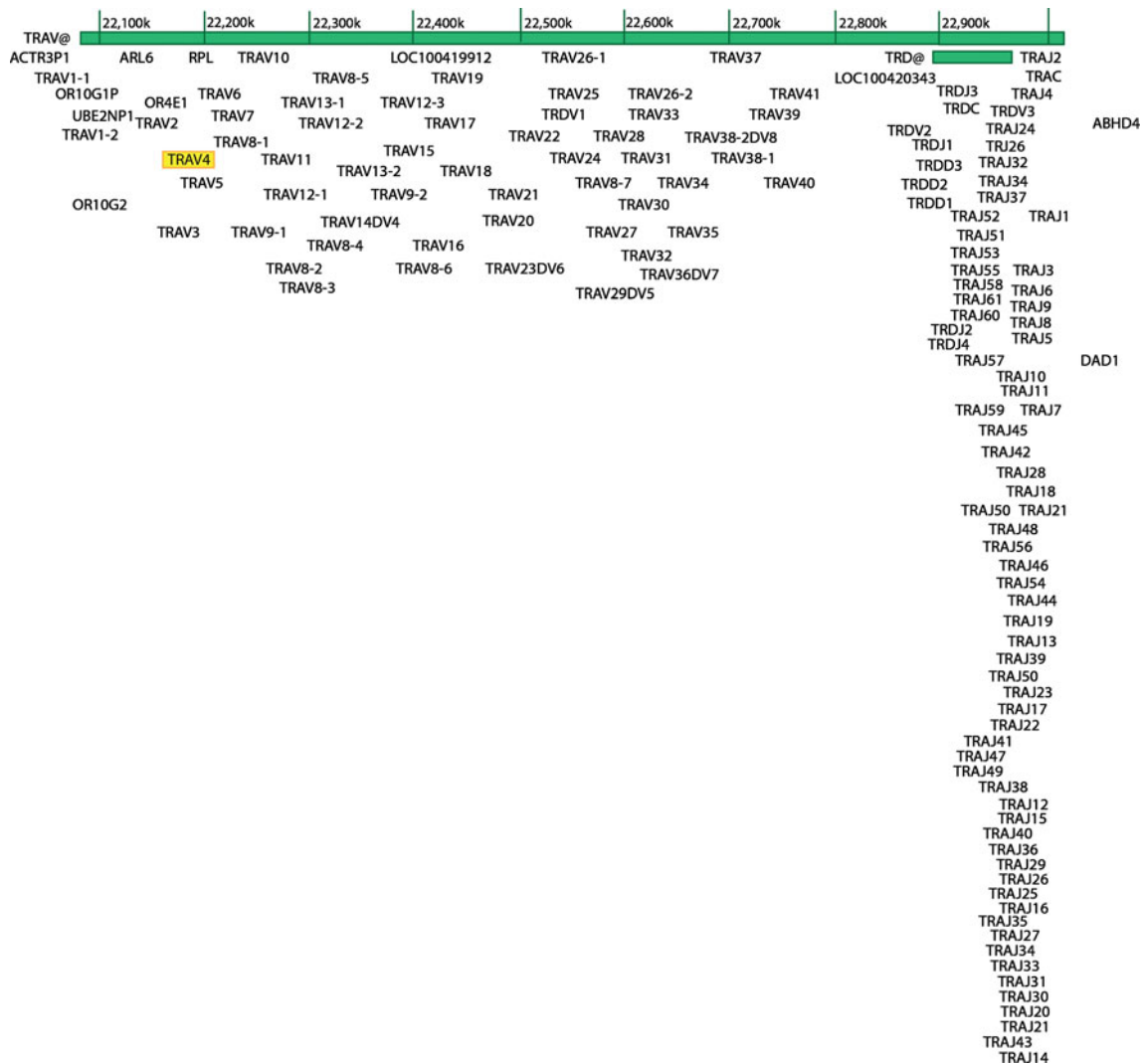
Fig. 3 The complexity of the *TRAV4* locus

or DMFS scores, slows down as one grows older, although many individuals may suffer from repetitive bouts of disease affection. These assumptions are indirectly supported by our results, which showed statistically significant associations between TRAV4 genetic variation and low caries experience in the youngest cohort study (the Turkish). The results continued to support this association in the cohorts from more underserved areas (Filipinos and Guatemalans), although no associations were found in the Brazilian cohort from Curitiba, which in contrast to the other studied groups is the least affected by caries. Since *TRAV4* appears to have higher expression in younger individuals with low caries experience in comparison with adults with low caries experience, we hypothesize that this gene is more likely to play a role in caries at younger ages and not so much later in life. In light of the high complexity of the TRAV locus, it is possible that other genes in the region may contribute to the association results of this study. Future research should focus on examining all the genes in the *TRAV4* locus to search for genetic variants that may be linked to mechanisms that naturally protect individuals against the caries attack.

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