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IRF6 Genetic Variation and Maternal Smoking During Pregnancy in Cleft Lip/Palate

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The goal of the present work was to revisit published data to test if genetic variation in interferon regulatory factor 6 (*IRF6*) is associated with children born with cleft lip with or without cleft palate (CL/P) for cases with positive history of maternal smoking. From the 573 individuals originally studied, this reanalysis focused on 57 who had a positive history of maternal smoking during pregnancy (39 born with CL/P and 18 born without CL/P). Seven *IRF6* markers (rs4844880, rs2235371, rs2013162, ra861019, rs2073487, rs642961, and rs658860) were tested for over-transmission of alleles and an alpha of 0.05 was considered statistically significant. All individuals born with CL/P were homozygous for the wild type allele of rs2235371 in comparison to just two individuals born without clefts ($p = 0.0000001$). For rs861019, individuals born with CL/P were more likely to have the variant allele ($p = 0.006$). A similar trend was seen for rs642961 ($p = 0.09$). The results suggest that statistical evidence of over-representation of *IRF6* alleles in individuals born with CL/P may be unveiled only when maternal smoking during pregnancy is used as the inclusion criterion in the analysis.

Keywords: cleft lip, cleft palate, smoking, interferon regulatory factors, polymorphis, genetic

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INTRODUCTION

Maternal cigarette smoking increases the likelihood of a child being born with cleft lip with or without cleft palate (CL/P) (1), and the population attributable fraction for maternal cigarette smoking was suggested to be between 6.1 and 9.9% (2, 3). A population attributable fraction denotes the fraction of cases that would not have occurred if exposure did not occur, and the association is causal (4). Most cases of CL/P have a multifactorial inheritance, with contributions from more than one gene and possibly from the environment as well (5).

We believe maternal cigarette smoking causing CL/P happens under a multifactorial inheritance framework (5), in which the combination of the presence of certain genetic variation in the individual who smokes leads to clefts. That is why there are many instances of mothers who smoked during pregnancy and who did not have babies with CL/P.

Previous work has tested for potential interactions between maternal cigarette smoking and genetic variants (6, 7), and showed that there are differences depending on if the population is Asian or European in origin (7). Interferon regulatory factor 6 (*IRF6*), a gene which, when mutated, causes Van der Woude and popliteal pterygium syndromes (8), has been included in these analyses because of evidence that it is associated with the occurrence of CL/P in several populations (9).

But in general, the proportion of infants exposed to maternal cigarette smoking during pregnancy included in these analyses are very low (6).

There are examples of previous work testing for an association between *IRF6* and CL/P that was designed excluding any cases where there was a history of maternal cigarette smoking during pregnancy (10). This approach eliminates any chance for testing gene-environment interactions but creates more discreet test groups and possibly decreases heterogeneity. However, data like maternal smoking during pregnancy are prone to suffer from recall bias, even if the question is asked at birth. It is well-known smoking has detrimental consequences to one's health, including a developing fetus. Not wanting to be judged, people will hide this information. To improve homogeneity, one can suggest that individuals tested should have been exposed to the same risk factor (11), therefore here we tested for association only children that had maternal cigarette smoking positive history. This approach was aimed at minimizing recall bias and at the same time it increases homogeneity. The aim of the present work was performing secondary analysis on a cohort of families that had a child born with CL/P and showed an association with *IRF6* to test if associations could still be detected if only individuals with mothers that smoked during pregnancy were considered.

METHODS

We performed secondary analysis of published data (12) and details of the studied cohort have been reported elsewhere. We examined 573 individuals, 158 born with CL/P and 161 non-related individuals with no history of syndromic CL/P. All participants were recruited at the Department of Pedodontics clinics, Istanbul University, Turkey. Individuals born with isolated forms of CL/P were invited to participate in this study between October 2007 and October 2009. We also invited at least one unrelated individual not born with CL/P for each cleft case recruited of the same age and sex during the same period. Individuals born with CL/P had an average age of 10.89 years of age and ranged in age from 3 to 23 years. Their ages were no different from the ages of the unaffected individuals (average age, 10.79 years, ranging from 3 to 23 years; $p = 0.84$). All study participants were examined in a dental office by the same professional (M.K.). Maternal smoking during pregnancy data were originally collected using a standard questionnaire. It was considered any answer that declared smoking was done during any time of pregnancy. These data matched the information available in the dental records. All participants signed an informed consent document prior to entering into this study. Parents consented for their offspring, and age-appropriate consent was obtained from all children older than 7 years of age. This protocol is approved by both the Istanbul University and University of Pittsburgh Institutional Review Boards. Genomic DNA samples were obtained from saliva. Genotyping of seven *IRF6* markers were originally performed by the Taqman method (13), with a 7900 automatic instrument and pre-designed probes (Applied Biosystems, Foster City, CA, USA) and were available for this study. These SNPs (rs4844880, rs2235371, rs2013162, rs861019, rs2073487, rs642961, and rs658860) were originally chosen based on linkage disequilibrium and allele frequency. The corresponding probes are marketed, respectively with the following assay numbers: C__2502442_10, C__15952140_10, C__2500165_10, C__2500178_10, C__2500179_1, C__2238941_20, and C__2500208_10.

Over transmission of alleles was tested using PLINK (14) and at first with an alpha set at 0.05 to detect all possible signals. Then, we applied a most strict criterion to account for multiple testing, lowering alpha to 0.007 (0.05/7).

RESULTS

Thirty-nine children were born with CL/P from mothers who smoked during their pregnancy and they were compared to eighteen children born without CL/P from mothers who smoked during their pregnancy. The rs2235371 variant allele was more likely to be present among children born without clefts whose mothers smoked during pregnancy. On the other hand, children born with clefts whose mothers smoked were more likely to have the variant allele of rs861019 and the same trend could be seen for rs642961 (**Table 1**).

DISCUSSION

The original report of an association between isolated CL/P and *IRF6* (9) particularly highlighted the rs2235371 marker. This marker is a non-synonymous change with a valine being substituted by an isoleucine at position 274. However, since the wild type allele was over-transmitted to the cases born with CL/P, it was concluded that it may not have functional consequences. In the present study, testing for association individuals that were exposed to maternal smoking, the same over-transmission of the wild type allele of rs2235371 was unveiled. It is of interest to mention that the initial analysis of the present study population with all individuals born with CL/P, independent of the mother having smoked during pregnancy or not (12), did show an association between *IRF6* rs4844880 and CL/P. For the V274I marker (rs2235371), the association could only be detected when the analysis was adjusted for maternal medication use and smoking during pregnancy. In the present reanalysis, the association could be clearly demonstrated when maternal cigarette smoking was used as the inclusion criterion. It was remarkable that all individuals born with clefts were homozygous for the wild type allele [the originally associated allele in (9)]. The original report (9) also showed association particularly for Filipinos and Vietnamese. The European groups studied did not show statistical significant associations likely due to the lower information content of the V274I change. The frequency of the *IRF6* V274I change in the public databases (consulted at www.ncbi.nlm.nih.gov/snp/rs 2235371) is reported to be 39% in Koreans and 38% in Vietnamese, whereas 1% in Estonians and Finns, 3% in Swedes, and 0.5% in Qatarians. A study group from South America (derived from the Latin-American Collaborative Study of Congenital Malformations—ECLAMC) also did not show association between clefts and the *IRF6* V274I

TABLE 1 | Genotyping frequency and summary association results.

Single nucleotide polymorphism (SNP)	Alleles	Children born with clefts whose mother smoked during pregnancy ($n = 39$)	Children born without clefts whose mother smoked during pregnancy ($n = 18$)	*p*-Value
rs4844880	AA	20	11	0.46
	AT	15	5	
	TT	2	0	
rs2235371	CC	39	2	0.00000001
	CT	0	13	
	TT	0	2	
rs2013162	AA	17	6	0.23
	AC	18	6	
	CC	3	4	
rs861019	AA	7	6	0.006
	AG	25	3	
	GG	6	7	
rs2073487	CC	16	7	0.65
	CT	18	6	
	TT	4	3	
rs642961	AA	23	16	0.09
	AG	13	2	
	GG	2	0	
rs658860	CC	25	16	0.18
	CT	12	2	
	TT	1	0	

Genotyping totals not matching are due to PCR (polymerase chain reaction) failures.

change originally (9). Only when these data were stratified by mitochondrial DNA haplotypes of Amerindians was that an association was unveiled (15). The V274I change may be therefore protective against clefts and *IRF6* and the higher frequency of clefts in Amerindians and Asians in comparison to Europeans may not be related to *IRF6*, which appears to impact equally all populations (16).

A trend not detected before (12) could be seen for rs642961, with cases born with CL/P having more frequently the variant allele. This marker has better heterozygosity in European groups (17) and was suggested to have functional consequences by disrupting the binding site of transcription factor AP-2alpha.

The role of maternal smoking in clefts may be through hypoxia (5) and we propose here that maternal smoking during pregnancy may also interact with *IRF6*. *IRF6*-impaired mice have several key aspects of tissue repair altered, including increased proliferation in the newly formed epidermis and maintenance of myofibroblasts in the granulation tissue (18). It is known that an important aspect of wound healing is oxygen supply and tension in the wound bed (19). IRF6 is expressed in hyperthophic chondrocytes, osteocytes, and bone matrix of craniofacial tissues. When IRF6 is absent, a reduction in the number of lacunae, embedded osteocytes in matrices, and a reduction in mineralization during bone formation can be seen (20). Similarly, hypoxia suppresses hypertrophic differentiation of chondrocytes (21). During facial development, maternal smoking may lead to fluctuations in oxygen supply that are critical at the time the lip and/or palate are having the connective tissue leveled and the middle-edge epithelial apoptosis. This imbalance in the presence of particular *IRF6* genetic variation may be the cause in a subset of cases of CL/P. *IRF6* rs2235371, rs642961, and/or rs861019 may be useful genomic markers for indicating increased risk of CL/P in pregnancies that are exposed to maternal smoking.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Istanbul University Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AV provided the concept, designed the study, interpreted and analyzed the data, and wrote the first draft of the manuscript. MK collected the data, obtained the genotypes, and critically revised the manuscript. EB compiled the data and critically revised the manuscript. FS and AM helped to design the study and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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