UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

FACULTAD DE MEDICINA

BIOLOGÍA EXPERIMENTAL

ESTUDIO MOLECULAR DE GENES CANDIDATOS EN PACIENTES MEXICANOS CON MICROTIA COMO EXPRESIÓN MÍNIMA DEL ESPECTRO FACIO-AURÍCULO-VERTEBRAL (EFAV)

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

M. EN C. ESTANDÍA ORTEGA BERNARDETTE

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CIUDAD UNIVERSITARIA, CD MX, 2023

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COORDINACIÓN DEL POSGRADO EN CIENCIAS BIOLÓGICAS

FACULTAD DE MEDICINA

OFICIO CPCB/142/2023

ASUNTO: Oficio de Jurado

M. en C. Ivonne Ramírez Wence
Directora General de Administración Escolar, UNAM
P r e s e n t e

Me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día **28 de noviembre de 2022** se aprobó el siguiente jurado para el examen de grado de **DOCTORA EN CIENCIAS** de la estudiante **ESTANDÍA ORTEGA BERNARDETTE** con número de cuenta **507210728** con la tesis titulada **"ESTUDIO MOLECULAR DE GENES CANDIDATOS EN PACIENTES MEXICANOS CON MICROTIA COMO EXPRESIÓN MÍNIMA DEL ESPECTRO FACIO-AURÍCULO-VERTEBRAL (EFAV)",** realizada bajo la dirección de la **DRA. ARIADNA ESTELA GONZÁLEZ DEL ANGEL**, quedando integrado de la siguiente manera:

Presidente:	DRA. MARISOL LÓPEZ LÓPEZ
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Vocal:	DRA. CARMEN ALAEZ VERSON
Vocal:	DRA. JAZMÍN ARTEAGA VÁZQUEZ
Secretario:	DR. JUAN CARLOS ZENTENO RUIZ

Sin otro particular, me es grato enviarle un cordial saludo.

A T E N T A M E N T E
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Ciudad Universitaria, Cd. Mx., a 01 de febrero de 2023

COORDINADOR DEL PROGRAMA

DR. ADOLFO GERARDO NAVARRO SIGÜENZA

POSGRADO EN CIENCIAS BIOLOGICAS
COORDINACIÓN

AGRADECIMIENTOS INSTITUCIONALES

Al Posgrado en Ciencias Biológicas-UNAM y a todos los que lo conforman, por su disposición y ayuda.

Al CONACYT, por el apoyo económico recibido en el doctorado a través de la beca otorgada.

A la Dra. Ariadna Estela González del Ángel, mi mentora a lo largo de toda mi formación como genetista, por haber contribuido de manera muy importante a mi desarrollo académico y personal a lo largo de este posgrado.

A los miembros de mi Comité Tutor, el Dr. Juan Carlos Zenteno Ruiz y el Dr. Samuel Canizales Quinteros, por su valiosa retroalimentación, asesoría e interés durante la realización de este proyecto de investigación.

AGRADECIMIENTOS PERSONALES

A mis hijos por ser mi mayor inspiración.

A Jerry, mi esposo, por motivarme siempre.

A mamá y hermanas por estar siempre a mi lado.

Al Dr. Miguel Angel Alcántara Ortigoza, mi maestro y asesor, por su apoyo e impulso en este camino del conocimiento y también, por ser un gran líder para nuestro grupo de investigación y hacer posible la realización y publicación de este proyecto.

A los colaboradores de este proyecto, Dra. Miriam Erandi Reyna Fabián, Dra. Liliana Fernández Hernández y Dr. José Antonio Velázquez Aragón por su pasión y compromiso.

A mis maestros por todas sus enseñanzas.

ÍNDICE

ESTUDIO MOLECULAR DE GENES CANDIDATOS EN PACIENTES MEXICANOS CON MICROTIA COMO EXPRESIÓN MÍNIMA DEL ESPECTRO FACIO-AURÍCULO-VERTEBRAL (EFAV)

RESUMEN

El diagnóstico clínico del Espectro Facio-Aurículo-Vertebral (EFAV) se establece cuando la microtia está presente en asociación con hipoplasia hemifacial (HH) y/o malformaciones oculares, vertebrales y/o renales. La etiología de los casos esporádicos y de los casos familiares de microtia puede ser desconocida, aunque se ha reportado herencia monogénica, autosómica dominante o recesiva y multifactorial en ambos grupos. Dada la evidencia sobre la contribución genética en el desarrollo de la microtia, las variantes en ciertos genes podrían ser causales o predisponer al desarrollo de esta malformación. Hasta la fecha, no existen estudios genéticos sobre microtia/EFAV en población mexicana, por lo que realizamos secuenciación de siguiente generación de los genes candidatos *HOXA2, TCOF1, SALL1, EYA1* y *TBX1* en 49 pacientes mexicanos no relacionados familiarmente, con diagnóstico de microtia/EFAV. Se identificaron 39 variantes en los genes *TCOF1, SALL1, EYA1 y TBX1, 17* de ellas fueron no sinónimas (15 de sentido erróneo, 1 microdeleción y 1 microduplicación, ambas en marco de lectura) clasificadas como benignas (B, n=12) y probablemente benignas (PB, n=5). La ausencia de variantes patogénicas en los genes analizados excluyó la posibilidad de una etiología monogénica de microtia/EFAV que involucrara a alguno de estos genes en nuestra población de estudio. Sin embargo, se identificó una interacción intergénica estadísticamente significativa (p-valor 0.001) entre la variante p.(Pro1099Arg) en TCOF1 [rs1136103] y la variante p.(Leu858=) en SALL1 [rs1965024]. Esta interacción génica podría sugerir una posible participación de estos dos genes en vías relacionadas con alteraciones craneofaciales como la vía del ácido retinoico. Nuestros hallazgos sugieren que futuros análisis sobre interacción génica podrían contribuir a la comprensión de la etiología de microtia/EFAV y a un asesoramiento genético más certero.

ABSTRACT

Clinical diagnosis of oculo-auriculo-vertebral spectrum (OAVS) is established when microtia is present in association with hemifacial hypoplasia (HH) and/or ocular, vertebral, and/or renal malformations. The etiology of sporadic cases and familial cases of microtia may be unknown, although monogenic, autosomal dominant or recessive, and multifactorial inheritance has been reported in both groups. Thus, it is likely that diverse gene variants could cause or predispose for the development of this malformation. No genetic study on microtia/OAVS has been published for a Mexican population to date. Therefore, we herein performed next-generation sequencing of the candidate genes, *HOXA2*, *TCOF1*, *SALL1*, *EYA1*, and *TBX1*, in 49 Mexican non-related microtia/OAVS patients. Thirty-nine variants were identified in *TCOF1*, *SALL1*, *EYA1*, and *TBX1*; of them, 17 were non-synonymous (15 missense, 1 in-frame microdeletion, and 1 in-frame microduplication) and were classified as benign (B, n=12) or likely benign (LB, n=5). The absence of pathogenic variants in any of those analyzed genes excluded the possibility of a monogenic etiology for microtia/OAVS involving any of these genes in the studied population. However, a statistically significant intergenic interaction (*p*-value 0.001) was identified between variants p.(Pro1099Arg) *TCOF1* [rs1136103] and p.(Leu858=) *SALL1* [rs1965024]. This intergenic interaction may suggest that the products of these two genes participate in pathways related to craniofacial alterations, such as the retinoic acid (RA) pathway. Our findings indicate that future comprehensive gene interaction analyzes could improve the etiologic understanding of microtia/OAVS and genetic counseling.

INTRODUCCIÓN

La microtia (HP:0008551) u “oreja pequeña” es una malformación congénita del pabellón auricular y del conducto auditivo externo que se origina por alteración en las estructuras derivadas del primer y segundo arcos faríngeos durante el período embrionario (Alasti y Van Camp, 2009; Barisic et al., 2014; Beleza-Meireles et al., 2014). Cuando la microtia se asocia con hipoplasia hemifacial (HH, HP:0011332; también denominada como microsomía hemifacial o craneofacial) y malformaciones oculares, vertebrales, cardiacas y/o renales, se sugiere el diagnóstico de Espectro Facio-Aurículo-Vertebral (EFAV, MIM #164210). Sin embargo, no existe un consenso aún sobre los criterios diagnósticos mínimos (Luquetti et al., 2012; Gendron et al., 2016; Bragagnolo et al., 2018; Glaeser, et al., 2020; Guida et al., 2021).

La microtia es considerada como una expresión mínima del espectro clínico de EFAV ya que ambas entidades tienen expresividad variable, involucro asimétrico de estructuras faciales, predominio de lateralidad derecha, mayor frecuencia en el género masculino y se ha observado la presencia de microtia o alteraciones relacionadas como pits o apéndices preauriculares en familias, con penetrancia incompleta (Rollnick y Kaye, 1983; Llano-Rivas et al., 1999; Tasse et al., 2007).

Se han asociado tanto factores genéticos y no genéticos en la etiología de microtia/EFAV. La exposición prenatal a alcohol, retinoides o diabetes materna se consideran factores ambientales causales de esta entidad (Foroud et al., 2012; Gendron et al., 2016; Berenger et al., 2018). Por otro lado, existe evidencia suficiente que apoya la idea de una contribución genética en el desarrollo de la microtia/EFAV, como: a) la identificación de familias con expresión variable y penetrancia incompleta de microtia con un patrón de herencia autosómico dominante (AD), autosómico recesivo (AR) o multifactorial (Llano-Rivas et al., 1999), b) una mayor concordancia entre gemelos monocigóticos contra dicigóticos (38.5% vs 4.5%, respectivamente) (Artunduaga et al., 2009), c) la diferencia en la prevalencia entre los grupos étnicos (Alasti y Van Camp, 2009), como hispanos (1.12/10,000), hispanos nacidos en EE.UU. (0.83/10,000), asiáticos (0.54/10,000) (Shaw et al., 2004), nativos de las Islas del Pacífico (4.61/10,000) y poblaciones de Filipinas (4.77/ 10,000) (Forrester y Merz, 2005), d) la existencia de modelos murinos que desarrollan microtia por variantes patogénicas en genes ortólogos a los genes mutados en pacientes con microtia (por ejemplo, *Hoxa2, Tcof1, Eya1 y Tbx1*) (Alasti et al., 2008; Luquetti et al., 2012; Brown et al., 2013; Piceci et al., 2017; Meddaugh y Zambrano, 2020; Si et al., 2020)

y e) la microtia ha sido observada dentro del espectro clínico de más de 50 síndromes cromosómicos y monogénicos (Luquetti et al., 2012; Bartel-Friedrich, 2015; Gendron et al., 2016).

En la mayoría de los pacientes con esta entidad compleja no se logra reconocer la etiología. Sin embargo, se han observado numerosas alteraciones cromosómicas en pacientes con microtia/EFAV (Chen et al., 2021) y diferentes grupos de investigación han realizado estudios genéticos mediante secuenciación de siguiente generación (*del inglés Next Generation Sequencing, NGS*), pero no se ha identificado un gen causal en la mayoría de los casos (Wang et al., 2017, 2019; Zamariolli et al., 2019; Yang et al., 2021). En la literatura existen estudios de secuenciación del exoma completo (del inglés *Whole Exome Sequencing*, WES) y microarreglos de SNPs (*del inglés Single Nucleotide Polymorphisms-array*) en pacientes con microtia/EFAV en los cuales se identificaron genes candidatos: *MYT1* (MIM*600379) (Lopez et al., 2016; Berenguer et al., 2017, Luquetti et al., 2020), *AMIGO2* (MIM*615690) (Venugopalan et al., 2019), *ZYG11B* (MIM*618673) (Tingaud-Sequeira et al., 2021), *ZIC3* (MIM**300265) (Trimouille *et al.*, 2020), *VWA1* (MIM* 611901) (Wang et al., 2020), *SF3B2* (MIM*605591) (Timberlake et al., 2021) y *EYA3* (MIM*601655) (Tingaud-Sequeira et al., 2021). Debido a la baja frecuencia de las variantes génicas identificadas en estos genes, se confirma la alta heterogeneidad genética de este espectro clínico (Tingaud-Sequeira et al., 2022). Se ha documentado que la exposición prenatal a ácido retinoico puede tener un efecto teratogénico con un cuadro clínico similar a microtia/EFAV (Berenguer et al., 2018), e interesantemente, los genes *MYT1* y *ZYG11B* están involucrados en la vía de señalización del ácido retinoico (Lopez et al., 2016; Tingaud-Sequeira et al., 2020).

Debido a que las poblaciones latinoamericanas, como la mexicana, tienen una de las prevalencias de microtia más altas del mundo (Shaw et al., 2004) y no existen estudios sobre factores genéticos en individuos de este origen étnico con esta entidad, secuenciamos 5 genes candidatos de microtia/EFAV: *HOXA2* (7p15.2), *TCOF1* (5q32), *SALL1* (16q12.1), *EYA1* (8q13.3) y *TBX1* (22q11.21).

HOXA2

HGNC: 5103, Entrez Gene: 3199, Ensembl: ENSG00000105996, MIM*604685, UniProtKB: O43364, NG_012078.1 RefSeqGene, NM_006735.3

HOXA2 (homeobox A2) es un gen homeótico conformado por 2,458 pb, 2 exones y un intrón. Da lugar a dos transcritos por *splicing* alternativo: HOXA2-001 de 1814 pb que es traducido a una proteína de 376 aminoácidos (NM_006735.3), isoforma 1, la cual es la única codificante y HOXA2-002 de 1993 pb el cual retiene el intrón y no se traduce (https://www.genecards.org/). Codifica para un factor de transcripción constituido por 376 aminoácidos y un dominio de unión al DNA (homeodominio). Se expresa en el mesénquima de la cabeza y crestas neurales (2°, 3°, 4° y 6° arcos faríngeos), sistema nervioso central y hueso en el periodo embrionario (Cox et al., 2014). *Hoxa2* controla la formación del pabellón auricular (sólo externo) a través de la vía de señalización de BMP *(bone morphogenetic protein)*, mediante la regulación de la expresión de *Bmp5 (bone morphogenetic protein 5), Bmp4 (bone morphogenetic protein 4)* y *Tsg (twisted gastrulation).* También regula la expresión de *Eya1*, por lo que se considera un regulador transcripcional fundamental en la morfogénesis del pabellón auricular (Minoux et al., 2013). Se ha observado en el embrión murino mutante de *Hoxa2* la presencia de duplicación "en espejo" de las estructuras auriculares, así como pequeños remanentes ectópicos de apéndices preauriculares (Cox et al., 2014). Existen cinco reportes en la literatura de pacientes con microtia con un patrón de herencia autosómico dominante y recesivo causada por una variante patogénica en el gen *HOXA2* (Alasti et al., 2008; Brown et al., 2013; Piceci et al., 2017; Meddaugh y Zambrano, 2020; Si et al., 2020).

TCOF1

HGNC: 11654, Entrez Gene 6949, Ensembl: ENSG00000070814, MIM*606847, UniProtKB: Q13428, NG_011341.1 RefSeqGene, NM_000356.3 isoforma B

El gen *TCOF1* (*treacle ribosome biogenesis factor 1*) está conformado por 42,670 bases y 26 exones. Se han descrito 15 transcritos por *splicing* alternativo, sólo 10 de ellos codifican para una proteína. El transcrito TCOF1-201 codifica para la isoforma 1, la más frecuente (NM_000356.3) (https://www.genecards.org/). La proteína *treacle* o melaza tiene 1488 aminoácidos, participa en la biogénesis ribosomal y juega un papel fundamental en la embriogénesis temprana de las estructuras derivadas de los 1º y 2º arcos faríngeos (Valdez et al., 2004; Dixon et al., 2006). En humanos, variantes patogénicas en el gen *TCOF1* condicionan el síndrome de Treacher Collins Franceschetti (TCS; OMIM#154500), que cursa con malformaciones de oído externo y medio, así como hipoacusia conductiva (Bowman et al.,

2012). El modelo murino del TCS (*Tcof1*$^{+/-}$) muestra una penetrancia y expresividad similar a las observadas en humanos (Dixon et al., 2006). Existe un caso con fenotipo de EFAV sin características de TCS con una variante patogénica en el gen *TCOF1* (Su et al., 2007).

SALL1

HGNC: 10524, Entrez Gene: 6299, Ensembl: ENSG00000103449, MIM*602218, UniProtKB: Q9NSC2, NG_007990.1RefSeqGene, NM_001127892.1 isoforma B

El gen *SALL1* (*spalt like transcription factor* 1), está constituido por 16,342 bases y 3 exones. Se han descrito cinco transcritos, cuatro de ellos codifican para una proteína y la más frecuente es la isoforma SALL-001 de 5146 pb que codifica para una proteína de 1324 aminoácidos (NM_001127892.1) (https://www.genecards.org/). En el embrión murino se expresa en el tejido neural, las yemas que dan lugar a las extremidades, arcos faríngeos, cavidad oral, meso/metanefros, tubérculo genital, orofarínge y cristalino (Buck et al., 2001). La co-expresión de *Six1 y Eya1* incrementa la actividad del promotor de *Sall1*, lo que sugiere una vía compleja de interacción entre estos genes (Chai et al., 2006). Variantes patogénicas en *SALL1* en humanos condicionan el síndrome de Townes-Brocks (TBS, OMIM #107480) caracterizado por pabellones auriculares malformados, apéndices preauriculares e hipoacusia neurosensorial, entre otras malformaciones. En algunos de los pacientes en los que se sobrelapa el cuadro clínico de TBS y EFAV se han identificado variantes génicas patogénicas y benignas en *SALL1* (Kosaki et al., 2007).

EYA1

HGNC: 3519, Entrez Gene: 2138, Ensemble: ENSG00000104313, MIM*601653, UniProtKB: Q99502, NG_011735.2 RefSeqGene, NM_000503

Pertenece a la familia *EYA* (*eyes absent)*, la cual está conformada por 4 genes *EYA* con diversas funciones regulatorias en el desarrollo*. EYA1* está localizado en 8q13.3, lo conforman 16 exones y codifica para un co-activador transcripcional. El gen *EYA1 (eyes absent 1)* tiene 11 transcritos, 5 isoformas y cuatro variantes (EYA1A-EYA1D) como resultado de *splicing* alternativo. EYA1C, una de las dos variantes de la isoforma 1, es el transcrito más largo y frecuente (NM_000503) (https://www.genecards.org/). La proteína EYA1 tiene 592 aminoácidos y funciona como cofactor transcripcional y fosfatasa durante el desarrollo de los mamíferos

(Wong et al., 2013). *EYA1* se expresa en condensaciones cartilaginosas lo que sugiere su participación en la formación o en el crecimiento del pabellón auricular y en estructuras óseas del oído medio (Mallo, 2001). Durante la morfogénesis del oído en mamíferos, Eya1 y Six1 forman un complejo de activación transcripcional para controlar proliferación, supervivencia celular y diferenciación (Wong et al., 2013). En modelos murinos homocigotos para un genotipo nulo en el gen *Eya1*, presentan malformaciones o ausencia de los canales semicirculares y la cóclea, mientras que los heterocigotos cursan con alteraciones moderadas o leves en las estructuras del oído (Zou et al., 2008). Dado que *EYA1* participa en la formación del oído externo y medio en los humanos y es el gen causal de entidades sindromáticas que cursan con alteraciones auriculares (síndromes branquio-oto-renal (BOR), OMIM #113650 y Branquio-ótico (BOS1), OMIM #602588) (Zou et al., 2008), se ha sugerido que pueda estar implicado en microtia aislada.

TBX1

HGNC:11592, Entrez Gene: 6899, Ensemble: ENSG00000184058, MIM*602054, UniProtKB: Q152R5, NG_009229.10 RefSeqGene, NM_005992.1 isoforma B

El gen *TBX1* (*T-box transcription fctor 1*) es un regulador transcripcional, está localizado en el *locus* 22q11.2 y lo conforman 13 exones.

TBX1 (NG_009229.10) genera tres isoformas (*TBX1A, TBX1B* y *TBX1C*) como resultado de *splicing* alternativo. La isoforma B es la más frecuente (NM_005992.1) (https://www.genecards.org/). TBX1 es un miembro de la familia de factores de transcripción dosis-sensibles, los cuales comparten un dominio palindrómico de unión a DNA llamado *T-box* y están involucrados en la regulación del desarrollo embrionario en vertebrados e invertebrados. En embriones murinos, *Tbx1* se expresa en los arcos faríngeos, bolsas faríngeas, vesículas óticas, mesénquima periótico, columna vertebral y yemas dentales. En los humanos, *TBX1* se expresa en 1º y 2º arcos faríngeos, cerebro, corazón y sistema músculo-esquelético (Vitelli et al., 2003). *Tbx1* y *her9 (hayri-relted 9),* se regulan y son inducidos por ácido retinoico e inhibidos por Hedgehog (Hh) (Radosevic et al., 2011).

Los ratones *Tbx1*$^{-/-}$ presentan alteraciones en oído externo, medio e interno (Vitelli et al., 2003). Dado que el gen *TBX1* participa en la formación del oído y su haploinsuficiencia ocurre en el síndrome velo-cardio-facial por la microdeleción 22q11.2 (OMIM #192430), podría ser factible

la existencia de variantes génicas en pacientes con microtia aislada, sin embargo, aún no existe evidencia de ello en la literatura.

Se seleccionaron específicamente los 5 genes mencionados porque variantes en estos puede contribuir a la presencia de microtia con base en la siguiente evidencia: a) existen modelos murinos *knock-out* para genes ortólogos que generan microtia (Gendron et al., 2016), b) se han identificado variantes patogénicas en *HOXA2* y *SALL1* en familias con microtia de herencia AD o AR (Kosaki et al., 2007; Alasti et al., 2008; Brown et al., 2013; Piceci et al., 2017; Meddaugh y Zambrano, 2020; Si et al., 2020), c) variantes patogénicas en estos genes candidatos son causales de síndromes monogénicos que presentan microtia (Luquetti et al., 2012; Gendron et al., 2016), d) estos genes se expresan durante la embriogénesis auricular (Vitelli et al., 2003; Dixon et al., 2006; Cox et al., 2014) y e) se ha sugerido que los productos de estos genes están involucrados en algunas vías metabólicas como es el caso de *HOXA2* que participa en la vía del ácido retinoico (Lopez et al., 2016).

Además, como la interacción gen-gen juega un papel importante en la etiología de las enfermedades complejas y no existen reportes en la literatura que hayan analizado las interacciones génicas potenciales en pacientes con microtia/EFAV, evaluamos la epistasis entre los genes estudiados en nuestra población mediante el método de reducción de dimensionalidad multifactorial (del inglés *Multifactorial Dimensionality Reduction*, MDR) (Moore et al., 2006, Moore y Williams, 2009; Hsieh et al., 2011).

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The Enigmatic Etiology of Oculo-Auriculo-Vertebral Spectrum (OAVS): An Exploratory Gene Variant Interaction Approach in Candidate Genes

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Abstract: The clinical diagnosis of oculo-auriculo-vertebral spectrum (OAVS) is established when microtia is present in association with hemifacial hypoplasia (HH) and/or ocular, vertebral, and/or renal malformations. Genetic and non-genetic factors have been associated with microtia/OAVS. Although the etiology remains unknown in most patients, some cases may have an autosomal dominant, autosomal recessive, or multifactorial inheritance. Among the possible genetic factors, gene–gene interactions may play important roles in the etiology of complex diseases, but the literature lacks related reports in OAVS patients. Therefore, we performed a gene–variant interaction analysis within five microtia/OAVS candidate genes (*HOXA2*, *TCOF1*, *SALL1*, *EYA1* and *TBX1*) in 49 unrelated OAVS Mexican patients (25 familial and 24 sporadic cases). A statistically significant intergenic interaction (p-value < 0.001) was identified between variants p.(Pro1099Arg) *TCOF1* (rs1136103) and p.(Leu858=) *SALL1* (rs1965024). This intergenic interaction may suggest that the products of these genes could participate in pathways related to craniofacial alterations, such as the retinoic acid (RA) pathway. The absence of clearly pathogenic variants in any of the analyzed genes does not support a monogenic etiology for microtia/OAVS involving these genes in our patients. Our findings could suggest that in addition to high-throughput genomic approaches, future gene–gene interaction analyses could contribute to improving our understanding of the etiology of microtia/OAVS.

Keywords: gene–gene interactions; microtia; Mexican population; Multifactor Dimensionality Reduction (MDR); next-generation sequencing; OAVS

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Citation: Estandia-Ortega, B.; Reyna-Fabián, M.E.; Velázquez-Aragón, J.A.; González-del Angel, A.; Fernández-Hernández, L.; Alcántara-Ortigoza, M.A. The Enigmatic Etiology of Oculo-Auriculo-Vertebral Spectrum (OAVS): An Exploratory Gene Variant Interaction Approach in Candidate Genes. *Life* **2022**, *12*, 1723. https://doi.org/10.3390/life12111723

Academic Editors: Antonio Diez-Juan and Alexey V. Polonikov

Received: 26 September 2022
Accepted: 24 October 2022
Published: 28 October 2022

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1. Introduction

Microtia (HP:0008551) is a congenital anomaly of heterogeneous etiology that arises from alterations in structures derived from the first and second pharyngeal arches during the embryonic period [1–3]. When microtia is associated with hemifacial hypoplasia (HH, HP:0011332) and ocular, vertebral, cardiac, and/or renal malformations, the diagnosis of oculo-auriculo-vertebral spectrum (OAVS, MIM #164210) is suggested. However, there is no agreement on the minimum diagnostic criteria for OAVS [4–8].

Microtia is a minimal expression of the OAVS clinical spectrum, since both entities have variable phenotypic expression, asymmetric involvement of facial structures, right-side predominance, and male predilection. Moreover, familial occurrence with incomplete penetrance may be seen for microtia and/or related anomalies, such as preauricular tags and pits [9–11].

Genetic factors and non-genetic factors have been associated with the etiology of microtia/OAVS. Prenatal exposure to alcohol, retinoids, or maternal diabetes are considered environmental causal factors of this entity [5,12,13]. The genetic contribution to the development of microtia/OAVS is supported by diverse lines of evidence, such as: (a) the identification of families with variable expression and incomplete penetrance segregating as an autosomal dominant (AD), autosomal recessive (AR), or multifactorial trait [10]; (b) the greater concordance between monozygotic versus dizygotic twins (38.5% vs. 4.5%, respectively) [14]; (c) the differences in its prevalence across ethnicities [1], such as in Hispanic (1.12/10,000), U.S.-born Hispanic (0.83/10,000), Asian (0.54/10,000) [15], Pacific Island native (4.61/10,000), and Philippine (4.77/10,000) [16] populations; (d) the finding that murine models develop microtia due to pathogenic variants (PV) in genes orthologous to those identified as mutated in some microtia patients (i.e., *Hoxa2*, *Tcof1*, *Eya1*, and *Tbx1*) [4,17–21]; and (e) the observation of microtia within the clinical spectrum of more than 50 chromosomal and monogenic syndromes [4,5,22]. Despite these findings, however, the etiology underlying microtia/OAVS in most patients remains unknown.

Recently, several chromosomal abnormalities associated with microtia/OAVS have been described [23]. Genetic studies using next-generation sequencing (NGS) have been performed in patients with this entity [24–27], but only in a few cases candidate genes have been identified. These include *MYT1* (MIM*600379) [23–25], *AMIGO2* (MIM*615690) [26], *ZYG11B* (MIM*618673) [27], *ZIC3* (MIM*300265) [28], *VWA1* (MIM*611901) [29], *SF3B2* (MIM*605591) [30], and *EYA3* (MIM*601655) [31]. Variants in these genes were identified at a low frequency, supporting the notion that this clinical spectrum has high genetic heterogeneity [32]. Interestingly, the products encoded by *MYT1* and *ZYG11B* are involved in the retinoic acid (RA) signaling pathway [23,27], and prenatal exposure to RA was reported to have a teratogenic effect with a clinical presentation similar to that of microtia/OAVS [13].

Gene–gene interactions have been shown to play important roles in the etiologies of complex diseases [33]. However, the literature lacks any report addressing potential gene interactions in patients with microtia/OAVS [34,35]. Given that Mexican and Hispanic populations show the highest prevalence of microtia worldwide [15], we performed an epistasis analysis [36] in a group of Mexican patients by the Multifactorial Dimensionality Reduction (MDR) method in five microtia/OAVS candidate genes. We assessed *HOXA2* (MIM*604685, 7p15.2), *TCOF1* (MIM*606847, 5q32), *SALL1* (MIM*602218, 16q12.1), *EYA1* (MIM*601653, 8q13.3), and *TBX1* (MIM*602054, 22q11.21), whose selection was supported by various lines of evidence (see Table 1).

Table 1. Evidence supporting the selection of the studied candidate microtia/OAVS genes.

Gene	Murine Knockout Models for Orthologous Genes with Microtia [5]	PV Have Been Identified in Familial Cases (AD or AR Inheritance) [17–21,37]	PV at These Loci Are Causative of Monogenic Syndromes That Present Microtia [4,5]	Expression during Ear Embryogenesis [38–40]	Participation in Retinoic Acid Pathway [23]
EYA1	+	NR	+	+	NR
HOXA2	+	+	+	+	+
SALL1	+	+	+	+	NR
TBX1	+	NR	+	+	NR
TCOF1	+	NR	+	+	NR

Abbreviations: + reported, PV pathogenic variants, AD autosomal dominant, AR autosomal recessive, NR not reported.

2. Materials and Methods

2.1. Study Population

Forty-nine unrelated Mexican patients (31 males, 18 females; ages 0 to 18 years old) who were evaluated between 2015 and 2019 at the National Institute of Pediatrics (Mexico) and given a clinical diagnosis of microtia/OAVS (microtia or anotia with or without HH, structural alterations in the spine and/or kidneys) were included. Patients who met the clinical criteria for microtia/OAVS but had congenital malformations distinct from those involving the spine and/or kidneys and/or an alteration in somatic growth (low or high height) or intellectual disability were excluded. Those with reported prenatal exposure to specific teratogens associated with microtia/OAVS (e.g., alcohol, retinoids, or maternal diabetes) were also excluded. Each patient underwent a systematic physical examination by clinical geneticists and a detailed family history was obtained (25 familial cases and 24 sporadic cases). Imaging studies (computed tomography of the inner ear, orthopantomography, complete spinal radiography, and renal ultrasound) were performed on the patients and their parents. Detailed clinical information related to all included patients was previously published [41]. This research protocol was approved and registered by the Ethics, Research and Biosafety Committees of the National Institute of Pediatrics (Mexico City, Mexico, registry number 004/2017).

2.2. NGS of the Five Microtia/OAVS Candidate Genes

Genomic DNA isolated from peripheral blood or buccal cell samples of all patients was analyzed with a targeted five-gene NGS panel consisting of *HOXA2* (NM_006735.3), *TCOF1* (NM_001135243.1), *SALL1* (NM_002968.2), *EYA1* (NM_000503.5), and *TBX1* (NM_080647.1). The studied sequences covered the promoter region, all coding exons, and the intron–exon boundaries (200 bp). NGS libraries were prepared with an IDT xGen lockdown probe customized panel kit according to the manufacturer's protocol. Libraries were sequenced on an Illumina MiSeq 2 × 150 platform (San Diego, CA, USA) through the Admera Health Company (South Plainfield, NJ, USA). Our in-house bioinformatics analysis pipeline included a quality evaluation and trimming of low-quality raw reads, alignment against the GRCh38 human reference sequence, and calling of single nucleotide variants (SNVs) and small insertion–deletion variants. The GATK Toolkit (version 4.2.6.1) [42] and the Alamut Batch (version 1.4), Focus (version 1.0), and Visual (version 2.7.2, Interactive Biosoftware, Rouen, France) software packages were used for variant annotation and filtering. All clinically relevant variants were confirmed by Sanger automated sequencing in the index cases and their available relatives. The identified gene variants were classified according to the criteria of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) [43].

2.3. Statistical and Gene Interaction Analyses

The allele frequencies of the variants identified in the five studied genes were obtained using the allelic counting method. We first determined if the variant was in Hardy–Weinberg equilibrium using the two-tailed Fisher's exact test, which assessed whether the distribution of genotypes was as expected in our population. When information was available, an association analysis was performed to compare the genotype frequencies in our study population with those reported in Mexican individuals from Los Angeles (1000 Genomes Project, phase 3; https://www.ncbi.nlm.nih.gov/genome/gdv/, accessed on 17 May 2022) using Armitage's trend test as applied by the deFinetti online software (https://ihg.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl, accessed on 17 May 2022).

To identify possible intergenic and/or intragenic interactions between the variants in the analyzed loci, we used the Multifactor Dimensionality Reduction (MDR) ver. 3.0.2 software (Vanderbilt University Medical School, Nashville, TN, USA) [36].

Additionally, the STRING software (http://string-db.org, accessed on 20 September 2022) was used to look for interacting partners of known craniofacial and RA-associated proteins, including those encoded by the five studied genes. The selected candidate

interacting protein partners were: AMIGO2, BAPX1, CHD7, CYP26B1, EYA1, EYA3, FGF3, FGF10, FGFR2, FRAS1, FREM2, GDF3, GRIP1, GSC, HDAC1, HDAC2, HOXA2, HOXA13, HOXD13, MLL2, MYT1, PHF5A, PITX2, PLCB4, RARB, SALL1, SF3B2, SIN3B, SIX1, SIX2, TBX1, TCOF1, TFAP2A, VWA1, ZIC3, and ZYG11B.

3. Results

The median read depth for the five-gene panel was 639X (range 86X–1940X), and the coverage was 99.9%. Thirty-nine gene coding variants were identified in *TCOF1*, *SALL1*, *EYA1*, and *TBX1*; of them, 17 were predicted to alter the amino acid sequence (non-synonymous). These included 15 missense mutations, 1 in-frame microdeletion, and 1 in-frame microduplication that were classified as benign (B, n = 12) and likely benign (LB, n = 5) according to ACMG/AMP criteria (see Table 2). Furthermore, 22 synonymous or non-coding variants were identified in our patients (see Table 3). No variant was identified in the coding region of the *HOXA2* gene.

Table 2. Comparison of allelic frequencies (AFs) for the 17 non-synonymous gene variants of *TCOF1*, *SALL1*, *TBX1*, and *EYA1* identified in our population versus those reported in the reference group (Mexicans from LA, 1000 Genomes Project Database).

ACMG/AMP Classification: [Criteria] *	Cases (n=)	cDNA	Protein	Reference SNP	Cases in Our Study				Reference Group				*p*-Value
					HoRA	Hetero	HoMA	AF	HoRA	Hetero	HoMA	AF	
				TCOF1 NM_000356.3									
LB: [BS1, BS2, BP1, BP4, BP6]	1	c.503C>T	p.(Thr168Met)	rs181203524	48	1	0	0.01	63	0	0	0	0.99
B: [BA1, BP1, BP4]	4	c.1762G>C	p.(Ala588Pro)	rs2071240	45	4	0	0.96	65	1	1	0.98	0.47
B: [BA1, BP1, BP4, BP6]	6	c.2429T>C	p.(Val810Ala)	rs7713638	43	6	0	0.94	57	9	1	0.92	0.55
B: [BA1, BP1, BP4, BP6]	4	c.3296C>G	p.(Pro1099Arg)	rs1136103	45	3	1	0.95	51	16	0	0.88	0.07
B: [BA1, BP1, BP4, BP6]	21	c.3938C>T	p.(Ala1313Val)	rs15251	28	20	1	0.78	38	22	7	0.73	0.45
B: [BP6, BS1, BS2, BP1, BP4]	1	c.4061G>C	p.(Gly1354Ala)	rs45491898	48	1	0	0.99	63	0	0	1	0.25
				SALL1 NM_002968.2									
B: [BS1, BS2, PP3]	1	c.400_411dup	p.(Lys134_Ser137dup)	rs750817837	48	1	0	0.01	64	0	0	0	0.99
B: [BA1, BP1, BP4, BP6]	4	c.475A>G	p.(Ser159Gly)	rs13336129	45	4	0	0.96	56	9	2	0.9	0.13
B: [BS1, BS2, PP3]	5	c.475_477del	p.(Ser159del)	rs113614842	44	5	0	0.95	65	0	0	1	**0.008**
LB: [BS1, BS2, BP1, BP4]	1	c.2804C>T	p.(Thr935Met)	rs755926434	48	1	0	0.01	63	0	0	0	0.99
B: [BS1, BS2, BP1, BP6, PP3]	1	c.3794G>A	p.Gly1265Glu)	rs149302006	48	1	0	0.01	63	1	0	0.007	0.84
B: [BA1]	49	c.3823G>A	p.(Val1275Ile)	rs4614723	0	0	49	0	0	0	67	0	NR
B: [BS1, BS2, BP1, BP4, BP6]	1	c.3872A>G	p.(Asn1291Ser)	rs74499562	48	1	0	0.01	62	2	0	0.015	0.72
				TBX1 NM_080647.1									
LB: [BP1, BP4, PP3]	1	c.68C>T	p.(Ala23Val)	rs1415687525	48	1	0	0.01	64	0	0	0	0.99
B: [BA1, BP1, BP4, BP6]	31	c.1189A>C	p.(Asn397His)	rs72646967	18	22	9	0.59	29	28	10	0.64	0.45
LB: [PM2, BS2, BP1]	1	c.1397C>T	p.(Ala466Val)	rs753613632	48	1	0	0.01	64	0	0	0	0.99
				EYA1 NM_000503.5									
LB: [BS2, BP6, PP2]	1	c.107C>T	p.(Thr36Ile)	rs727503048	48	1	0	0.01	64	0	0	0	0.99

Numbers in bold indicate *p*-value < 0.05. * Classified according to ACMG/AMP criteria [43]. Bracketed data indicates all criteria met by the variant. Abbreviations: AF: allelic frequency, HoRA: homozygous for the reference allele, Hetero: heterozygous, HoMA: homozygous for the minor allele, (n=): number of cases, NR: not previously reported.

Table 3. Detailed information on the synonymous variants observed in our population.

Cases (n=)	cDNA	Protein	Reference SNP	Cases in Our Study				Reference Group				*p*-Value
				HoRA	Hetero	HoMA	AF	HoRA	Hetero	HoMA	AF	
			TCOF1 NM_001135243.1									
1	c.630A>G	p.(Thr210=)	rs765654624	48	1	0	0.99	NR	NR	NR	NR	NR
5	c.1578C>T	p.(Pro526=)	rs2071238	44	5	0	0.95	54	9	1	0.91	0.32
1	c.1761G>T	p.(Gly587=)	rs7701163	48	1	0	0.99	56	8	0	0.94	**0.04**
5	c.1842A>G	p.(Ser614=)	rs2071239	44	5	0	0.95	54	9	1	0.91	0.32
			SALL1 NM_002968.2									
2	c.390G>A	p.(Pro130=)	rs75156807	47	2	0	0.98	62	2	0	0.98	0.78
1	c.570A>G	p.(Val190=)	rs1317946303	48	1	0	0.99	NR	NR	NR	NR	NR
1	c.1674G>A	p.(Pro558=)	rs747355231	48	1	0	0.99	NR	NR	NR	NR	NR
3	c.2178G>A	p.(Arg726=)	rs144019351	46	3	0	0.97	62	2	0	0.98	0.44
2	c.2343G>A	p.(Leu781=)	rs60270998	47	2	0	0.98	64	0	0	1	0.1
41	c.2574C>T	p.(Leu858=)	rs1965024	8	18	23	0.35	7	33	24	0.37	0.75
7	c.3456C>T	p.(His1152=)	rs11645288	42	5	2	0.91	50	12	2	0.88	0.47

Table 3. *Cont.*

Cases (n=)	cDNA	Protein	Reference SNP	Cases in Our Study				Reference Group				*p*-Value
				HoRA	Hetero	HoMA	AF	HoRA	Hetero	HoMA	AF	
				TBX1 NM_080647.1								
1	c.75G>T	p.(Gly25=)	rs72646952	48	1	0	0.99	62	2	0	0.98	0.72
1	c.135G>A	p.(Pro45=)	NR	48	1	0	0.99	NR	NR	NR	NR	NR
2	c.297G>A	p.(Ala99=)	rs72646953	47	2	0	0.98	62	2	0	0.98	0.78
31	c.420T>C	p.(Phe140=)	rs41298814	18	22	9	0.59	29	27	8	0.66	0.27
26	c.664C>T	p.(Leu222=)	rs2301558	23	24	2	0.71	37	22	5	0.75	0.53
31	c.933A>G	p.(Ala311=)	rs41298840	18	22	9	0.59	29	27	8	0.66	0.27
4	c.1059A>G	p.(Ala353=)	rs13054377	45	4	0	0.96	59	4	1	0.95	0.83
				EYA1 NM_000503.5								
1	c.585A>T	p.(Ile195=)	rs780672889	48	1	0	0.99	NR	NR	NR	NR	NR
22	c.813A>G	p.(Thr271=)	rs1445398	27	19	3	0.74	34	28	2	0.75	0.92
33	c.1278C>T	p.(Gly426=)	rs4738118	16	24	9	0.57	31	26	7	0.69	0.078
40	c.1755T>C	p.(His585=)	rs10103397	9	28	12	0.47	15	35	14	0.51	0.54

Reference group: Mexicans from LA, 1000 Genomes Project Database. Numbers in bold indicate p-value < 0.05. Abbreviations: AF: allelic frequency, HoRA: homozygous for the reference allele, Hetero: heterozygous, HoMA: homozygous for the minor allele, (n=): number of cases, NR: not previously reported.

3.1. Association Analysis

When we compared the allelic frequencies (AFs) of benign (B) or likely benign (LB) variants observed in our population with those reported in Mexican individuals from Los Angeles (1000 Genomes Project), a statistically significant difference ($p < 0.05$) was observed only for the p.(Ser159del) *SALL1* variant, which was found specifically in microtia/OAVS patients (n = 5, 3 familial and 2 sporadic cases). Since the missense p.(Val1275Ile) *SALL1* variant was present in a homozygous state in all cases and all individuals of the reference group, we did not perform an association analysis for this variant (see Table 2). Comparing the AFs of synonymous variants among our patients with those of the reference group, a statistically significant difference ($p < 0.05$) was observed only for the p.(Gly587=) *TCOF1* variant (see Table 3).

3.2. MDR Interaction Analysis

We analyzed possible interactions among the 17 genotypes of the variants found in *TCOF1*, *SALL1*, *EYA1*, and *TBX1* for our 49 microtia/OAVS cases and those present in the 63 individuals belonging to the reference group of a Mexican population from Los Angeles (1000 Genomes Project). Five synonymous variants (*TCOF1*: p.(Thr210=); *SALL1*: p.(Pro558=) and p.(Val190=); *EYA1*: p.(Ile195=); and *TBX1*: p.(Pro45=)) could not be subjected to MDR interaction analysis due to the lack of information in the reference group.

One significant gene–gene interaction related to the presentation of microtia/OAVS was identified: that between the non-synonymous p.(Pro1099Arg) *TCOF1* variant and the synonymous p.(Leu858=) *SALL1* variant (Figure 1). In this MDR interaction analysis, the balance accurate cross-validation testing result was 0.8175 and the cross-validation consistency value was 10/10. In a permutation analysis performed with 10,000 repetitions, the obtained p-value was statistically significant ($p < 0.001$).

Furthermore, using the network created by STRING, we added the intergenic interaction that we identified in this study between the variants p.(Pro1099Arg) *TCOF1* (rs1136103) and p.(Leu858=) *SALL1* (rs1965024) and incorporated in this network the following based on reported evidence for other gene–gene interactions: (a) The co-expression of *SIX1* and *Eya1* synergistically regulates the expression of *SALL1* during kidney development [44] and also play keys roles in ear determination [45,46]. (b) In the mouse limb bud, *Hoxa13* and *Hoxd13* inhibit the expression of *Sall1* [47]. In mouse embryonic stem cells, *Sall1* appears to inhibit various *Hox* genes, including *Hoxd13* and *Gsc* [48]. (c) Although it is not known whether *Sall1* has a regulatory relationship with *Hoxa2* in the branchial arches, the former is expressed early in head mesenchyme and then becomes restricted around the first branchial cleft in close proximity of *Hoxa2*, which encodes an important transcription factor in the external ear morphogenesis of mice [40]. (d) *Hoxa2* coordinates the downregulation of *Gsc*, which acts as a transcriptional repressor in wild-type cartilage during mouse embryogenesis [49]. (e) In zebrafish, *gsc* downregulates the expression of *bapx1* in the second pharyngeal arch [50,51]. (f) Both increased and decreased RA signaling could induce craniofacial abnor-

malities, such as those found in OAVS [13]. (g) *MYT1* overexpression reportedly induces the downregulation of RA receptor β (RARB), whereas mutated *MYT1* does not [23] (see Figure 1).

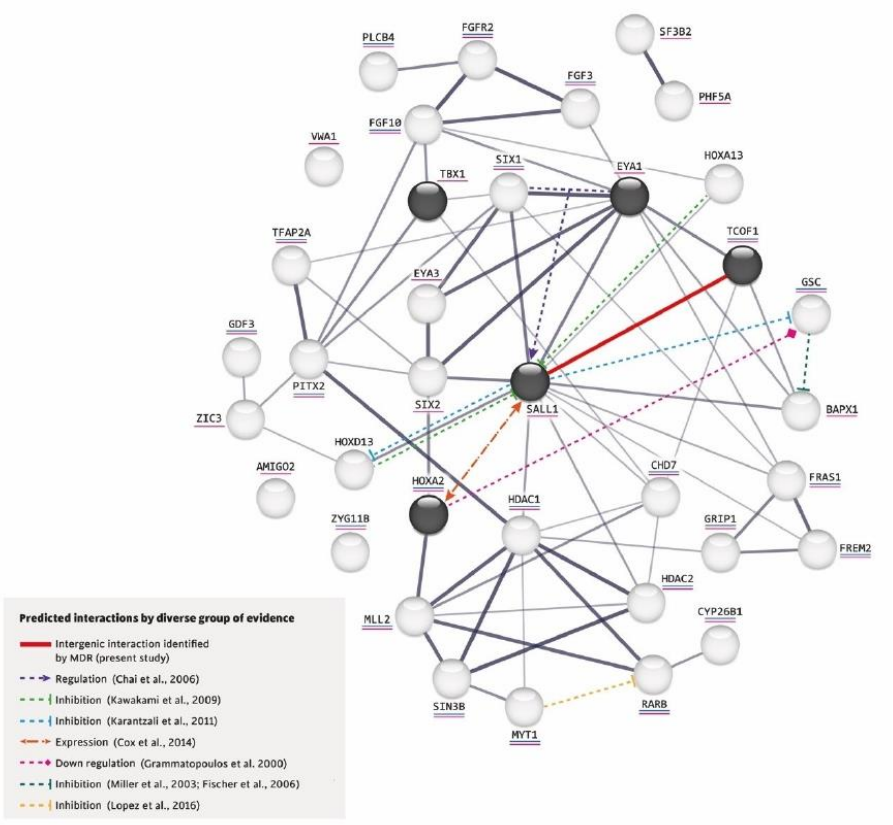

Figure 1. Interactions predicted by diverse lines of evidence [23,40,44,47–51].

This interaction network was built by the STRING V.11.0 software (https://string-db.org/, accessed on 20 September 2022) and includes the proteins encoded by the five genes studied herein (in dark circles) plus those related to RA (underlined in blue) and craniofacial disorders (underlined in pink). The thickness of a gray line indicates the strength of the data compatibility based on the STRING evidence. The solid red line indicates the intergenic interaction that we herein identified between p.(Pro1099Arg) *TCOF1* (rs1136103) and p.(Leu858=) *SALL1* (rs1965024) variants, and the colored dotted lines represent the interactions documented in the literature.

4. Discussion

Since microtia/OAVS shows a heterogeneous etiology, incomplete penetrance, and variable expressivity, its clinical and molecular diagnoses have proven challenging. In the literature, the inclusion criteria for patients with this spectrum are diverse and not always well described or defined, which could be considered a limitation when interpreting and discussing results. We consider that the clinical inclusion criteria utilized herein allowed us to study a more homogeneous population and thereby avoid biases.

Some groups have previously analyzed genetic factors related to microtia/OAVS [22–24,26–28,52–56], but no previous gene–gene interaction analysis has been per-

formed in these patients. Given the genomic differences found across populations and the high prevalence of this disorder in Latin-American populations worldwide, it is important to obtain a more precise knowledge in patients with microtia/OAVS of this ethnic origin. Gaining a better understanding of the genetic etiology of this malformation could enable clinicians to provide more accurate genetic counseling to families [17–21,37].

4.1. Association Analysis

When we performed the association study between the variants identified in our patients and the reference group, we identified that the in-frame p.(Ser159del) microdeletion of *SALL1* appears to be a risk factor for microtia, as evidenced by the statistically significant difference in the AF between our patients and the reference group and the presence of this variant in only our microtia/OAVS cases. Similar associations have been identified between variants of certain genes and the risk of developing malformations, such as congenital heart defects, biliary atresia, pyloric stenosis, hypospadias, and microtia [53,54,57].

The genome-wide association study (GWAS) approach has been successful in identifying new susceptibility loci for common structural congenital defects, such as oral clefts, congenital heart defects, biliary atresia, pyloric stenosis, hypospadias, craniosynostosis, and clubfoot [57]. However, congenital ear abnormalities, including anotia/microtia, have not previously been addressed by GWAS. The sole exception to this was a genome-wide linkage analysis performed on two families with OAVS [58]. In one family, the authors identified a highly suggestive linkage to a region harboring the *GSC* (Goosecoid homeobox) gene, which was considered to be a good candidate gene for this entity. However, coding-region changes and gross rearrangements were excluded in these two OAVS familial cases and in 120 additional sporadic cases [58].

Synonymous variants may influence the development of various human diseases, including birth defects [59,60]. A statistically significant difference was identified for the p.(Gly587=) *TCOF1* variant. The AF for this variant was greater in the reference group, suggesting that it confers protection against or a decreased risk for microtia/OAVS in our Mexican population. There is no single mechanism by which a synonymous change could exert a biological effect. Accumulating evidence shows that biological systems take advantage of the degeneracy of the genetic code to control gene expression, protein folding efficiency, and coordinated expression across several gene families. The most obvious and well-characterized mechanism by which synonymous changes can exert a deleterious biological effect is by perturbing pre-mRNA splicing [59]. However, a synonymous variant could also be in linkage disequilibrium with deleterious functional variants located nearby. To our knowledge, the p.(Gly587=) of *TCOF1* could be the first described synonymous variant associated with the microtia/OAVS trait.

4.2. MDR Interaction Analysis

In a very recent review of genetic and non-genetic factors involved in the development of microtia/OAVS, there was no mention of data related to gene–gene interactions [32]. As the interaction analysis for benign and/or synonymous variants could suggest a genetic protection or susceptibility factor for complex traits [34], such as ear malformations, we decided to apply an MDR analysis, which is a nonparametric model-free method for identifying epistasis using the identified variants [61]. This strategy identified a single statistically significant intergenic interaction between the non-synonymous p.(Pro1099Arg) *TCOF1* variant and the synonymous p.(Leu858=) *SALL1* variant (Figure 1). At the statistical level, combining the *TCOF1* and *SALL1* genotypes allowed us to discriminate between cases and controls, indicating that there is an interaction between these two genes. Although this interaction has not previously been reported, the TCOF1 and SALL1 proteins are related to craniofacial disorders [23] (Figure 1). The available evidence generally supports the involvement of the analyzed genes/proteins and various other genes/proteins in the development of microtia/OAVS. Notably, *SALL1* interacts with most of these genes/proteins and thus appears to play a central role. In addition, it was the gene in which the most

non-synonymous variants were identified in our group of patients (Figure 1), suggesting that *SALL1* warrants future study in this regard.

Moving forward, the inclusion of a larger number of microtia/OAVS-related genes or the use of whole-exome sequencing should identify new variants that can be considered in future gene–gene interaction studies [62]. Epigenetic inheritance also has been suggested as a possible pathogenic mechanism [5]. For example, a histone acetylation-dependent imbalance in the allelic expression of the key craniofacial development gene, *BAPX1* (also called *NKX3-2*, MIM*602183), was observed in five patients with OAVS [51]. Thus, the contribution of epigenetic mechanisms to the etiology of microtia/OAVS deserves attention in future genetic studies.

The published evidence and our present findings collectively support the complexity of ear embryogenesis and microtia/OAVS development, which involves the temporal and spatial expression of different proteins and signaling by multiple pathways. We did not identify any pathogenic variant in the five studied genes, but we found a gene–gene interaction between *TCOF1* and *SALL1*. This highlights the need to identify other genes and genotype interactions that contribute to the etiology of craniofacial disorders, including microtia/OAVS [33]. Future research is also needed to assess the involvement of the RA pathway in the genetic etiology of microtia/OAVS.

5. Conclusions

Although gene–gene interactions are known to play an important role in the etiology of many complex diseases, no previous study has addressed gene interactions in patients with microtia/OAVS. Our finding of a gene interaction between *TCOF1* and *SALL1* in a group of Mexican patients with this entity supports the complex nature of ear embryogenesis and the development of microtia/OAVS. Further research is warranted, such as the inclusion of more candidate loci, which should lead to the identification of new gene–gene interactions underlying microtia/OAVS.

Author Contributions: B.E.-O.: Research (literature review), structuring and review of the research protocol, recruitment, and selection of candidate patients to be included in the study, interpretation of genotypes, analysis and discussion of results, writing of the original draft, review and editing of subsequent versions. M.E.R.-F.: Research (literature review), structuring and review of the research protocol, molecular methodology, NGS bioinformatic analyses, interpretation of genotypes, analysis and discussion of results, writing of the original draft, review and editing of subsequent versions. J.A.V.-A.: Interpretation of genotypes in cases and reference groups, statistical analysis, study of association and interaction between the identified variants, analysis and discussion of results, revision and editing of manuscript. A.G.-d.A.: Structuring and review of the research protocol, recruitment, and selection of candidate patients to be included in the study, clinical methodology, analysis and discussion of results, and revision and editing of manuscript. L.F.-H.: Research (literature review), structuring and review of the research protocol, recruitment and selection of candidate patients, molecular methodology, analysis and discussion of results, revision and editing of manuscript. M.A.A.-O.: Structuring and review of the research protocol, molecular methodology, interpretation of genotypes, analysis and discussion of results, funding administration, project management, supervision, revision and editing of manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Instituto Nacional de Pediatría, Secretaría de Salud (Recursos Fiscales 2015–2019, Programa E022 Investigación y Desarrollo Tecnológico en Salud, Ciudad de México, México).

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved and registered by the Ethics, Research and Biosafety Committees of the National Institute of Pediatrics (Mexico, registry number 004/2017).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The genotypic datasets generated in this study have been uploaded to the Leiden Open Variation Database (LOVD) version 3.0 (https://www.lovd.nl/3.0/home, accessed on 20 September 2022).

Acknowledgments: We thank Juan Carlos Zenteno Ruíz, Samuel Canizales Quinteros, and Carlos Sabas Cruz Fuentes for their valuable comments and critical review of the manuscript. We also thank Katherine Fabián Aviléz for designing the figures that accompany the text.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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Received: 28 June 2021 | Revised: 3 December 2021 | Accepted: 3 January 2022
DOI: 10.1002/ajmg.a.62678

ORIGINAL ARTICLE

AMERICAN JOURNAL OF medical genetics PART A WILEY

Proposed clinical approach and imaging studies in families with oculo-auriculo-vertebral spectrum to assess variable expressivity

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Abstract

A diagnosis of oculo-auriculo-vertebral spectrum (OAVS) is established when microtia is present in association with hemifacial hypoplasia (HH) and/or ocular, vertebral, and/or renal malformations. There is no consensus on which imaging studies should be used to rule out variable expressivity and distinguish "sporadic" from "familial" patients. This observational and descriptive study was performed in a Mexican population of 51 patients (32 males, 19 females, 0–18 years old) with microtia/OAVS, and their available parents. A clinical history, genealogy, and physical examination were obtained from all included patients, as were a computed tomography (CT) scan of the ear, audiological evaluation, orthopantomography, complete spine radiography, and renal ultrasound. The same approach was completed in their available parents (51 mothers and 40 fathers), excluding the CT scan and audiological evaluation. By genealogy, 53% of patients were classified as "sporadic"; of the "familial" patients, at least 79.1% had suggestion of a multifactorial inheritance. In the available parents, orthopantomography, complete spine X-ray, and renal ultrasound identified the following OAVS-related manifestations: HH (16.2%, $n = 14/86$), vertebral alterations (10.9%, $n = 10/91$), and renal anomalies (2.2%, $n = 2/90$). Our evaluation of the parents allowed three patients to be reclassified from "sporadic" to "familial" (5.8%, $n = 3/51$). Our proposed clinical and imaging approach allowed the identification of variable expressivity that more clearly distinguished between "sporadic" and "familial" OAVS patients, which is of utmost importance in providing proper genetic counseling to these families.

KEYWORDS
clinical approach, microtia, OAVS, variable expressivity

1 | INTRODUCTION

Microtia (HP:0008551) occurs with a worldwide incidence of 1/4000–1/6500 live births, with an expected prevalence of 0.83–17.44/10,000 births. These rates vary according to ethnic origin: The documented risk is higher in Asians, Pacific Islanders, Chileans, Ecuadorians, and Mexico-born Hispanics, and lower in Caucasians and African Americans (Canfield et al., 2009; Gendron et al., 2016; Luquetti et al., 2012; Suutarla et al., 2007). Microtia is more frequent in males (2:1), and unilateral (49.1–93%) and right-side (46–70%) presentations predominate (Alasti & Van Camp, 2009; Suutarla et al., 2007). However, the condition shows a high degree of

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phenotypic variability (Hunter et al., 2009). Conductive hearing impairment (HP:0000405) is identified in 84.8%–96.1% of patients with microtia. This is mainly due to atresia or stenosis of the external auditory canal (EAC, HP:0000413, HP:0000402), and is related to sensorineural hearing impairment (HP:0000410) in 3.3%–9% of individuals (Suutarla et al., 2007).

When microtia is associated with hemifacial hypoplasia (HH, HP:0011332, also referred to as hemifacial microsomia but more recently as craniofacial microsomia) and ocular, vertebral, cardiac, and/or renal malformations, the diagnosis of oculo-auriculo-vertebral spectrum (OAVS) is suggested. However, there is no agreement on the minimal diagnostic criteria for OAVS (Gendron et al., 2016; Luquetti et al., 2012). There is controversy in the literature regarding whether microtia and OAVS are different entities, with several research groups proposing that microtia is a minimal expression of the OAVS clinical spectrum (Cox et al., 2014; Llano-Rivas et al., 1999; Luquetti et al., 2012).

Microtia/OAVS shows significant etiologic heterogeneity. In syndromic patients, chromosomal, Mendelian, or teratogenic etiologies have been documented (Alasti & Van Camp, 2009; Gendron et al., 2016). The non-syndromic and "sporadic" forms are usually of unknown etiology. In patients with a family history, the cause may be monogenic autosomal dominant (AD, HP:0000006) or autosomal recessive (AR, HP:0000007), with a smaller proportion appearing to exhibit multifactorial inheritance (HP:0001426) (Alasti & Van Camp, 2009; Llano-Rivas et al., 1999; Muñoz-Pedroza & Arenas-Sordo, 2013).

Some authors have suggested clinical approaches for patients with microtia and/or craniofacial microsomia (Cohen et al., 2017; Heike et al., 2013; Koening et al., 2018; Wang et al., 2001; Zim et al., 2017). However, we currently lack any consensus on which imaging studies (orthopantomography, full spine X-ray, renal ultrasound) should be performed in patients with microtia/OAVS and their parents or other relatives as part of a comprehensive clinical approach to rule out variable expressivity and adequately classify "sporadic" versus "familial" patients.

The aim of this study was to describe the facial, auditory, vertebral, and renal manifestations of patients with microtia/OAVS and their parents, and to use a complete physical examination and imaging studies to identify variable expressivity and accurately distinguish between "sporadic" and "familial" forms. Our proposed clinical approach could help clinicians provide proper genetic counseling to microtia/OAVS families.

2 | METHODS

2.1 | Study population

2.1.1 | Patients

Fifty-one unrelated patients (32 males, 19 females; ages 0–18 years old) who were evaluated between 2015 and 2019 at the National Institute of Pediatrics (Mexico) and given a clinical diagnosis of microtia/OAVS (microtia or anotia with or without HH, structural alterations in spine and/or kidneys) were included. Patients who met the clinical criteria for microtia/OAVS but had congenital malformations distinct from those involving the spine and/or kidneys and/or exhibited alteration in somatic growth (low or high height) or intellectual disability were excluded. Those with reported prenatal exposure to specific teratogens associated with microtia/OAVS (e.g., alcohol, retinoids, or maternal diabetes) were also excluded.

Each patient underwent a systematic physical examination performed by experienced medical geneticists that included an external structural examination of the eye along with recording of a detailed family history. A patient was considered "familial" if an auricular anomaly (microtia, preauricular pit, or appendage) was present in a 1st, 2nd, 3rd, and/or 4th degree relative with or without history of consanguinity or endogamy. "Consanguinity" was considered when there was a marriage between two descendants of the same ancestor, generally no more remote than the union between second cousins; endogamy or "inbreeding" was defined as intracommunity marriage (Bittles, 2008). After the complete physical examination was carried out, a computed tomography (CT) scan of the inner ear, audiological studies, orthopantomography, full spine X-ray, and renal ultrasound were performed in each index patient.

Microtia was described with respect to laterality and grade according to the classification proposed by Hunter et al. (2009), as well as by the presence or absence of stenosis or atresia of the EAC. When microtia was grade I, the length of the auricle was measured with a measuring tape from the upper edge of the helix to the lower edge of the lobe on the unaffected versus affected side; measurement was not performed in patients with microtia grades II and III, since anatomical reference points are lost in such cases (Bozkir et al., 2006). Microtia was considered if the length of the auricle was below the 3rd centile based on the graph of Feingold and Bossert (1974). Malformations in the external, middle, and inner ear were delineated by CT of the ear, and conductive and/or sensorineural hearing impairment were documented by audiometry or auditory brainstem evoked potentials (ABEP). The results of orthopantomography were interpreted using the measures described in Figure S1, Supporting Information. Evaluation of the full spine X-ray and renal ultrasound was used to rule out associated malformations at these levels. The structures of the ears and face (front and side) of each patient were photographed at the first clinical evaluation.

2.1.2 | Parents

Clinical examinations were performed on all mothers ($n = 51/51$) and 40 available fathers. Ear lengths were measured as described for patients (Bozkir et al., 2006; Feingold & Bossert, 1974). If a parent presented microtia, their characteristics were assessed as described above. In addition to the genealogy and clinical examination, imaging studies (orthopantomography, full spine X-ray and renal ultrasound) were performed on the available parents to examine the variable

expressivity in detail. To interpret the orthopantomography and define HH as variable expressivity, the methodology and reference measurements employed for patients were used. Regarding scoliosis, we defined an abnormal curvature of the spine as >10° (Jada et al., 2017). Photographs of the face and the ears were taken too.

A 1st degree relative was considered affected when she/he presented: (1) an auricular anomaly (microtia, preauricular pit, or appendage), or (2) two or more clinical manifestations of OAVS without auricular abnormality. When only HH, scoliosis, or renal cyst was observed without family history, this was considered to be a sporadic event (HP:0003745) instead of multifactorial inheritance, given that such alterations can occur at high frequency in the general population.

Genetic counseling was provided to the families based on the obtained genealogical and clinical results.

The frequencies identified in our population for certain clinical manifestations were compared with those reported from other populations using the chi-square test, with a statistical difference assumed at a significance level of 0.05.

This project was authorized and registered (004/2017) by the Research, Ethics and Biosafety Committees of the National Institute of Pediatric (Mexico) and the written informed consent was obtained from at least one parent of each participant.

3 | RESULTS

3.1 | Pedigree

According to the pedigree analysis, 47% ($n = 24/51$) patients had a family history of microtia/OAVS; in 19/24 (79.1%) of these patients, a multifactorial mode of inheritance was suggested by the presence of at least one 1st, 2nd, 3rd, and/or 4th degree affected relative. This subgroup included those with or without history of consanguinity or endogamy, which was reported in only 3/24 "familial" patients (EFAV-22, 87, 162). In 20.8% ($n = 5/24$) of the "familial" patients, other inheritance patterns (besides multifactorial) might be considered. An AR inheritance was suggested in 3/5 families (EFAV-40, -67, -120); in each of these families the sister of the proband had only a preauricular appendage, but of the three affected sisters, none was evaluated through imaging studies. In the remaining two families that showed potential for Mendelian inheritance (EFAV-79, -144), one of the parents was affected, so an AD inheritance could be considered. Finally, in three patients without other affected family members, consanguinity between parents (EFAV-84), consanguinity and inbreeding (EFAV-102), and inbreeding (EFAV-107) were reported (Table 1).

3.2 | Patients

Regarding the clinical presentation, 62.7% ($n = 32/51$) were male, and unilateral microtia (86.2%, $n = 44/51$) was more frequent than bilateral microtia. Of the individuals with unilateral microtia, the right side (63.6%, $n = 28/44$) and grade III (75%, $n = 33/44$) were the most frequent; only one patient showed microtia grade I, and none of anotia was identified.

In 98% ($n = 50/51$) of the patients, stenosis or atresia of the EAC was present; among them, the CT ear scan revealed that ossicular chain abnormality or aplasia (HP:0004452, HP:0009910) was present in 80% ($n = 40/50$), whereas fusion of the vestibule and right lateral semicircular canal was observed in only one patient. Ipsilateral conductive hearing impairment was diagnosed in 84.3% of patients ($n = 43/51$); in 11.7% ($n = 6/51$) of them, it was associated with sensorineural hearing impairment. Normal hearing was documented in only two patients (EFAV-110 with grade I right microtia; EFAV-141 with grade II bilateral microtia without abnormalities of the ossicular chain on CT scan). We did not identify epibulbar dermoid cyst in any patient.

Orthopantomography was done in 45/51 patients (4 did not cooperate during the study and 2 could not attend to carry it out). Among them, HH was identified in 42.2% ($n = 19/45$). With respect to other malformations, costal or vertebral anomalies were found in 12% ($n = 6/50$), and renal hypoplasia (HP:0000089) was observed in only 3.9% ($n = 2/51$) (Figure 1).

We analyzed each patient and classified them into those with: (1) only with auricular affection (ear, EAC, ossicular chain, hearing) and (2) microtia with facial, costal/vertebral, or renal level involvement. They were distributed as follows: auricular $n = 21$ (41.1%); auricular and facial (HH) $n = 16$ (31.3%); auricular, facial, and vertebral or costal $n = 3$ (5.8%); auricular and vertebral or costal $n = 3$ (5.8%); auricular and renal $n = 1$ (1.9%) and, unclassified $n = 7$ (6 orthopantomography and 1 full spine X-ray were not available).

The overall clinical findings in our patients are described and compared with those from other populations in Table 2. We identified statistically significant differences in the frequencies of: unilateral microtia reported in the American population (86.2% in this work vs. 61%; $p = 0.047$) (Rollnick et al., 1987) and German population (vs. 49.1%; $p = 0.0001$) (Tasse et al., 2005); atresia/stenosis of EAC in Mexican patients (98% vs. 55%; $p = 0.0001$) (Llano-Rivas et al., 1999), Greeks (vs. 53%; $p = 0.0001$) (Touliatou et al., 2006), and Europeans (vs. 25.1%; $p = 0.0001$) (Barisic et al., 2014); inner ear defects in Italians (2% vs. 20%; $p < 0.01$) (Brotto et al., 2017); conductive hearing impairment in Finns (84.3% vs. 96.1%; $p = 0.0046$) (Suutarla et al., 2007); and conductive hearing impairment associated with sensorineural hearing impairment in Mexican population (11.7% vs. 3.4%; $p = 0.0368$) (Llano-Rivas et al., 1999).

3.3 | Parents

Orthopantomography, full spine X-ray, and renal ultrasound were performed as indicated in Figure 1.

In 13/51 mothers who had been previously considered unaffected, the following phenotypic alterations were observed: HH ($n = 4$); HH and scoliosis (HP:0002650, $n = 1$, EFAV-70); HH and pyeloureteral dilatation of the upper third of the right ureter at 17 mm in the anteroposterior axis ($n = 1$, EFAV-93); an extra

TABLE 1 Inheritance patterns interpreted before and after studying parents

Case	Family history of microtia	Consanguinity/ endogamy	Inheritance pattern	Associated anomalies		Inheritance pattern after cabinet studies performed in parents
				Mother	Father	
EFAV-22	Paternal cousin (3rd degree)	E	M	Lumbar scoliosis	–	M
EFAV-26	Paternal uncle (2nd degree)	–	M	Extra collapsed hemivertebra between T8 and T9	–	M
EFAV-28	–	–	S	–	–	S
EFAV-32	Maternal uncle and maternal great aunt (2nd, 3rd degree)	–	M	Thoracic scoliosis	HH	M
EFAV-35	–	–	S	HH	–	S
EFAV-38	Father and maternal cousin (1st, 3rd degree)	–	M	HH	HH	M
EFAV-40	Sister (1st degree)	–	AR, M	–	OP	AR, M
EFAV-42	Paternal great uncle and aunt (3rd degree)	–	M	–	NV	M
EFAV-45	–	–	S	Thoracolumbar scoliosis	NV	S
EFAV-47	–	–	S	–	–	S
EFAV-50	Maternal cousin (3rd degree)	–	M	–	NV	M
EFAV-52	–	–	S	–	–	S
EFAV-55	–	–	S	–	NV	S
EFAV-57	–	–	S	–	HH and cleft vertebrae (C3–C4)	AD, M
EFAV-64	–	–	S	–	–	S
EFAV-67	Sister (1st degree)	–	AR, M	–	–	AR, M
EFAV-70	–	–	S	HH and lumbar scoliosis	–	AD, M
EFAV-75	–	–	S	–	NV	S
EFAV-77	Paternal great-grandfather (3rd degree)	–	M	–	NV	M
EFAV-79	Father (1st degree)	–	AD, M	HH	–	AD, M
EFAV-82	–	–	S	–	–	S
EFAV-84	–	C	S	–	–	S
EFAV-87	Half sister (2nd degree)	C	M	–	NV	M
EFAV-90	–	–	S	–	RP	S
EFAV-93	–	–	S	HH and pyeloureteral dilatation	–	AD, M
EFAV-96	Mother, brother and maternal uncle (1st, 4th degree)	–	M	–	–	M
EFAV-99	Maternal uncle and paternal uncle (4th degree)	–	M	–	Lumbar scoliosis	M
EFAV-102	–	C/E	S	–	–	S

TABLE 1 (Continued)

Case	Family history of microtia	Consanguinity/ endogamy	Inheritance pattern	Associated anomalies		Inheritance pattern after cabinet studies performed in parents
				Mother	Father	
EFAV-105	–	–	S	–	NV	S
EFAV-107	–	E	S	–	HH	S
EFAV-110	Mother, father (1st degree); paternal aunt and cousin (2nd, 3rd degree); maternal aunts (3rd degree)	–	M	–	HH	M
EFAV-113	–	–	S	–	NV	S
EFAV-115	Maternal aunt (6th degree)	–	S	–	–	S
EFAV-117	Maternal cousin (3rd degree)	–	M	–	–	M
EFAV-120	Sister (1st degree)	–	AR, M	–	–	AR/M
EFAV-126	–	–	S	–	HH	S
EFAV-129	Maternal uncle (4th degree)	–	M	–	–	M
EFAV-132	Maternal niece (4th degree)	–	M	–	Thoracic scoliosis	M
EFAV-135	–	–	S	–	–	S
EFAV-138	Paternal great grandfather uncle, uncle and cousin (4th, 5th degree)	–	M	Lumbar scoliosis	–	M
EFAV-141	–	–	S	HH	–	S
EFAV-144	Mother, maternal grandmother and great aunt (1st, 2nd, 3rd degree)	–	AD, M	–	–	AD, M
EFAV-147	–	–	S	–	NV	S
EFAV-149	–	–	S	–	NV	S
EFAV-151	Maternal uncle (4th degree)	–	M	–	HH	M
EFAV-154	Paternal grandfather uncle and two paternal cousins (3rd degree)	–	M	OP	OP	M
EFAV-156	–	–	S	–	–	S
EFAV-159	–	–	S	–	–	S
EFAV-162	Paternal niece (4th degree)	E	M	–	–	M
EFAV-165	Paternal aunt (2nd degree); maternal cousins (5th degree)	–	M	–	HH	M
EFAV-168	–	–	S	Simple cyst, OP	OP	S

Abbreviations: AD, dominant inheritance; AR, recessive inheritance; C, consanguinity; E, endogamy; HH, hemifacial hypoplasia; M, multifactorial inheritance; NV, not valued; OP, orthopantomography pending; RP, renal ultrasound pending; S, sporadic event.

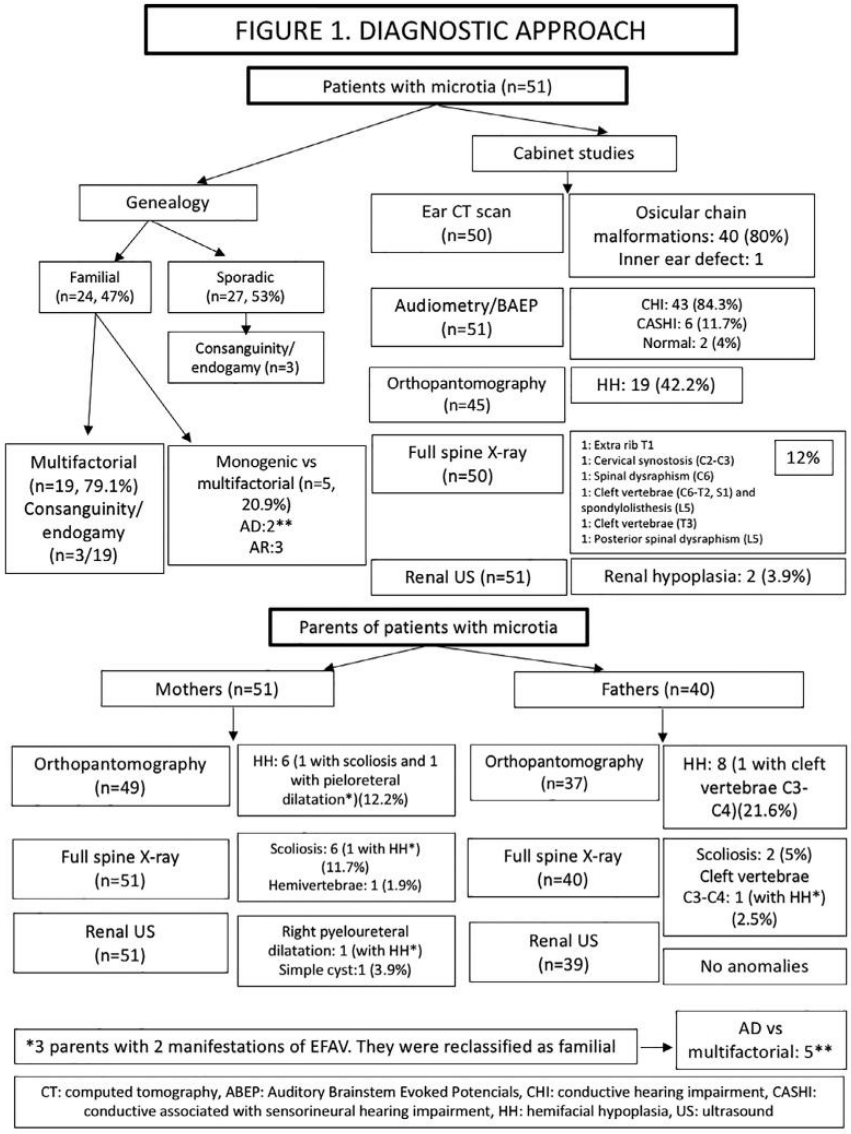

FIGURE 1 Diagnostic approach

hemivertebra (HP:0008467) collapsed between T8-T9 ($n = 1$); spinal scoliosis ($n = 5$); and unilateral simple renal cyst (HP:0012581, $n = 1$). In 10/40 fathers (8 previously considered unaffected), the following associated anomalies were recognized: HH was documented in 7/37 fathers (2 with preauricular pit or appendage; EFAV-38, -110); 1/37 had HH and vertebral clefting (HP:0008428) of C3-C4 (EFAV-57); and 2/40 had spinal scoliosis. No renal alteration was found in fathers ($n = 0/39$) (Figure 1).

Imaging evaluation of parents allowed us to reclassify 3/27 (11.11%) patients from their genealogy-based designation of "sporadic" to "familial" with possible AD or multifactorial inheritance (EFAV-57, -70, -93), further enabling us to offer them appropriate genetic counseling (shaded in Table 1).

4 | DISCUSSION

OAVS is characterized by marked variability in expression (Elalaloui et al., 2010). Its diagnosis is suggested when microtia is associated with HH and ocular, vertebral, cardiac, and/or renal malformations, but there is no agreement on the minimal diagnostic criteria for OAVS. It has been suggested that clinicians should perform renal ultrasound in patients with syndromic and nonsyndromic microtia, as 16%–29% of such patients have kidney abnormalities (Koening et al., 2018; Wang et al., 2001). At least one research group found that spinal X-ray was not justified due to the lack of associated vertebral abnormalities in their study population (Zim et al., 2017). However, another group recommended that complete spinal X-ray and a renal

TABLE 2 Clinical findings observed in our population compared to other populations

Characteristic	Our population	Rollnick et al. (1987)	Vento et al. (1991)	Okajima et al. (1996)	Llano-Rivas et al. (1999)	Tasse et al. (2005)	Touliatou et al. (2006)	Suutarla et al. (2007)	Brotto et al. (2017)	Barisic et al. (2014)
	$n = 51$ % (p) Mexico	$n = 294$ % (p) USA	$n = 154$ % (p) USA	$n = 592$ % (p) Japan	$n = 145$% (p) Mexico	$n = 53$ % (p) Germany	$n = 17$ % (p) Greece	$n = 190$ % (p) Finland	$n = 35$ % (p) Italy	$n = 259$ % (p) EUROCAT
Male sex	62.7	64 ($p > 0.05$)	NR	64.7 (>0.05)	60 (>0.05)	60.4 (>0.05)	NR	NR	NR	NR
Unilateral microtia	86.2	*61 (0.047)*	NR	90.9 (>0.05)	75 (0.1174)	*49.1 (0.0001)*	NR	88.4 (0.6347)	NR	NR
Right sided microtia	63.6	69.6 (0.6)	NR	64.3 (>0.05)	52 (0.1199)	46.2 (0.0647)	NR	59.5 (0.4952)	NR	NR
Atresia/stenosis EAC	98	NR	NR	92 (>0.05)	*55 (0.0001)*	NR	*53 (0.0001)*	NR	NR	*25.1 (0.0001)*
Ossicular chain malformations	80	NR	NR	NR	NR	NR	NR	NR	74 (0.2419)	NR
Inner ear defect, fusion of vestibule and right lateral semicircular canal	2	NR	NR	NR	NR	NR	NR	NR	*20 (<0.01)*	NR
Conductive hearing impairment	84.3	NR	NR	NR	84.8 (1.0)	85 (1.0)	NR	*96.1 (0.0046)*	NR	NR
Conductive and sensorineural hearing impairment	11.7	NR	NR	NR	*3.4 (0.0368)*	NR	NR	9.0 (0.5917)	NR	NR
Epibulbar dermoid cyst	0	4.4	20.7	NR	NR	20.7	12	NR	NR	7.7
Hemifacial hypoplasia	42.2	*68.7 (0.0006)*	NA	NR	55 (0.1675)	*83 (0.0001)*	59 (0.2581)	NR	NR	49 (0.3328)
Costal or vertebral defects, Extra rib T1, cervical synostosis (C2-C3), spinal dysraphism (C6), cleft vertebrae (C6-T2, S1) and spondylolisthesis (L5), cleft vertebrae (T3), posterior spinal dysraphism (L5)	12	18.7 (0.221)	18.8 (0.1917)	NR	22 (0.1215)	19 (0.2696)	18 (0.4146)	NR	NR	*24.3 (0.0373)*
Renal anomalies, renal hypoplasia	3.9	5 (1.0)	NR	NR	3.4 (1.0)	16.9 (0.0522)	*23 (0.0307)*	NR	NR	*15.8 (0.0251)*

Note: Statistically significant differences among specific populations are italicized.
Abbreviation: NR, not reported.

TABLE 3 Previously reported and suggested clinical approaches in patients with microtia and/or hemifacial microsomia

	Cabinet studies				
	Phenotype inclusion criteria	CT of the ears	Orthopantomography	Full spine x-ray	Renal ultrasound
Wang et al. (2001), $n = 42$ (33 syndromic, 9 isolated), 12/42 (29%) had renal anomalies (11 syndromic, 1 isolated)	Isolated preauricular pits, cup ears, or any other ear anomaly accompanied by other malformation or dysmorphic feature, a family history of deafness, auricular and/or renal malformation, or a maternal history of gestational diabetes	Not evaluated	Not evaluated	Not evaluated	It should be performed
Zim et al. (2017), $n = 514$ (145 had undergone renal ultrasound and 81 had cervical spine X-rays), no cervical spine or structural renal abnormality was identified	Isolated microtia and/or aural atresia	Not evaluated	Not evaluated	Routine screening is not required	Routine screening is not required
Koening et al. (2018), $n = 80$ (51 syndromic, 29 isolated), 13/80 (16%) had renal anomalies (11 syndromic, 2 isolated)	All patients with microtia (syndromic and non-syndromic)	Not evaluated	Not evaluated	Not evaluated	It should be performed
Heike et al. (2013) (review)	Craniofacial microsomia	Not evaluated	Not evaluated	Screening at 3 years of age	Screening at diagnosis
Cohen et al. (2017), $n = 89$, 40/86 (47%) had vertebral anomalies, 25/86 (29%) had genitourinary anomalies	Hemifacial microsomia	Not evaluated	Not evaluated	Screening at diagnosis	Screening at diagnosis
Our population (this work), $n = 51$, 40/50 (80%) had ossicular chain abnormality, 19/45 (42.2%) had hemifacial hypoplasia, 6/50 (12%) had costal/vertebral anomalies, 2/51 (3.9%) had genitourinary anomalies	Microtia/OAVS	Screening at diagnosis	Screening at 3 years of age	Screening at diagnosis	Screening at diagnosis

ultrasound should be performed when craniofacial microsomia is detected, because vertebral (47%) and genitourinary (29%) abnormalities were clearly noted in such cases (Cohen et al., 2017) (Table 3). At present, there is also no consensus on which imaging studies should be requested from parents and relatives of microtia patients to rule out minimal expression of OAVS.

In our pediatric-age study population, the following microtia characteristics documented in patients were consistent with those previously reported in the literature: male sex predominance (62.7% in this work vs. 58%–65% in the literature), unilateral presentation (86.2% vs. 49.1%–93%), and right laterality (63.6% vs. 46–70%). In addition, due to atresia or stenosis of the EAC (98% vs. 55%–93%), conductive hearing impairment was identified in the majority of the patients (84.3% vs. 84.8%–96.1%), a proportion of whom also had sensorineural auditory deficits (11.7% vs. 3.3%–9%) (Eavey, 1995; Llano-Rivas et al., 1999; Okajima et al., 1996; Rollnick et al., 1987; Suutarla et al., 2007; Tasse et al., 2005) (Table 2). With respect to the specific populations studied in previous reports, however, our findings showed significant population-level differences in the frequencies of unilateral microtia, atresia/stenosis of the EAC, inner ear defects, conductive hearing loss, and conductive hearing loss associated with sensorineural hearing loss (Table 2). This reaffirms the importance of studying other genetically heterogeneous populations, such as that in Mexico.

Regarding the degree of severity, 50/51 patients presented microtia II or III and 98% had stenosis or atresia of the EAC. This may reflect that our National Institute of Pediatrics is a third-level hospital at which patients with the most severe malformations seek surgical treatment. It would be unlikely for patients with microtia I to present at this institute, given that they have structurally and functionally normal ears that do not require highly specialized management. Our lack of patients with anotia (HP:0009892) or microtia grade IV, which is the most severe form of this malformation and is identified in

2%–44.5% of patients (Forrester & Merz, 2005; Suutarla et al., 2007), could reflect that these conditions have a very low prevalence in our population, as previously noted for some other ones (Liu et al., 2018).

Unexpectedly, none of our patients presented with epibulbar dermoid cyst, which has been observed in 4%–20% of microtia/OAVS patients from other populations (Barisic et al., 2014; Rollnick et al., 1987; Tasse et al., 2005; Touliatou et al., 2006; Vento et al., 1991). However, we did not use ocular defects (e.g., epibulbar dermoid cyst) as an inclusion criterion, as has been done in other studies (Strömland et al., 2007). This was because we herein focused on describing the manifestations associated with microtia/OAVS. Thus, although epibulbar dermoid cysts are considered part of OAVS, the absence of patients with epibulbar dermoid cyst in the present study could be attributed to its low frequency in our population and/or the utilized criteria, rather than misdiagnosis. This phenomenon could also be related to small sample size analyzed herein: If we consider the reported frequency of epibulbar dermoid cyst (4%–20%) with a confidence interval of 4%–95%, we would expect between 0.7 and 8 patients in our population ($n = 51$). Therefore, the absence of this clinical feature could be expected by chance.

The OAVS-associated malformations observed in our population were similar to those reported in the literature. They included mid-ear ossicular chain abnormality or aplasia (80% in the present work vs. 74%) (Brotto et al., 2017), HH (42.2% vs. 49%–83% in the literature) and renal anomalies (3.9% vs. 3.4%–23%) (Barisic et al., 2014; Llano-Rivas et al., 1999; Rollnick et al., 1987; Tasse et al., 2005; Touliatou et al., 2006; Vento et al., 1991). Only one patient in the present work had fusion of the vestibule and lateral semicircular canal in the right side (2% vs. 20%), suggesting that inner ear anomaly might be an infrequent manifestation in our microtia/OAVS patients, even though at least one report listed this as an OAVS-associated anomaly (Vendramini et al., 2007). We observed vertebral abnormality at a lower percentage than previously reported (12% vs. 18%–24.3%) (Barisic et al., 2014; Llano-Rivas et al., 1999). An ocular examination by an ophthalmologist, an echocardiography, and/or neuroimaging were not performed, so we cannot rule out eye defects other than epibulbar dermoid cyst, congenital heart defects (5%–27.8%), and/or central nervous system abnormalities (10.4%–29%), which have also been associated with OAVS (Barisic et al., 2014).

The etiology of "sporadic" non-syndromic microtia is usually unknown, and patients with familial history of microtia may show inheritance as AD, AR, and even (to a lesser extent) multifactorial traits (Llano-Rivas et al., 1999). Familial history of microtia/OAVS was reported in 47% ($n = 24/51$) of our patients, so more than half were identified as "sporadic" forms, as described in the literature (Llano-Rivas et al., 1999; Mastroiacovo et al., 1995). Multifactorial inheritance was suggested in 37% of all families ($n = 19/51$) based on aggregation of affected relatives and clinical findings in parents. AD and AR inheritance patterns were considered possible in the remaining familial patients, such as when microtia/OAVS was observed in a 1st degree relative (9.8%, $n = 5/51$), although multifactorial inheritance cannot be ruled out in such cases. Consanguinity and/or endogamy were reported in only 3/24 "familial" patients (EFAV-22, 87, 162); and in the parents of three patients with no affected family member (EFAV-84, -102, -107) and thus were classified as "sporadic."

By considering an affected 1st degree relative to be those with two or more OAVS-related clinical manifestations even without auricular abnormality, we identified three affected parents, two of them presenting HH and abnormalities of the spine (EFAV-57, -70) and one had scoliosis and kidney abnormality (EFAV-93) (Figures S2–S4). We also identified 20/91 parents (21.9%) that each had a single anomaly (HH, $n = 11$; hemivertebrae, $n = 1$; scoliosis, $n = 7$; renal cyst, $n = 1$); however, they were not considered to be affected by OAVS, as these alterations can occur at high frequency in the general population. For example, HH occurs in 1/3,000–5,000 live births (Birgfeld & Hieke, 2019), hemivertebra in 1–10/10,000 live births (Johal et al., 2016), idiopathic spine scoliosis in 0.47%–5.2% of the population (Jada et al., 2017), and renal cysts in 27% (Mensel et al., 2018).

Notably, our approach allowed us to reclassify three patients (11.1%, $n = 3/27$) from their genealogy-based classification of "sporadic" to "familial" after studying their parents (EFAV-57, -70, -93). The father of patient EFAV-57 presented with left HH (6-mm difference between both condyles with mandibular branches) and cleft vertebrae (C3-C4). The mother of patient EFAV-70 showed left HH and lumbar scoliosis. The mother of EFAV-93 presented left HH (8-mm difference between both condyles with mandibular branches) and pyeloureteral dilatation of the upper third of the right ureter (Figures S2–S4). Thus, although these patients were referred without family history of OAVS, our analysis revealed that AD or multifactorial inheritance should be considered.

After we studied the parents for clinical variable expressivity, the overall percentage of "familial" OAVS in our population was 52.9% ($n = 27/51$). Among the "familial" cases, 70.3% ($n = 19/27$) appeared to represent a multifactorial inheritance pattern, while the remaining could represent monogenic (5 AD, 3 AR) or multifactorial inheritance. The predominance of families with a possible multifactorial inheritance pattern (37.2%, $n = 19/51$) resembled that observed in a Hungarian population, in which 4.0% (14/354) of patients with non-syndromic microtia had affected 1st degree relatives (Paput et al., 2012), but contrasts with the 33.8% monogenic (without discarding the possibility of multifactorial inheritance in some familial cases) and <1% multifactorial inheritance patterns previously described in Mexican patients who attended our same institution (Llano-Rivas et al., 1999). These discrepancies might reflect that our criteria for registering a case as "familial" were the presence of a 1st, 2nd, 3rd, and/or 4th degree relative with auricular abnormality (microtia, preauricular pit, or appendage) with or without history of consanguinity or endogamy, and/or our procedure of recording of family history of OAVS, performing a detailed clinical evaluation, and using imaging studies to evaluate facial, vertebral, and renal abnormalities in parents.

Based on our present results, we suggest that the clinical diagnosis of patients with microtia/OAVS should include a complete physical examination plus CT scan of the ears, orthopantomography, audiological studies, full spine X-ray, and renal ultrasound (Table 3). Performing

ear CT and audiometry/ABEP in all parents might have allowed us to increase the detection of "familial" individuals, but were cost-prohibitive. The usefulness of ear CT scan and audiometry/ABEP in further delineating variable expressivity in familial microtia/OAVS should be assessed further in terms of their costs and benefits. In addition, ophthalmologic, cardiac, and/or neurologic evaluation should be considered to certainly rule out OAVS-related abnormalities.

In conclusion, our proposed imaging assessment of parents appeared effective in improving the proper classification of microtia/OAVS patients into "sporadic" or "familial" forms and helping identify inheritance patterns for improved genetic counseling.

ACKNOWLEDGMENTS

This work was supported by the Instituto Nacional de Pediatría, Secretaría de Salud (Recursos Fiscales 2015–2019, Programa E022 Investigación y Desarrollo Tecnológico en Salud, Ciudad de México, México). We thank Dr. José Velázquez-Aragón for conducting the statistical analysis of the clinical data and Dr. Jazmín Arteaga-Vázquez for her valuable comments and critical review of this article.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

DATA AVAILABILITY STATEMENT

All authors confirm the absence of shared data. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Estandia-Ortega, B., Fernández-Hernández, L., Alcántara-Ortigoza, M. A., & González-del Angel, A. (2022). Proposed clinical approach and imaging studies in families with oculo-auriculo-vertebral spectrum to assess variable expressivity. *American Journal of Medical Genetics Part A*, 1–11. https://doi.org/10.1002/ajmg.a.62678

DISCUSIÓN

Dado que la microtia/EFAV presenta penetrancia incompleta y expresividad variable, su diagnóstico clínico y molecular representa un desafío. Actualmente no se ha logrado un consenso sobre los criterios diagnósticos para integrar esta entidad y en la literatura los criterios de inclusión en los estudios sobre microtia suelen ser heterogéneos, lo que representa una limitación al interpretar y discutir el fenotipo de los pacientes. En este estudio de investigación no incluimos casos con defectos distintos de los que involucran la columna y/o riñones, ni tampoco con alteración en el crecimiento somático (talla baja o alta) o discapacidad intelectual. Si bien es cierto que existen varias malformaciones asociadas con microtia/EFAV, como los defectos cardíacos congénitos (Barisic et al., 2014; Ramprasad et al., 2020), nuestros criterios de inclusión se establecieron con el objetivo de evitar la incorporación de pacientes con síndromes genéticos que cursan con microtia, distintos a EFAV (Luquetti et al., 2012; Bartel-Friedrich, 2015).

Se sabe que tanto factores genéticos y no genéticos contribuyen a la presencia de microtia/EFAV (Gendron et al., 2016). Debido a lo anterior, en este estudio, aquellos pacientes con exposición prenatal a teratógenos específicos como alcohol, retinoides o diabetes materna fueron excluidos, ya que estos factores no genéticos o ambientales han sido considerados como una causa de esta entidad (Foround et al., 2012; Gendron et al., 2016; Berenger et al., 2018).

Con relación a los factores genéticos relacionados con la microtia existen algunos estudios en la literatura que los abordan (Tingaud-Sequeira et al., 2021). Sin embargo, es relevante estudiar a pacientes de origen latinoamericano con microtia debido a la falta de información al respecto y a la diferencia que existe entre el genoma de las distintas poblaciones.

Nosotros seleccionamos como estrategia molecular la secuenciación de las regiones codificantes de un panel de 5 genes relacionados con microtia/EFAV con base en la evidencia reportada en la literatura (Luquetti et al., 2012; Gendron et al., 2016; Vitelli et al., 2003; Dixon et al., 2006; Cox et al., 2014). En 4/5 genes analizados se identificaron variantes no sinónimas e *indels* clasificadas como variantes B o LB, así como variantes sinónimas. Sin embargo, no se observaron variantes patogénicas, probablemente patogénicas o VUS en este panel, por lo que sería importante expandir el número de genes a estudiar o realizar WES en nuestros pacientes con microtia/EFAV para tratar de comprender la etiología de esta entidad (Xue et al., 2015).

En el análisis de asociación de la FA de las variantes B o LB identificadas en nuestra población considerando la FA reportada en individuos mexicanos de Los Ángeles (Proyecto 1000 Genomas), se observó una diferencia estadísticamente significativa en una variante: *SALL1* c.475_477del o p.(Ser159del) [rs113614842]. Dado que esta variante sólo se observa en el grupo de nuestros casos, este cambio podría considerarse como un factor de riesgo para esta malformación, como se ha descrito para otras variantes génicas en pacientes con defectos cardíacos congénitos, atresia biliar, estenosis pilórica, hipospadias y microtia (Wang et al., 2017, 2019; Lupo et al., 2019).

Con relación a las variantes sinónimas, se sabe que pueden tener consecuencias funcionales a pesar de que el cambio no altere la secuencia de aminoácidos de la proteína. No existe un mecanismo único por el cual estos cambios ejerzan un efecto biológico, sin embargo, el mecanismo mejor caracterizado por el cual los cambios sinónimos dan como resultado un impacto biológico claro es a través de alteraciones en el *splicing* del pre-ARNm. Con base en lo anterior, las variantes sinónimas pueden influir en el desarrollo de enfermedades humanas, incluidos defectos congénitos como microtia y defectos cardíacos congénitos (Hunt et al., 2014; Dixit et al., 2019). Con relación a las variantes sinónimas identificadas en nuestra población, se identificó una frecuencia alélica estadísticamente significativa más baja en la variante *TCOF1* c.1761G>T (rs7701163) al compararla con el grupo de referencia. Esta podría ser una variante que confiera protección para microtia/ EFAV, en lugar de riesgo, como se ha reportado para variantes en otros genes asociadas a otras malformaciones (Alonso-Montes et al., 2018; Lupo et al., 2019).

El abordaje molecular mediante GWAS (del inglés *Genome Wide Association Study*) ha tenido éxito en la identificación de nuevos *loci* de susceptibilidad para defectos congénitos estructurales comunes, como hendiduras orales, defectos cardíacos congénitos, pie equinovaro, hipertrofia pilórica, entre otros (Lupo et al., 2019). Entre los defectos que no se han estudiado mediante GWAS se encuentran las anomalías congénitas del oído, incluida la anotia / microtia, con la excepción de un reporte sobre escaneo del genoma completo y análisis de ligamiento en dos familias con EFAV. El gen candidato más probable fue *GSC* (goosecoid, OMIM * 138890) pero al analizar 2 casos familiares y 120 esporádicos de esta entidad, no se identificaron variantes patogénicas en este gen (Kelberman et al., 2001).

A diferencia de otras poblaciones en las que se han identificado variantes patogénicas de microtia/EFAV en casos familiares (Alasti et al., 2008; Luquetti et al., 2012; Brown et al., 2013; Piceci et al., 2017, Meddaugh y Zambrano, 2020; Si et al., 2020), nosotros no identificamos variantes codificantes en el gen *HOXA2*, ni en los casos esporádicos ni en los familiares. En nuestra población, este gen parece ser poco polimórfico y no responsable del fenotipo estudiado.

El método de MDR se ha utilizado como una estrategia no paramétrica para identificar combinaciones de factores genéticos con un efecto pequeño y/o factores ambientales que son predictivos de un fenotipo clínico (Moore et al., 2006; Moore y Williams, 2009; Gui et al., 2013; Velázquez-Aragón et al., 2016). Este tipo de análisis nos permitió identificar una interacción intergénica significativa entre 2 de las 39 variantes ubicadas en los genes analizados: la variante p. (Pro1099Arg) *TCOF1* [rs1136103] y la variante p.(Leu858=) *SALL1* [rs1965024] en los pacientes con microtia/EFAV y dado que no existen estudios en la literatura que hayan realizado este abordaje en pacientes con microtia/EFAV, esta podría considerarse la primera publicación con este enfoque.

Es de interés, el papel central de *SALL1* ya que interactúa con la mayoría de los genes/proteínas mencionados (Figura 1). Esta evidencia debería ser considerada en futuros estudios, ya que éste fue el gen con más variantes no sinónimas identificadas en nuestro grupo de pacientes, una de sus variantes se asoció con mayor riesgo para microtia y además mostró una interacción con otro de los genes analizados (*TCOF1)*.

La evidencia publicada y nuestros hallazgos nos permitieron integrar una red de interacciones que respalda la complejidad de la embriogénesis del oído y el desarrollo de microtia/EFAV ya que sugiere el involucro de la expresión temporal y espacial de diferentes proteínas y la señalización a través de múltiples vías como la del ácido retinoico.

Por lo tanto, aunque no identificamos ninguna variante patogénica en los cinco genes estudiados en nuestros pacientes, y sólo encontramos la interacción entre *TCOF1* y *SALL1*, este estudio destaca la necesidad de identificar otros genes y genotipos, así como sus interacciones, para definir su contribución a la etiología de trastornos craneofaciales, incluyendo microtia/EFAV.

CONCLUSIONES

La mayoría de los pacientes con microtia/EFAV permanecen sin una etiología identificable, por lo que se requieren mayores conocimientos sobre esta entidad, para que el abordaje clínico y molecular sea más preciso. Se han observado numerosas alteraciones y algunos genes causales pero se sugiere extender el estudio de los factores genéticos a través de otras estrategias como el análisis de interacciones entre genes.

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