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Associations Between *TGFA/TGFB3/MSX1* Gene Polymorphisms and Congenital Non-Syndromic Hearing Impairment in a Chinese Population

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Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEF **Jihong Du**
CDEF **Jianhua Deng**

Department of Otorhinolaryngology, The First People's Hospital of Kunshan,
Kunshan, Jiangsu, P.R. China

Corresponding Author: Jianhua Deng, e-mail: dengjianhua_djh@126.com
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Background: The aim of this study was to investigate whether the *TGFA/TGFB3/MSX1* gene polymorphisms and haplotypes lead to individual differences between congenital non-syndromic hearing impairment (NSHI) patients and normal people in a Chinese population and to analyze the risk factors for NSHI.

Material/Methods: Between December 2010 and September 2014, 343 congenital NSHI patients were recruited as cases, and 272 healthy subjects were recruited as controls. Denaturing high-performance liquid chromatography (DHPLC) was used to identify genotypes, SHEsis software was used to conduct gene linkage disequilibrium and haplotype analyses, and regression analysis was performed to identify risk factors for congenital NSHI.

Results: The distribution of genotype frequencies and allele frequencies of *TGFA* rs3771494, *TGFB3* rs3917201 and rs2268626, and *MSX1* rs3821949 and rs62636562 were significantly different between the case and the control groups (all $P < 0.05$). *TGFA/TGFB3/MSX1* gene rs3771494, rs1058213, rs3917201, rs2268626, rs3821949, and rs62636562 haplotype analysis showed that haplotype CCGTAC and TTACGT might be protective factors (both $P < 0.001$), while TTGCGC might be a risk factor for the normal population ($P < 0.001$). The other risk factors include paternal smoking, advanced maternal age, maternal sickness history, maternal contact with pesticides or similar drugs, maternal abortion history, maternal medication history, maternal passive smoking history during pregnancy, rs3771494 CT, rs2268626 CC and TC, and rs3821949 GG and AG genotypes were risk factors (all $P < 0.05$), while maternal vitamin supplements during pregnancy, rs3917201 GA, rs62636562 TT and CT genotypes were protective factors for congenital NSHI (all $P < 0.05$).

Conclusions: rs3771494, rs3917201, rs2268626, rs3821949 and rs62636562 might be associated with congenital NSHI.

MeSH Keywords: **Polymorphism, Genetic • Transforming Growth Factor alpha • Transforming Growth Factor beta3**

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Background

As a most common human sensory system defect, hearing impairment affects social, educational, and intelligence development, as well as speaking, expressing, comprehension, and psycho-social growth of individuals in a negative way [1]. Hearing impairment is a great threat to public health worldwide and nearly 10% people in the world have mild to moderate hearing impairment [2]. The incidence of congenital hearing impairment is 0.4–1.1 per 1000 births in the United States and 2 per 1000 births in Turkey [3]. There are 2 kinds of hearing impairment: syndromic hearing impairment with some other specific abnormalities accounting for 30%, and non-syndromic hearing impairment (NSHI) (70%) without additional abnormalities [4]. Both genetic and environmental factors are responsible for hearing impairment; 50% of hearing impairment in developed countries is attributable to genetic factors, and the majority is congenital NSHI [5]. Genetic factors are important for the pathogenesis of hearing impairment. Approximately 60% of congenital hearing impairment in developed countries has genetic causes; this proportion has been increasing due to the improvement of public health conditions and consequent reduction of hearing impairment linked to other causes [6]. Thus it is of great importance to study the genetic factors in congenital NSHI.

Belonging to the family of epidermal growth factors (EGFs), transforming growth factor alpha (TGF- α), encoded by *TGFA* gene, can be activated by binding to cellular signaling-related receptors [7]. TGF- α can function as pleiotropic molecules during development and pathological processes like cancer progression and wound healing [8]. The transforming growth factor-beta 3 (TGF- β 3) is a multifunctional peptide that exhibits diverse biological activities in cell growth and differentiation as well as regulation of extracellular matrix production [9]. *TGFB3* gene encoding the TGF- β 3 protein is a candidate gene of great influence for cleft lip and palate in humans and has a broad spectrum of biological activities [10]. Human *Msh homeobox 1* (*MSX1*) gene, mapped to chromosome 4, is expressed during early tooth development, and mutations of the *MSX1* gene are involved in human isolated tooth agenesis with nail dysplasia or with cleft lip and palate [11]. *MSX1* gene is abundantly expressed in inductive cell-cell interaction sites of the embryo and thus plays an important role during early development [12].

However, no study has been conducted on the associations between the *TGFA/TGFB3/MSX1* gene polymorphisms and congenital NSHI. Thus, we aimed to identify the associations between the polymorphisms of *TGFA/TGFB3/MSX1* genes and congenital NSHI, and to systematically study the risk factors in the development of the congenital NSHI.

Material and Methods

Study participants

Between December 2010 and September 2014, 343 congenital NSHI patients who were diagnosed by a comprehensive hearing examination and received treatment at Department of Otorhinolaryngology, the First People's Hospital of Kunshan, Jiangsu province were enrolled to be the case group [13]. The patients' mothers (n=310) were enrolled as the mother group, and the patients' fathers (n=277) were enrolled as the father group. There were 248 complete core families (father + mother + child). Exclusion criteria were: (1) the patient's hearing impairment was caused by infections in the middle ear, inner ear or intracalvarium, including non-suppurative otitis media, suppurative otitis media, otitis labyrinthica, and encephalitis; (2) the patient's hearing impairment appeared after definite trauma to head, temporal bones, or ears; (3) the patient's hearing impairment was caused by significant vibration-based damage of the inner ear; (4) the patients had syndromic hearing impairment accompanied with other organ or system diseases; (5) patients with conductive hearing impairment caused by unexplained causes; (6) the patient's hearing impairment was due to inner ear vascular disease caused by diabetes, autoimmune diseases, etc.; (7) patients with hearing impairment accompanied with central nervous system diseases and mental disorders. A total of 272 healthy subjects who received physical examinations were enrolled as the control group. The study was in line with medical ethics standards and the standards of the Declaration of Helsinki [14], and approved by the Ethics Committee of the First People's Hospital of Kunshan. Informed consents were received from patients or their families.

Data collection

The baseline data of all the study subjects were obtained through questionnaire surveys including gender, age, paternal smoking (PS), advanced maternal age (AMA), maternal sickness history (MSH), maternal contact with pesticides or similar drugs (MCPSD), maternal abortion history (MAH), maternal medication history (MMH), maternal vitamin supplements (MVS), and maternal passive smoking history (MPSH) during pregnancy.

Audiology diagnosis

Audiological tests included auditory brainstem response (ABR), auditory steady state response (ASSR), acoustic immittance, and pure tone audiometry (PTA) (only for people with older age and able to cooperate). GSI 70 hearing screening instrument (GSI, Illinois, USA) and brainstem auditory-evoked potential testing system (HIS Company, Colorado, USA) were used for measurements. ABR test was carried out in an electrically shielded soundproof room. Electrodes were in a silver-silver

Table 1. Primer sequences of *TGFA/TGFB3/MSX1* gene.

SNP	Primer sequences (5'-3')	Amplification length
TGFA		
rs3771494	F: ACGTTGGAGTAGAGGAGGAG	411 bp
	R: AAGCCAATGTGGTATTTTTA	
rs1058213	F: CGGTCGGTACCTAAGGTCCG	271 bp
	R: ACAGCATTAAATGTAGAAGTC	
TGFB3		
rs3917201	F: CAGTCTCCTTCTTCGC	314 bp
	R: TTAGCAACACTCCTCCT	
rs2268626	F: GTCTGGACCACAGTGGGGACA	332 bp
	R: TGAGCAGGATGCTGTTCAGG	
MSX1		
rs3821949	F: ACCCCCGCTTCAGGCAGAT	392 bp
	R: GTCCAAGGGTCACAACAC	
rs62636562	F: GCCTCTCCTTCCTCTCG	300 bp
	R: AGGGAGCAAAGAGGTGAAA	

SNP – single nucleotide polymorphisms; bp – base pairs; F – forward; R – reverse; *TGFA* – transforming growth factor alpha; *TGFB3* – transforming growth factor-beta 3; *MSX1* – Msh homeobox 1.

chloride disk type, a recording electrode was placed in the middle of the forehead, and a reference electrode was placed to test ear mastoid with nasal root pointing to the ground. A short “click” sound was used to conduct stimuli with alternating waves at a superposition times of 1500 times, a filtering range of 30–1500 Hz, a scan time of 30 ms, and a maximum stimulation intensity of 105 dBnHL. The ASSR test used pure tone signal at a carrier wave frequency of 0.25–8 kHz, a modulation frequency of 70–110 Hz, and a maximum stimulation intensity of 120 dBHL, and the reaction signal was extracted by the computer software. Acoustic immittance testing was performed as follows: a probe of correct size was chosen and placed sealed in the external auditory canal, and the hole was on the right of the eardrum. The tympanogram was drawn at an initial pressure test of +200 daPa, a terminal pressure of –400 daPa, with a pressure change rate of 50 daPa/s and the direction from positive to negative. Suspicious individuals or individuals with abnormal acoustic immittance test results were given temporal bone thin layer CT scan at scan parameters of 120 kV, 180 mA, 1 mm thick, and a total of 20 layers. PTA was conducted in a standard soundproof room. According to GB/T 16403-1996 standard requirements, a rise method and a descent method were used to conduct PTA twice at 0.5–6 kHz, respectively. Two hearing threshold results were compared; the PTA hearing threshold difference at each frequency should be less than or equal to 5 dBHL to exclude pseudo-hearing impairment or exaggerated hearing impairment. Based on the

standards recommended by the World Health Organization (WHO) in 2005 [15], hearing ability was determined depending on whether there existed hearing impairment, and the extent and nature of the hearing impairment (average hearing thresholds of 0.5 kHz, 1 kHz, 2 kHz, and 4 kHz) were used as standards. The degrees of hearing impairment were classified as: normal hearing: lower than or equal to 25 dBHL; mild hearing impairment: 26–40 dBHL; moderate hearing impairment: 41–70 dBHL; severe hearing impairment: 71–90 dBHL; and extremely severe hearing impairment: >90 dBHL. Syndromic hearing impairment referred to patients with hearing impairment as well as lesions in other locations, such as eyes, bone, kidney, and skin, while non-syndromic hearing impairment exhibited only hearing impairment.

DNA extraction and genotyping

Peripheral blood samples of all study subjects were collected, mixed with glucose, citric acid and sodium citrate for anticoagulation, and stored at –80°C. Then, the peripheral blood samples were thawed slowly at 4°C, taken out to room temperature after the blood samples were completely thawed, and were carefully extracted when the temperature of the blood samples and room temperature were balanced. The blood clots and precipitates were carefully shattered in the extraction process to avoid the interference of subsequent extraction operation. DNA was extracted by DNA kit strictly following the instructions

Figure 1. DHPLC profile patterns of *TGFA* genotypes and the sequencing results of PCR products. **(A)** DHPLC profile patterns of rs3771494 and the sequencing results of PCR products; **(A1)** The bi-peak indicated the heterozygous CT genotype and the single peaks indicated CC and TT genotypes; **(A2)** The single peak indicated CC genotype and the bi-peak indicated TT genotype; **(A3)** The mutation sequencing peak pattern of C and T alleles; **(B)** DHPLC profile patterns of rs1058213 and the sequencing results of PCR products.; **(B1)**: The bi-peak indicated the heterozygous CT genotype and the single peaks indicated the CC and TT genotypes; **(B2)**: The single peak indicated TT genotype and the bi-peak indicated CC genotype; **(B3)** The mutation sequencing peak pattern of C and T alleles; DHPLC – denaturing high-performance liquid chromatography; PCR – polymerase chain reaction; *TGFA* – transforming growth factor alpha.

(Qiagen Company, Germany). The DNA extraction steps were: (1) 1.0 ml of cell lysis buffer was extracted and loaded into a 2 ml Eppendorf tube (EP tube), and 400 μ l blood sample was added into the corresponding EP tube. The mixture was mixed upside-down for 20-30 times until no blood clot blocks appeared, and centrifuged at 16 000 rpm for 4 min (2). The supernatant was carefully discarded, and the remaining samples were drained by absorbent papers. We added 20 μ l of protein denaturing buffer solution, and the mixture was shocked and mixed evenly immediately when the buffer solution was added each time until no cell clusters appeared; when the buffer solution was all added, we carefully checked whether there were incomplete cleavage samples (3). The mixture was centrifuged briefly at 65°C water bath for 20 min, during which about 1/3 of the length of the tube or more was kept above the liquid surface water bath to prevent the lid from opening due to heating and water and other impurities from entering into the tube (4). After brief centrifugation, 200 μ l of isopropanol was added, and the mixture was turned up and down several times, until flocculent precipitate was observed (5). The mixture was centrifuged at 16 000 rpm for 4 min, the supernatant was carefully discarded and drained by absorbent papers

for 30 s, and then the white DNA precipitate can be observed at the bottom of the tube (6). We added 200 μ l 75% ethanol without stirring up the pellet precipitate, and the mixture was centrifuged at 16 000 rpm for 5 min (7). The supernatant was carefully discarded and drained by absorbent papers for 20 s, and 110 μ l DNA lysis buffer solution was added. The mixture was kept in the water bath at 65°C for 20 min, and placed in a shaker for 12 h. Finally, the electrophoresis was conducted, and the sample concentration was measured. The remaining samples were stored at –80°C.

Single-nucleotide polymorphisms (SNPs) information of *TGFA*, *TGFB3* and *MSX1* genes were retrieved from National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/Snp/>), with *TGFA* gene rs3771494 and rs1058213, *TGFB3* gene rs3917201 and rs2268626, and *MSX1* gene rs3821949 and rs62636562 selected. Premier 5.0 software was used to design and verify polymerase chain reaction (PCR) amplification primers for each site, which were synthesized by Shanghai Sangon Biological Engineering Technology Co. (Shanghai, China). The primer sequences were shown in Table 1. PCR reactions were conducted in a Mastercycler gradient PCR instrument

Figure 2. DHPLC profile patterns of *TGFB3* genotypes and the sequencing results of PCR products. **(A)** DHPLC profile patterns of rs3917201 and the sequencing results of PCR products; **(A1)** The bi-peak indicated the heterozygous GA genotype and the single peaks indicated GG and AA genotypes; **(A2)** The single peak indicated GG genotype and the bi-peak indicated AA genotype; **(A3)** The mutation sequencing peak pattern of G and A alleles; **(B)** DHPLC profile patterns of rs2268626 and the sequencing results of PCR products; **(B1)** The bi-peak indicated the TC heterozygous genotype and the single peaks indicated the TT and CC genotypes; **(B2)** The single peak indicated TT genotype and the bi-peak indicated CC genotype; **(B3)** The mutation sequencing peak pattern of T and C alleles; DHPLC – denaturing high-performance liquid chromatography; PCR – polymerase chain reaction; *TGFB3* – transforming growth factor-beta 3.

(Eppendorf, Germany). The reaction system consisted of: 100 ng of genomic DNA, 2.5 μ l 10 $\times$ PCR buffer, 1.5 μ l dNTPs (each 2.5 mM), primers (10 pmol/ μ l) and Taq enzyme (2 U/ μ l), and water making the system volume 25 μ l. The PCR protocol was: denaturation at 95°C for 5 min, 35 cycles of 95°C for 30 s, 52°C for 1 min, and 72°C for 30 min, followed by a final extension at 72°C for 5 min. After the completion of the reaction, 5 μ l of the PCR product and 1 μ l of 6 $\times$ electrophoresis Loading Buffer were mixed evenly, and mixture was spotted on the agarose gel (ethidium bromide stained). The electrophoresis was conducted at 120V for 30 min. A gel imager was used to assess the amount and lengths of PCR amplification products. Another 25 μ l of each PCR product was placed in 96-well plates of WAVE system for denaturing high performance liquid chromatography (DHPLC) analysis under partial denaturing conditions. The column temperature was 59.3°C and the mobile phase flow rate was 0.9 ml/min. All genotyping was performed in 2 steps: first, bi-peak DHPLC showed heterozygous genotype and second, PCR products that presented single peak conducted an equal amount mixture with homozygous sample confirmed by sequencing, and then the mixture conducted DHPLC detection. The allelic mutations were confirmed by sequencing. The DHPLC profile patterns and sequencing results of PCR products are shown in Figures 1–3.

Statistical analysis

SPSS 21.0 statistical software (SPSS Inc, Chicago, IL, USA) was used for data analysis. The categorical data were presented as percentage or rate, and the genotype frequencies of the groups were compared using the chi-square test. Odds ratios (OR) and 95% confidence intervals (95%CI) were also calculated. SHEsis software analysis was used to analyze linkage disequilibrium and haplotype. Logistic regression analysis was used to identify the risk factors for the congenital NSH patients. All tests used a 2-sided test, and a $P < 0.05$ indicated statistical significance.

Results

Comparisons of baseline characteristics

As shown in Table 2, there was no significant difference in age or gender between the case group and the control group (both $P > 0.05$). The proportions of PS, AMG, MSH, MCPSD, MAH, MMH, and MPSH during pregnancy were significantly higher in the case group than those in the control group (all $P < 0.05$). The proportion of MVP during pregnancy was significantly lower in the case group ($P < 0.05$).

Figure 3. DHPLC profile patterns of *MSX1* genotypes and the sequencing results of PCR products. **(A)** DHPLC profile patterns of rs3821949 and the sequencing results of PCR products; **(A1)** The bi-peak indicated the heterozygous AG genotype and the single peaks indicated AA and GG genotypes; **(A2)** The single peak indicated GG genotype and the bi-peak indicated AA genotype; **(A3)** The mutation sequencing peak pattern of A and G alleles; **(B)** DHPLC profile patterns of rs62636562 and the sequencing results of PCR products; **(B1)** the bi-peak indicated the heterozygous CT genotype and the single peaks indicated CC and TT genotypes; **(B2)** the single peak indicated CC genotype and the bi-peak indicated TT genotype; **(B3)** the mutation sequencing peak pattern of C and T alleles; DHPLC – denaturing high-performance liquid chromatography; PCR – polymerase chain reaction; *MSX1* – Msh homeobox 1.

TGFA polymorphisms and congenital NSHI

Hardy-Weinberg genetic equilibrium law showed that genotypes of all the sites in the control group were in line with the Hardy-Weinberg genetic equilibrium ($P > 0.05$), thus the controls can be considered to have been selected from a balanced population with group representation. Genotype and allele frequencies of *TGFA* rs3771494 were significantly different in the case group, the father group, and the mother group from those in the case group (all $P < 0.05$). The frequencies of C and T alleles of *TGFA* rs3771494 showed significant differences in the control group and the case group (both $P < 0.05$), and T allele might be a risk for congenital NSHI (OR=1.538, 95%CI=1.199–1.972). There was no significant difference in the *TGFA* rs1058213 genotype or allele frequency between the control group and the case group, or between the father group and the control group (all $P > 0.05$), while the *TGFA* rs1058213 genotype and allele frequencies showed significant differences in the mother group and the control group (both $P < 0.05$) (Table 3).

TGFB3 polymorphisms and congenital NSHI

Compared with the control group, the genotype and allele frequencies of *TGFB3* rs3917201 were significantly different in the

case group (both $P < 0.05$), but showed no significant difference in the father or the mother group (all $P > 0.05$). The frequencies of G and A alleles of rs3917201 were significantly different in the control group and the case group (both $P < 0.05$), and A allele carrier had lower risk of congenital NSHI (OR=0.623, 95%CI=[0.484, 0.802]). The control group and the case group, the control group and the father group, and the control group and the mother group showed significant differences in *TGFB3* rs2268626 genotype and allele frequencies (all $P < 0.05$). T and C alleles of rs2268626 showed significant differences between the control group and the case group (both $P < 0.05$), and C allele might have increased congenital NSHI risk (OR=1.705, 95%CI=[1.322, 2.197]) (Table 4).

MSX1 polymorphisms and congenital NSHI

Compared with the control group, the genotype and allele frequencies of *MSX1* rs3821949 showed significant differences in the case group, the father group, and the mother group (all $P < 0.05$). The frequencies of A and G alleles of rs3821949 were significantly different between the control group and the case group (both $P < 0.05$), and G allele might have increased the risk of congenital NSHI (OR=1.619, 95%CI=[1.260, 2.080]). The genotype and allele frequencies of *MSX1* rs62636562 were

Table 2. Comparisons of baseline characteristics between the case group and the control group.

	Case group	Control group	t/ χ^2	P
Age	10.8±2.4	11.1±2.0	1.553	0.121
Gender				
Male	138	169	2.257	0.133
Female	110	103		
PS				
Yes	159	140	8.484	0.004
No	89	132		
AMA (more than 35 years old)				
Yes	172	146	13.42	0.001
No	76	126		
MSH				
Yes	168	10	192.3	<0.001
No	80	262		
MCPSD				
Yes	128	80	26.64	<0.001
No	120	192		
MAH				
Yes	132	42	83.18	<0.001
No	116	230		
MMH				
Yes	135	5	182.4	<0.001
No	113	267		
MVS				
Yes	15	114	89.45	<0.001
No	233	158		
MPSH				
Yes	176	140	13.88	0.001
No	72	132		

PS – paternal smoking; AMA – advanced maternal age; MSH – maternal sickness history; MCPSD – maternal contact with pesticides or similar drugs; MAH – maternal abortion history; MMH – maternal medication history; MVS – maternal vitamin supplements; MPSH – maternal passive smoking history.

Table 3. Comparisons of genotype and allele frequencies of *TGFA* rs3771494 and rs1058213.

Genotypes	Father	Mother	Case	Control
rs3771494				
CC	56 (22.58)	53 (21.37)	57 (22.98)	84 (30.88)
TT	118 (47.58)	115 (46.37)	125 (46.37)	102 (37.50)
CT	74 (29.84)	80 (32.26)	66 (30.65)	86 (31.62)
χ^2	6.570*	7.532*	9.044*	Ref.
<i>P</i>	0.037*	0.023*	0.011*	–
C	186 (37.50)	186 (37.50)	180 (36.29)	254 (46.69)
T	310 (62.50)	310 (62.50)	316 (63.71)	290 (53.31)
χ^2	8.980*	8.980*	11.54*	Ref.
<i>P</i>	0.003*	0.003*	0.001*	–
C vs. T				
OR	1.460*	1.333*	1.538*	Ref.
95%CI	1.139~1.870*	1.043~1.702*	1.199~1.972*	–
rs1058213				
CC	68 (27.42)	73 (29.44)	73 (29.44)	82 (30.15)
TT	54 (21.77)	30 (12.10)	47 (18.95)	58 (21.32)
CT	126 (50.81)	145 (58.46)	128 (51.61)	132 (48.53)
χ^2	0.482*	8.953*	0.630*	Ref.
<i>P</i>	0.923*	0.011*	0.730*	–
C	262 (52.82)	291 (58.67)	274 (45.97)	296 (54.41)
T	234 (47.18)	205 (41.33)	222 (54.03)	248 (45.59)
χ^2	0.264*	1.913*	0.072*	Ref.
<i>P</i>	0.608*	0.167*	0.788*	–
C vs. T				
OR	1.066*	0.841*	0.967*	Ref.
95%CI	0.835~1.361*	0.658~1.075*	0.757~1.235*	–

Ref. – references; OR – odds ratio; 95%CI – 95% confident interval; * Compared with the control group; *TGFA* – transforming growth factor alpha.

Table 4. Comparisons of genotype and allele frequencies of *TGFB3* rs3917201 and rs2268626.

Genotypes	Father	Mother	Case	Control
rs3917201				
GG	91 (36.69)	98 (39.52)	118 (47.58)	89 (32.72)
AA	42 (16.94)	42 (16.94)	36 (14.52)	60 (22.06)
GA	115 (46.37)	108 (43.54)	94 (37.90)	123 (45.22)
χ^2	2.365*	3.483*	12.860*	Ref.
<i>P</i>	0.306*	0.323*	0.002*	–
G	297 (59.88)	304 (61.29)	330 (66.53)	301 (55.33)
A	199 (40.12)	192 (38.71)	166 (33.47)	243 (44.67)
χ^2	2.196*	3.787*	13.640*	Ref.
<i>P</i>	0.138*	0.151*	0.001*	–
G vs. A				
OR	0.830*	0.782*	0.623*	Ref.
95%CI	0.648~1.060*	0.611~1.002*	0.484~0.802*	–
rs2268626				
TT	59 (23.79)	56 (22.58)	48 (19.35)	70 (11.03)
CC	124 (50.00)	122 (49.19)	139 (56.05)	102 (38.97)
TC	65 (26.21)	70 (28.23)	61 (24.60)	100 (50.00)
χ^2	9.416*	7.544*	18.16*	Ref.
<i>P</i>	0.009*	0.023*	0.001*	–
T	173 (48.79)	182 (47.18)	157 (40.89)	240 (36.03)
C	313 (51.21)	314 (52.82)	339 (52.62)	304 (63.97)
χ^2	7.759*	2.435*	17.080*	Ref.
<i>P</i>	0.005*	0.015*	< 0.001*	–
T vs. C				
OR	1.350*	1.362*	1.705*	Ref.
95%CI	1.053~1.732*	1.062~1.747*	1.322~2.197*	–

Ref. – references; OR – odds ratio; 95%CI – 95% confident interval; * Compared with the control group; *TGFB3* – transforming growth factor-beta 3.

Table 5. Comparisons of genotype and allele frequencies of *MSX1* rs3821949 and rs62636562.

Genotypes	Father	Mother	Case	Control
rs3821949				
AA	47 (18.95)	46 (18.55)	45 (18.15)	77 (28.31)
GG	113 (45.56)	128 (51.61)	123 (49.59)	100 (36.76)
GA	88 (35.48)	74 (29.84)	80 (32.26)	95 (34.93)
χ^2	7.227*	12.780*	10.970*	Ref.
<i>P</i>	0.027*	0.002*	0.004*	–
A	182 (36.69)	166 (33.47)	170 (34.27)	249 (45.77)
G	314 (63.31)	330 (66.53)	326 (65.73)	295 (54.23)
χ^2	8.811*	16.380*	14.260*	Ref.
<i>P</i>	0.003*	< 0.001*	0.001*	–
A vs. G				
OR	1.456*	1.678*	1.619*	Ref.
95%CI	1.136~1.867*	1.305~2.158*	1.260~2.080*	–
rs62636562				
CC	83 (33.47)	90 (36.29)	90 (36.29)	100 (36.76)
TT	35 (14.11)	50 (20.16)	36 (14.52)	87 (31.99)
CT	130 (52.42)	108 (43.55)	122 (49.19)	85 (31.25)
χ^2	32.120*	12.180*	27.240*	Ref.
<i>P</i>	< 0.001*	0.002*	< 0.001*	–
C	296 (59.68)	288 (58.06)	302 (40.89)	259 (61.58)
T	200 (40.32)	208 (41.94)	194 (39.11)	285 (38.42)
χ^2	15.180*	11.370*	18.410*	Ref.
<i>P</i>	< 0.001*	0.001*	< 0.001*	–
C vs. T				
OR	0.614*	0.656*	0.584*	Ref.
95%CI	0.480 ~0.785*	0.514~0.839*	0.456~0.747*	–

Ref. – references; OR – odds ratio; 95%CI – 95% confident interval; * Compared with the control group; *MSX1* – Msh homeobox 1.

Table 6. Linkage disequilibrium analysis of TGFA/TGFB3/MSX1 gene SNPs.

SNP	D' / r ²				
	rs1058213	rs3917201	rs2268626	rs3821949	rs62636562
rs3771494	0.977/0.564	1.000/0.464	0.937/0.758	0.903/0.769	0.994/0.546
rs1058213	–	0.781/0.480	1.000/0.509	1.000/0.556	0.769/0.554
rs3917201	–	–	1.000/0.400	1.000/0.437	0.762/0.487
rs2268626	–	–	–	0.953/0.830	0.894/0.381
rs3821949	–	–	–	–	0.934/0.455

SNP – single nucleotide polymorphisms; D – Linkage disequilibrium coefficient; r – correlation coefficient; TGFA – transforming growth factor alpha; TGFB3 – transforming growth factor-beta 3; MSX1 – Msh homeobox 1.

Table 7. Comparisons of haplotypes of TGFA/TGFB3/MSX1 gene between the case group and the control group.

Haplotype	Case (freq.)	Control (freq.)	χ ²	P	OR (95%CI)
CCGCGC	18.00 (0.036)	10.00 (0.018)	3.040	0.081	1.983 (0.906~4.342)
CCGTAC	151.00 (0.304)	224.96 (0.414)	15.394	<0.001	0.593 (0.456~0.770)
TTACGT	128.06 (0.258)	211.96 (0.390)	22.743	<0.001	0.521 (0.398~0.682)
TTGCGC	34.41 (0.069)	10.00 (0.018)	16.138	<0.001	3.936 (1.924~8.053)

Freq. – frequency; OR – odds ratio; 95%CI – 95% confident interval; TGFA – transforming growth factor alpha; TGFB3 – transforming growth factor-beta 3; MSX1 – Msh homeobox 1.

significantly different between the control group and the case group, the control group and the father group, and the control group and the mother group (all $P<0.05$). C and T alleles of rs62636562 showed different distributions between the control group and the case group (both $P<0.05$), and T allele might have decreased the risk of congenital NSHI (OR=0.584, 95%CI=0.456–0.747) (Table 5).

Haplotype analysis

SHEsis analysis software was used to analyze the linkage disequilibrium and haplotype of the 6 sites of rs3771494, rs1058213, rs3917201, rs2268626, rs3821949, and rs62636562. The results showed that there was strong linkage disequilibrium between these 6 sites (Table 6). Haplotypes with a frequency less than 3% were excluded from the haplotype analysis, thus the CCGCGC haplotype was excluded. Haplotype CCGTAC and TTACGT might be protective (both $P<0.001$), while haplotype TTGCGC might be a risk factor for the normal population ($P<0.001$) (Table 7).

Logistic regression analysis

Whether study participants (case group and control group) had congenital NSHI was regarded as the dependent variable,

whereas PS, AMG, MSH, MCPSD, MAH, MMH, MVS, and MPSH during pregnancy, along with rs3771494, rs3917201, rs2268626, rs3821949, and rs62636562 mutant and non-mutant genotypes were taken as the independent variables of the logistic regression model. Multivariable non-conditional logistic regression analysis was conducted with regression coefficients estimated and OR and 95%CI calculated. OR >1 indicated the corresponding factor to be a risk factor, while OR <1 indicated protective factors. PS, AMG, and MSH, MCPSD, MAH, MMH, and MPSH during pregnancy were risk factors for congenital NSHI (all $P<0.05$; OR >1), while MVS during pregnancy was a protective factor for congenital NSHI ($P<0.05$, OR <1). Besides, rs3771494 CT genotype, rs2268626 CC and TC genotypes, and rs3821949 GG and AG genotypes were risk factors for congenital NSHI (rs3771494 CT: OR=4.089, 95%CI=(1.393,11.998), $P=0.010$; rs2268626 CC: OR=14.598, 95%CI=(4.288,49.697), $P<0.001$; rs2268626 TC: OR=8.100, 95%CI=(2.193,29.925), $P=0.002$; rs3821949 GG: OR=3.413, 95%CI=(1.163,10.018), $P=0.025$; rs3821949 AG: OR=4.346, 95%CI=(1.395,13.543), $P=0.011$), while rs3917201 GA genotype, rs62636562 TT and CT genotypes were protective factors (rs3917201 GA: OR=0.194, 95%CI = (0.076,0.493), $P=0.001$; rs62636562 TT: OR=0.341, 95%CI=(0.132,0.876), $P=0.041$; rs62636562 CT: OR=0.098, 95%CI=(0.031, 0.308), $P<0.001$) (Table 8).

Table 8. Multivariate non-conditional logistic regression analysis for congenital NSHI.

	B	S.E.	Wald	df	Sig.	OR	95% CI	
							Lower	Upper
PS	0.908	0.429	4.471	1	0.034	2.479	1.069	5.750
AMA	0.917	0.424	4.673	1	0.031	2.502	1.089	5.748
MSH	4.914	0.601	66.917	1	< 0.001	136.213	41.963	442.153
MCPSD	1.198	0.523	5.242	1	0.022	3.313	1.188	9.235
MAH	2.474	0.475	27.131	1	< 0.001	11.869	4.679	30.108
MMH	5.288	0.874	36.631	1	< 0.001	198.000	35.721	1097.491
MVS	-2.664	0.617	18.672	1	< 0.001	0.070	0.021	0.233
MPSH	0.966	0.493	3.836	1	0.050	2.627	0.999	6.909
rs3771494			6.655	2	0.036			
rs3771494 (1)	1.103	0.593	3.460	1	0.063	3.012	0.943	9.626
rs3771494 (2)	1.408	0.549	6.574	1	0.010	4.089	1.393	11.998
rs3917201			11.908	2	0.003			
rs3917201 (1)	-0.491	0.562	0.763	1	0.382	0.612	0.203	1.842
rs3917201 (2)	-1.642	0.477	11.836	1	0.001	0.194	0.076	0.493
rs2268626			18.515	2	< 0.001			
rs2268626 (1)	2.681	0.625	18.396	1	< 0.001	14.598	4.288	49.697
rs2268626 (2)	2.092	0.667	9.844	1	0.002	8.100	2.193	29.925
rs3821949			6.937	2	0.031			
rs3821949 (1)	1.228	0.549	4.995	1	0.025	3.413	1.163	10.018
rs3821949 (2)	1.469	0.580	6.419	1	0.011	4.346	1.395	13.543
rs62636562			16.063	2	< 0.001			
rs62636562 (1)	-1.077	0.482	4.995	1	0.025	0.341	0.132	0.876
rs62636562 (2)	-2.326	0.585	15.791	1	< 0.001	0.098	0.031	0.308

NSHI – non-syndromic hearing impairment; B – partial regression coefficient; S.E. – standard error; df – degree of freedom; Sig. – significance; OR – odds ratio; 95%CI – 95% confidence interval; PS – paternal smoking; AMA – advanced maternal age; MSH – maternal sickness history; MCPSD – maternal contact with pesticides or similar drugs; MAH – maternal abortion history; MMH – maternal medication history; MVS – maternal vitamin supplements; MPSH – maternal passive smoking history; rs3771494 (1), the comparison result between CC and TT genotype; rs3771494 (2) the comparison result between CT and CC genotype; rs3917201 (1), the comparison result between AA and GG genotype; rs3917201 (2), the comparison result between GG and GA genotype; rs2268626 (1), the comparison result between CC and TT genotype; rs2268626 (2), the comparison result between TC and CC genotypes; rs3821949 (1), the comparison result between GG and AA genotype; rs3821949 (2), the comparison result between AG and AA genotype; rs62636562 (1), the comparison result between CC and TT genotype; rs62636562 (2), the comparison result between CT and CC genotype.

Discussion

For the first time, our study investigated the associations between the *TGFA/TGFB3/MSX1* gene polymorphisms and congenital NSHI. Our main results showed that the distribution of allele and genotype frequencies of rs3771494 in *TGFA* gene, rs3917201 and rs2268626 in *TGFB3* gene, and rs3821949 and rs62636562 in *MSX1* gene were statistically different between the case and control groups. Haplotype analysis further confirmed that CCGTAC and TTACGT might be protective factors, while haplotype TTGCGC might be a risk factor for the normal population, indicating that these 5 sites might cooperate together to participate in the development of congenital NSHI.

The delivery of TGF- α to the cell surface and cleavage from the cell surface are both critical to TGF- α -mediated EGF receptor activation and are dysregulated in many diseases [7]. The *TGFA/EGFR* autocrine signaling loop sustained the proliferation of papillary thyroid carcinoma cells, and *TGFA* transcripts were significantly higher in papillary thyroid carcinoma metastases and trended upwards with tumor stage, indicating that *TGFA* was associated with advanced tumor stage and poor clinical outcome [16]. EGF/TGF- α is believed to regulate palatal epithelial cell proliferation and differentiation, and the mutations in the *TGFA* gene might contribute to cleft lip or palate [17,18]. Furthermore, possible effects of gene-environment interaction between markers in *TGFA* and maternal smoking, alcohol consumption, and vitamin use were found [19].

TGF- β was revealed to be involved in proliferation, differentiation, motility, adhesion, and death of normal cells and it can act as a promoter of tumors, invasion, and metastasis due to its role in stimulating angiogenesis and epithelial to mesenchymal transformation [20,21]. TGF- β was also found to play a role in embryonic development and wound healing in many molecular pathways, including tissue repair [22]. In addition, SfaN1 polymorphism in *TGFB3* gene may be a good screening marker for patients with non-syndromic cleft lip with or without cleft palate [23]. Mutations in *TGFB3* are also responsible for aortic aneurysmal disease [24].

As transcriptional repressors, *MSX1* regulatory proteins are widely expressed in many organs and are involved in the craniofacial and limb modulation and nervous system development [25]. *MSX1* is especially expressed at epithelial-mesenchymal interaction in odontogenesis during the bud and cap stages of tooth development, and the C terminus of *MSX1* may play an important role in tooth agenesis [26].

Taken together, *TGFA/TGFB3/MSX1* genes are important in embryonic development and in the process of organ development, thus *TGFA/TGFB3/MSX1* gene polymorphisms might be related with congenital NSHI. Logistic regression analysis

further confirmed that rs3771494 CT genotype, rs2268626 CC and TC genotypes, and rs3821949 GG and AG genotypes were risk factors, while rs3917201 GA genotype, rs62636562 TT and CT genotypes were protective factors for congenital NSHI.

Another important result of our logistic regression analysis showed that paternal and maternal unhealthy lifestyle and poor maternal health condition and medication during pregnancy might increase the risk of congenital NSHI, while maternal vitamin supplements during pregnancy may prevent congenital NSHI. Prenatal smoke exposure causes obstetrical complications and might be independently associated with higher pure-tone hearing thresholds and an increase in the incidence of unilateral low-frequency hearing impairment, suggesting that in utero exposure to tobacco smoking may harm the auditory system [27]. Infants that have been prenatally exposed to cocaine are also at increased risk for hearing impairment because cocaine can alter neurosensory transmission through the brainstem and might cause damage to the organ of Corti during critical periods of development, which is important for transducing mechanical sound vibrations into nerve impulses [28]. A case report showed that cisplatin used during the second and third trimesters of pregnancy might lead to fetal ototoxicity and severe hearing loss [29]. It has also been reported that vitamin A is an important factor for normal fetal inner ear development, and vitamin A deficiency exacerbates the severity of ear infections and even hearing loss [30].

Conclusions

Our study showed that *TGFA/TGFB3/MSX1* gene polymorphisms were associated with congenital NSHI. CCGTAC and TTACGT haplotypes might be protective factors, while TTGCGC haplotype might be a risk factor for congenital NSHI. In addition, environmental factors, such as smoking, maternal sickness history, maternal medication history, and maternal vitamin supplements, are also associated with the development of congenital NSHI. Thus, the pathogenesis of congenital NSHI is a combination of both genetic and environmental effects. Further investigations are needed to define the detailed mechanisms of the associations between these 3 gene polymorphisms and congenital NSHI.

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Competing interests

The authors have declared that no competing interests exist.

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