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SHORT REPORT

A novel *de novo* mutation in *MYT1*, the unique OAVS gene identified so far

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Oculo-auriculo-vertebral spectrum (OAVS) is a developmental disorder characterized by hemifacial microsomia associated with ear, eyes and vertebrae malformations showing highly variable expressivity. Recently, *MYT1,* encoding the myelin transcription factor 1, was reported as the first gene involved in OAVS, within the retinoic acid (RA) pathway. Fifty-seven OAVS patients originating from Brazil were screened for *MYT1* variants. A novel *de novo* missense variant affecting function, c.323C>T (p.(Ser108Leu)), was identified in *MYT1*, in a patient presenting with a severe form of OAVS. Functional studies showed that *MYT1* overexpression downregulated all RA receptors genes (*RARA, RARB, RARG*), involved in RA-mediated transcription, whereas no effect was observed on *CYP26A1* expression, the major enzyme involved in RA degradation, Moreover, *MYT1* variants impacted significantly the expression of these genes, further supporting their pathogenicity. In conclusion, a third variant affecting function in *MYT1* was identified as a cause of OAVS. Furthermore, we confirmed *MYT1* connection to RA signaling pathway.
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INTRODUCTION

Oculo-auriculo-vertebral spectrum (OAVS) is a developmental disorder characterized by hemifacial microsomia associated with ear, eyes and vertebrae malformations, showing highly variable expressivity. Various other organs malformations are commonly retrieved.[1] Goldenhar syndrome (GS) could be considered as the most severe form of OAVS phenotype.[2]

Its etiology remains unknown even if environmental factors, including embryonic exposure to retinoic acid (RA)[3] and genetic factors were documented.[4,5] Recently, our group identified MYT1 (NM_004535.2) encoding the myelin transcription factor 1 as the first gene implicated in OAVS. Indeed, we found two heterozygous variants affecting function in *MYT1*, one nonsense *de novo* c.25C>T (p.(Arg9*)) and one inherited missense c.314C>T (p.(Ser105Leu)), in two unrelated patients among a cohort of 169 OAVS patients.[2]

The recruitment of 57 new Brazilian OAVS patients allowed us to identify a novel variant in *MYT1*. Moreover, functional studies showed that the repressor role of *MYT1* on several genes involved in the RA pathway was altered by missense variants affecting function.

MATERIALS AND METHODS

Fifty-seven patients were recruited from the Medical Genetic Center of the Genetic and Morphology Department of the Universidade Federal de São Paulo, Brazil. An ethics committee (CPP: DC2012/76) approved this study. SNP-arrays (genome-wide human SNP array 6.0, CytoScan Array 750k and HD, Affymetrix, Santa Clara, CA, USA) were performed in all patients.

Exons of the *MYT1* gene were amplified using a standard protocol.[2] PCR fragments were sequenced by GS Junior technology (ICM, Paris, France). Variants were confirmed by Sanger sequencing (Genewiz, France) and then registered in LOVD database (www.LOVD.nl/MYT1 under following individual ID 00095945 and 00095942, 00095943/00095955 for variants previously described[2]).

The pCS2+-*MYT1* (*MYT1*-WT) was obtained from GeneCust (Ellange, Luxemburg). The *MYT1*-p.Ser108Leu and *MYT1*-p.Ser105Leu constructs corresponded to c.323C>T (p.Ser108Leu) and c.314C>T (p.Ser105Leu) variants, respectively, were generated by site-directed mutagenesis.[2] Cell culture, transient transfections and RT-qPCR were performed as previously described.[2] Quantitative expression of *RARA, RARB, RARG* and *CYP26A1* was determined using the $2^{-\Delta\Delta}$Ct method with *GUSB* and *RPLPL0* as reference genes (primers available on request).

RESULTS

All recruited patients presented hemifacial microsomia, characterized by mandibular/malar or maxillar hypoplasia, and microtia (including 23% with anotia), which was associated with preauricular tags (40%) and/or dysplastic ears (63%). Eye abnormalities were observed in 74% of patients mainly due to abnormal orbital position (63%). Vertebral defects were found in 89% of patients. Furthermore, cardiac and renal anomalies were present in 47 and 16% of patients, respectively (Table 1). No pathogenic CNV were identified among this cohort. *MYT1* screening revealed a *de novo* missense variant c.323C>T (p.(Ser108Leu)) in a GS patient born from unrelated and healthy parents (Figure 1a). Both parents did not carry the missense variant identified in the index patient. *In silico* analysis predicted a pathogenic effect of the c.323C>T (p.(Ser108Leu)) variant, which is reported 2/120 646 in ExAC database but notably it was not found in latino population. The patient presented a right anotia with preauricular tags and cervical pits, right stenosis of the external auditory meatus and a left grade III microtia (Figure 1b) with right conductive (65–75 dB) and left mixed (50–70 dB) hearing loss. Moreover, right hemifacial microsomia with moderate soft tissue deficiency, a small mandible and

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Table 1 Clinical description of the 57 Brazilian patients compared to the cohort already published[2] as reference and clinical pattern for the 3 OAVS patients presenting variants in *MYT1*

Description	*Brazilian patients*		*Described cohort*[2]		*Patient c.25C>T, (p.(Arg9*))*[2]	*Patient c.314C>T, (p.(Ser105Leu))*[2]	*Brazilian patient c.323C>T (p.(Ser108Leu))*
Total patients	57	100%	169	100%			
Sex, F	21	37%	91	54%	+	–	+
Sex, M	36	63%	78	46%	–	+	–
OAVS familial history	5	9%	19	11%	–	–	–
Twinning	5	9%	8	5%	–	–	–
Gestational diabetes	4	7%	5	3%	–	–	–
Psychomotor delay	11	19%	16	9%	–	–	–
Ear abnormality	57	100%	166	98%	+	+	+
Anotia	13	23%	18	11%	–	–	+R
Microtia	57	100%	97	57%	+R	–	+L
Preauricular Tag	23	40%	101	60%	+R	+L	+L
Preauricular pit	1	2%	10	6%	–	–	+L
Ear dysplasia	36	63%	126	75%	+R	+L	+B
Hearing loss	45	79%	86	51%	+	+	+
Conductive hearing loss	40	70%	47		+R	+B	+R
Sensorineural hearing loss	26	46%	9	5%	–	–	–
'Mixed' Hearing loss	21	37%	6	4%	–	–	+L
Hemifacial microsomia	56	98%	139	82%			
Microsomia R	31	54%	38	22%	+	–	+
Microsomia L	23	40%	41	24%	–	+	–
Mandibular, malar, maxillar hypoplasia	56	98%	106	63%	+	+	+
Eye abnormality	42	74%	64	38%	+	–	+
Epibulbar dermoid	7	12%	42	25%	+R	–	–
Coloboma	5	9%	16	9%	–	–	–
Microphtalmia	18	32%	19	11%	–	–	–
Abnormal orbital position	35	61%	ND	ND	–	–	+
Micrognathism/retrognathism	44	77%	31	18%	–	–	–
Hypertelorism	6	11%	14	8%	–	–	–
Other dysmorphia	5	9%	55	33%	–	–	–
Macrocephaly, microcephaly	2	4%	0	0%	–	–	–
Vertebral abnormality	51	89%	67	40%	+	+	+
Cardiac malformation	27	47%	48	28%	+ (VSD)	–	+ (ASD/VSD)
Renal malformation	9	16%	14	8%			
Cerebral malformation	12	21%	8	5%	+ (seizures)	–	+ (accentuation of groove and cracks)
Other abnormality	34	60%	16	9%	–	–	–
Macrostomia	27	47%	19	11%	–	–	–
Orofacial cleft	18	32%	42	25%	–	–	–

Abbreviations: +, presence of criterion; –, absence of criterion; ND, not determined.

a right glenoid fossa with short ramus mandibular branch involvement were observed (Figures 1b and c). She had an abnormal orbital position. Spinal X-rays revealed cervical vertebrae fusion with reduced spaces from C5 to C7, and from L3 to S1. She had atrial and ventricular septal defects. She also presented an accentuation of grooves and cracks of the brain, a sign of neonatal hypoxia, and a micrognathism (Table 1).

Following *MYT1*-WT overexpression, expressions of *RARA*, *RARB* and *RARG* were found decreased by 2.91-, 1.3- and a 3.84-fold, respectively (Figures 2a—c). As a functional test, the effect of *MYT1*-p.Ser108Leu and *MYT1*-p.Ser105Leu (previously described[2]) overexpression on RARs expression was studied. Both mutants had a significantly lower inhibitory effect on *RARA* and *RARG* expression than *MYT1*-WT (Figures 2a and c). However, *MYT1*-p.Ser105Leu inhibited *RARB* expression to a significantly lesser extent than MYT1-WT, while *MYT1*-p.Ser108Leu induced a significantly higher decrease (Figure 2b).

Additionally, we looked for the effect of MYT1 on a gene involved in RA-catabolism. *MYT1*-WT overexpression did not induce any significant change on *CYP26A1* expression. However, *MYT1*-p.Ser105Leu increased its expression whereas *MYT1*-p.Ser108Leu form decreased it (Figure 2d).

DISCUSSION

We described here 57 new patients presenting with a typically heterogeneous OAVS. However, they presented a higher percentage of ocular and vertebral defects, and consequently, more patients were considered with GS than in previous reports: 40% *versus* 16%[2] or 7.5%.[6]

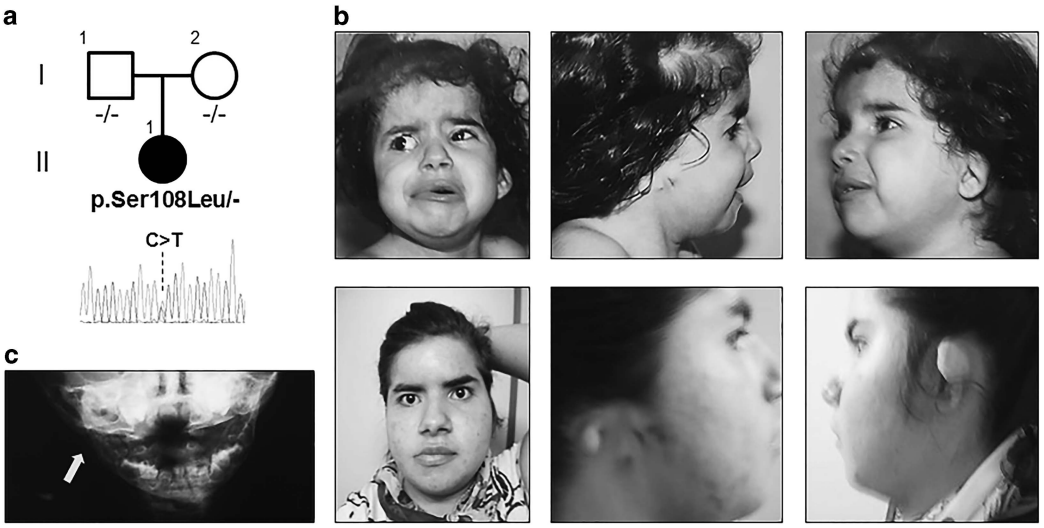

Figure 1 Pedigree and photographs of the patient carrying the c.323C>T (p.Ser108Leu) variant in *MYT1*. (**a**) Family tree and electrophoregram showing the *de novo* heterozygous missense variant. (**b**) Photographs of the proband showing facial asymmetry, right anotia and left microtia at age 1 and 17 years (top and bottom panels, respectively). (**c**) X-rays of proband at 1 year showing hypoplasia the right mandibular branch (arrow).

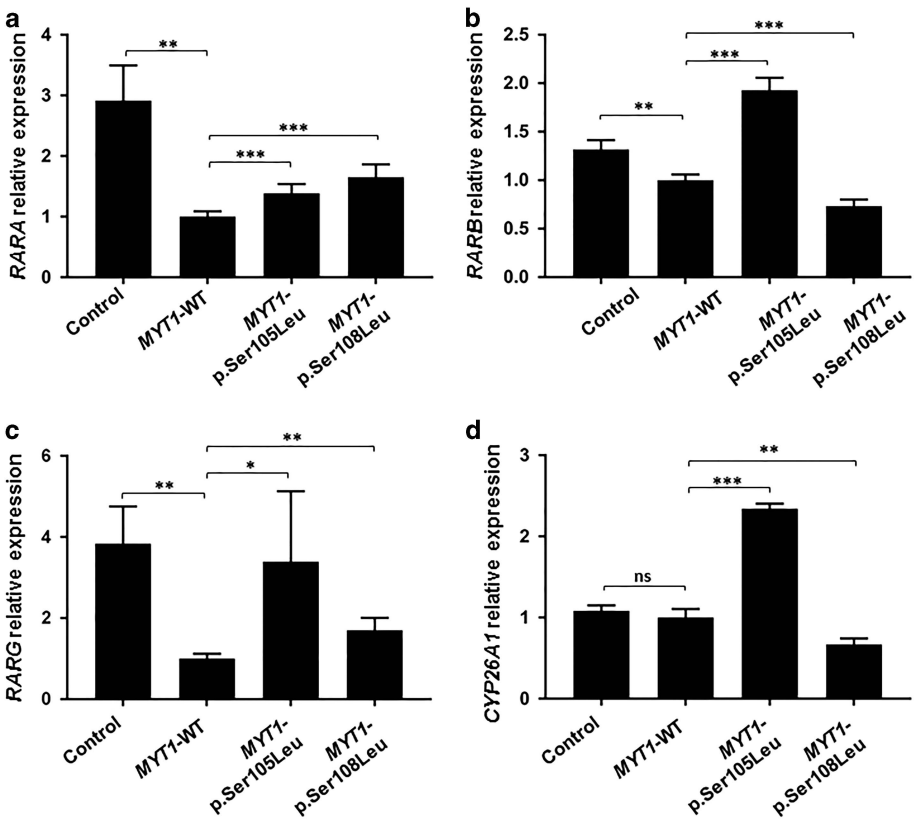

Figure 2 *MYT1* variants altered regulation of *RAR*s genes and *CYP26A1*. RT-qPCR analysis of *RARA* (**a**), *RARB* (**b**), *RARG* (**c**) and *CYP26A1* (**d**) are shown following overexpression of either empty, *MYT1*-WT, *MYT1*-p.Ser105Leu or *MYT1*-p.Ser108Leu pCS2+ expression vector. The relative transcript level was calculated as fold change using the $2^{-\Delta\Delta Ct}$ method ($n=3$–6). Statistical significance was evaluated using *t*-test (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

Recently, our group described *MYT1*, a transcription factor involved in neurogenesis,[7] as the first gene involved in OAVS.[2] In the present study, we identified a novel *de novo* missense variant affecting function, c.323C>T (p.(Ser108Leu)) in *MYT1* in a GS patient. Interestingly, this new variant represents the same amino acid substitution (Serine into Leucine), only 3 residues next to the c.314C>T (p.(Ser105Leu)) variant.[2] Even if no functional domain was described, the close vicinity of these two mutated residues in OAVS patients suggests a potential role for this region.

Considering that embryonic RA exposure could induce OAVS features, we previously described that RA, the natural RAR ligand, induced *MYT1* expression but also that MYT1 was a repressor of

RARB expression *in vitro*.[2] Very recently, it was shown that Myt1 bound genomic proximal region of *Rara* and repressed its expression, supporting a direct regulation.[7] Thus, we further explored MYT1 effect on all *RARs*, and found that MYT1 had a repressor effect on their expression.

We thereby studied the effect of the two *MYT1* missense variant affecting function on the expression of RARs. The overexpression of both mutated forms inhibited *RARA* and *RARG* expression to a lesser extent than *MYT1*-WT. However, the c.323C>T (p.Ser108Leu) variant inhibited *RARB* expression to a significantly higher extent than *MYT1*-WT, whereas c.314C>T (p.Ser105Leu) had the opposite effect.[2] Although these results appeared confusing, RARs dysregulations are involved into craniofacial defects either due to up- or downregulation. Indeed, loss and gain-of-function variants occurring in the *RARB* gene both cause anophthalmia and diaphragmatic hernia.[8] Moreover, *RARA* expression was upregulated in fibroblasts from the secondary palate of a patient presenting a cleft/lip palate.[9] Additionally, treatment with specific Rara or Rarb agonists led to craniofacial abnormalities in murine embryos[10,11] and injection of Rarg dominant negative mRNA in Xenopus embryos led to a partial resistance to RA.[12]

Subsequently, we investigated the effect of MYT1 on genes involved in RA degradation, thus regulating RA availability. In HEK293 (human embryonic kidney) cells, *CYP26A1* is the major enzyme of RA catabolism.[13] *MYT1-WT* overexpression had no detectable impact on *CYP26A1* expression. As observed for *RARB* expression, *MYT1*-p.Ser105Leu overexpression led to an upregulation of *CYP26A1* expression, whereas *MYT1*-pSer108Leu led to a downregulation of both genes. Therefore, the deregulation of *RARB* by *MYT1*-mutated forms seems to be compensated by *CYP26A1* deregulation in order to control RA availability, thus restoring a correct RA signaling pathway. *Rarb* and *Cyp26a1* were shown to be upregulated after RA treatment in murine embryo,[14] thus supporting a regulating loop between these two genes. Moreover, downregulation of *Rarb* and *Cyp26a1* (but also of *Rara*)[15] was observed in LgDel-22q11 mice and patients with OAVS features carried 22q11 deletions.[5,16] As shown with RARG,[17] our results suggest that RARB could participate in *CYP26A1* regulation. Moreover, *Cyp26A1*$^{-/-}$ embryos present malformations of the hindbrain,[18] where *Myt1, Cyp26A1 and Rarb* are expressed,[18,19] and where RA signaling pathway activity is detected.[20]

Taking together, our results show that MYT1 regulates genes involved in the RA pathway, and thus participates in its negative feedback. Variants affecting function in *MYT1* could lead to a disruption of RA signaling and may be responsible for OAVS features. Partial contradictory effects of the two *MYT1* variants could be associated by the paradoxical teratogenic mechanism evoked for RA. Indeed, ATRA exposure leads to rapid changes in enzymes involved in RA metabolism and induces a longer-term RA-deficiency.[14] Thus, future investigations will allow to better understand how variants in *MYT1* impact RA signaling, and especially if OAVS features could be related to one specific RAR or to the combined effect of them but also to study how MYT1 could modulate the expression of genes involved in RA-synthesis due to the importance of *Raldhs* in embryonic development.[21,22]

In conclusion, we reported a novel *de novo* variant in *MYT1* gene, thus confirming its involvement in OAVS. The low frequency of variants (1 out of 57 and 2 out of 169[2]) supports the high genetic heterogeneity of this spectrum. The identification of new candidate genes will increase the understanding of the syndrome and more broadly of embryonic development processes. In particular, genes involved in RA pathway could be a good target for future investigation in OAVS.[22]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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