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Brief Report

Rare variants in the epithelial cadherin gene underlying the genetic etiology of nonsyndromic cleft lip with or without cleft palate

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ABSTRACT

Nonsyndromic orofacial cleft (NSOFC) is a complex disease of still unclear genetic etiology. To investigate the contribution of rare epithelial cadherin (*CDH1*) gene variants to NSOFC, we target sequenced 221 probands. Candidate variants were evaluated via *in vitro*, *in silico*, or segregation analyses. Three probably pathogenic variants (c.760G>A [p.Asp254Asn], c.1023T>G [p.Tyr341*] and c.2351G>A [p.Arg784His]) segregated according to autosomal dominant inheritance in four nonsyndromic cleft lip/palate (NSCL/P) families (Lod score: 5.8 at θ=0; 47% penetrance). A fourth possibly pathogenic variant (c.387+5G>A) was also found, but further functional analyses are needed (overall prevalence of *CDH1* candidate variants: 2%; 15.4% among familial cases). *CDH1* mutational burden was higher among probands from familial cases when compared to that of controls (P=0.002). We concluded that *CDH1* contributes to NSCL/P with mainly rare, moderately penetrant variants, and *CDH1* haploinsufficiency is the likely etiological mechanism.

Key words: *CDH1*, oral clefts, gastric cancer, two-hit model, rare variant

Nonsyndromic cleft lip with or without cleft palate (NS CL/P) and nonsyndromic cleft palate only (NS CPO) are two complex disorders within the nonsyndromic orofacial cleft (NS OFC) spectrum [Gorlin, et al., 2001]. While the genetic etiology of NS CPO is largely unclear, genetic *loci* have been systematically implicated in NS CL/P, such as common low-risk 8q24, 10q25 and *IRF6* variants [Rahimov, et al., 2008; Birnbaum, et al., 2009; Mangold, et al., 2010, Brito et al., 2012a, Brito et al., 2012b]. However, given the high heritability attributed to NS CL/P [Hu, et al., 1982; Calzolari, et al., 1988; Brito, et al., 2011], searching for alternative genetic variants or mechanisms is necessary to bridge the missing heritability gap of these malformations.

Rare germline variants in the gene encoding the adhesion molecule epithelial cadherin, *CDH1* (MIM# 192090), have long been associated with diffuse gastric cancer and lobular breast cancer [van Roy and Berx, 2008]. Most recently, C*DH1* mutations have been reported in OFC patients, in association with gastric cancer [Frebourg, et al., 2006; Kluijt, et al., 2012; Benusiglio, et al., 2013] or not [Vogelaar, et al., 2013; Bureau, et al., 2014]. These findings raise the questions as to what the proportion of NS OFC cases underlain by *CDH1* variants and their attributed penetrance is, and which types of mutations or mechanisms lead to OFC, cancer, or both phenotypes.

Here, we performed a variant screening for *CDH1* (NM_004360.3) coding region in 221 NS OFC probands (affected by NS CL/P [n=189] or NS CPO [n=32], either from non-familial [n=138] or familial cases [n=83]; Supp. Table S1). Sequencing was performed by using next generation sequencing (NGS - exome or targeted gene sequencing) and Sanger sequencing (SS), and applied for 65 and 156 probands, respectively. Additional NGS or SS was performed for extra members of familial cases, when available. When exome sequencing was performed in affected members of the same family, we filtered out variants with minor allele frequency greater than 1% in public databases (1000 Genomes Project, and NHLBI ESP exomes) and in our in-house database of 609 Brazilian control exomes (Supp. Methods). Among the 221 probands, we identified a total of 47 variants, of which 12 were absent in our controls (2 missense, 1 nonsense and 9 noncoding or synonymous variants; Supp. Table S2). Variants were submitted to the LOVD database, at http://www.lovd.nl/CDH1.

The novel missense variant c.760G>A (p.Asp254Asn, exon 6) was the most likely causative variant among the main candidates detected by exome analysis (mean coverage of 60x; average of 25,140 variants called for each individual; Supp. Table S3) in families F3788 and F617 (4 affected individuals sequenced in each family; Supp. Fig. S1a and S1b). Both families segregate NS CL/P, and haplotype analysis of the exome data did not support a close

relationship between these families (data not shown). SS of additional 2 affected and 8 unaffected members from these two families supported segregation in accordance with an autosomal dominant model with incomplete penetrance estimated at 53%. Assuming this penetrance, a Lod score of 4.8 was obtained at recombination fraction (θ) 0, under an allele frequency of 0.0001. The other novel missense variant, c.2351G>A (p.Arg784His, exon 15), was found in the proband of family F1387 through SS. Segregation with NS CL/P was evidenced by its presence in 3 affected relatives (Supp. Fig. S1c), and penetrance was estimated at 62%. Further, the loss-of-function variant c.1023T>G (exon 9), predicted to create a stop codon at position 341 of *CDH1* (p.Tyr341*), was found in the proband of family F7618 (Supp. Fig. S1d) through SS. Segregation with NS CL/P was suggested by its presence in an affected first cousin once removed. Although this represents the first association between this variant and NS CL/P, an association with hereditary diffuse gastric cancer (HDGC) has been previously observed [Guilford et al., 2010]. Penetrance was estimated at 31% in this family. Considering the 4 pedigrees, an overall 47% penetrance of NS CL/P was estimated, with a maximum Lod score of 5.86 at θ=0 (individual Lod scores - F3788: 2.3; F617: 2.5; F1387: 0.9; F7618: 0.2).

Among the remaining 9 noncoding or synonymous novel variants, only 2 variants were significantly scored by *in silico* tools for pathogenicity prediction (Supp. Table S4). Variant c.387+5G>A, which possibly decreases exon 3 splice donor site recognition, was found in a NS CL/P proband from a non-familial case (parental DNA unavailable for testing whether it is a *de novo* variant). We considered this variant as possibly pathogenic, although further functional studies are necessary. Variant c.2514C>T (exon 16), present in 2 unrelated probands (one isolated and one familial case), was discarded as pathogenic as it did not segregate with NS CL/P in the familial case.

E-cadherin consists of 3 major domains: a short cytoplasmic, a single transmembrane and a large extracellular domain, with five repetitive subdomains [Paredes, et al., 2012]. The p.Asp254Asn substitution is located at a calcium-binding site comprised by the amino acid sequence Asp-Gln-Asn-Asp, at position 254-257 of CDH1 [Tepass, et al., 2000]. This site, in turn, is located in the outmost extracellular subdomain, which plays a major role in the molecular adhesive properties between cadherin trans-dimers [Shapiro, et al., 1995]. Calcium binding in the extracellular subdomains is necessary for the cis-dimerization of E-cadherin, and for conferring rigidity to the extracellular domain [Nagar, et al., 1996; Pertz, et al., 1999]. Importantly, an amino acid substitution in a nearby calcium-binding site has been reported to completely suppress the cellular adhesive properties of E-cadherin *in vitro* [Ozawa, et al., 1990]. The p.Arg784His substitution is located in the cytoplasmic domain, which is important for the assembly of catenins and for promoting cellular signaling [Nelson and Nusse, 2004]. Analyses with *in silico* tools indicated that these 2 missense variants are located in highly conserved regions of E-cadherin, and probably impair protein function (Supp. Table S4). To determine their pathogenic potential *in vitro*, we transiently transfected CHO (Chinese Hamster Ovary, ATCC number: CCL-61) cells, which are negative for E-cadherin expression, with vectors encoding the wild-type (WT) E-cadherin and variants p.Asp254Asn and p.Arg784His (Supp. Methods). As revealed by Western blot and immunocytochemistry analysis, Asp254Asn cells showed decreased total E-cadherin protein expression ($p=0.00053$; Fig. 1a), as well as reduced amount of E-cadherin located in the plasma membrane (Fig.1b), when compared with cells expressing the WT protein. Furthermore, mutant cells were unable to form cellular aggregates and exhibited a scattered phenotype, contrary to the WT cells, thus clearly indicating impaired adhesive function (Fig. 1c). Even though no structural impact was predicted in the mutated CDH1 protein (performed with FoldX, http://foldx.crg.es/: $\Delta\Delta G = -0.81$kcal/mol), our *in vitro* assays suggest that

p.Asp254Asn may lead to premature degradation, as shown for other cancer-related *CDH1* pathogenic variants [Simoes-Correia, et al., 2008; Simoes-Correia, et al., 2012; Figueiredo, et al., 2013]. The functional effect of this variant could also be related to disturbances in calcium ion binding, given its location. Arg784His cells, in turn, showed no observable difference from WT cells in total E-cadherin amount, its location in the plasma membrane, and its adhesive behavior (Fig. 1a, 1b and 1c). However, this result should not be sufficient to rule out the pathogenicity of this variant, since this *in vitro* assay may not be able to detect other types of functional effects, such as changes in interactions with other proteins, altering subsequent signaling pathways.

Families F3788 (p.Asp254Asn) and F1387 (p.Arg784His), the only two we were able to re-ascertain for cancer family history, include mutation carriers aged up to 70 years old without cancer. This observation suggests that, under certain circumstances, *CDH1* variants might cause NS CL/P alone. The invasive potential of Asp254Asn and Arg784His cells, investigated by an *in vitro* Matrigel matrix invasion assay, was similar to that of WT cells (Fig. 1d). Thus, it is possible that some E-cadherin mutations increase the risk of NS CL/P alone, while others increase risk of gastric cancer (mutations associated with higher invasiveness). However, it is of note that the current landscape of *CDH1* mutations associated with gastric cancer and CL/P does not suggest any preferential distribution of mutations along the E-cadherin molecule (Fig. 2).

The overall prevalence of rare, possibly pathogenic *CDH1* variants here reported was 2% (5 out of 221 NS OFC probands). To date, the *CDH1* mutational repertoire in the literature associated with OFC includes 10 different mutations. Six of these mutations have been reported in families also segregating gastric cancer (4 affecting mRNA splicing, 1 nonsense and 1 frameshift deletion) [Frebourg, et al., 2006; Kluijt, et al., 2012; Benusiglio, et al., 2013] and 4 were found in individuals with uncertain history of gastric cancer (one

nonsense [Bureau, et al., 2014] and 3 missense in a European cohort [Vogelaar et al., 2013]). Revisiting the list of variants described in the European cohort, we observed that the missense variant c.88C>A, which was reported in 2 patients from that study, was also found in 2 of our Brazilian controls; after removing this variant, the prevalence of possibly pathogenic *CDH1* mutations in the European cohort becomes 2%, instead of the previously reported 5% and now similar to our estimate. Furthermore, considering that 2 of the possibly pathogenic mutations here reported (p.Asp254Asn and p.Tyr341*) and most of the 10 above-mentioned mutations are predicted to cause *CDH1* loss-of-function, haploinsufficiency in critical stages of embryonic development seems to be the most likely mechanism by which rare variants in *CDH1* lead to OFC.

To investigate whether the group of 221 NS OFC probands presents a higher burden of *CDH1* rare variants compared to that of 609 Brazilian controls, we performed a gene-based Sequence Kernel Association Test (SKAT) [Wu et al., 2011]. A complementary two-tailed Fisher's exact test was performed to compare the proportion of individuals carrying at least one rare *CDH1* variant between probands and controls. Only variants with minor allele frequency <1% and with non-neutral prediction in at least one *in silico* tool were selected for these tests. To avoid methodological bias in the tests, we only included variants from regions that were covered by both SS and NGS (with minimum coverage of 25x; Supp. Table S5). No significant differences in variant enrichment were detected by SKAT when comparing the 221 NS OFC probands with our 609 control exomes (p=0.25). Similarly, no significant difference was detected in the number of individuals carrying these variants (two-tailed Fisher's exact test p=0.85; patients: 9/221 [4%]; controls: 28/609 [5%]). Most of the probably pathogenic variants here reported (p.Asp254Asn, p.Tyr341* and p.Arg784His) were found in familial cases with at least 2 affected members aside from the probands (4 out of 26 families matching the same condition, or 15.4%). Considering only probands from families with at

least 2 additional affected individuals, significant differences were detected by SKAT (P=0.002) and by two-tailed Fisher's exact test (p=0.002; patients: 6/26 [23%]; controls: 28/609 [5%]). These findings suggest that the most noteworthy *CDH1* etiological contribution to NS OFC arises from the fraction of NS CL/P cases involving moderate penetrance, which is best represented by familial cases. Since the previously suggested association between common variants and NS CL/P [Letra, et al., 2009; Hozyasz, et al., 2014] has not been supported by a large meta-analysis with GWAS data [Ludwig et al., 2012], rare variants seem to be the major contribution of *CDH1* to NS CL/P etiology.

Given the lack of correlation between type/location of *CDH1* rare pathogenic variants and NS CL/P or HDGC (Fig. 2), we speculate that a common underlying molecular mechanism could explain both phenotypes. Penetrance of *CDH1* germline mutations implicated in HDGC depends on a second hit, which frequently occurs via promoter hypermethylation of the nonmutated allele, possibly triggered by environmental factors [Oliveira et al., 2009; Zeng et al., 2015]. In this regard, a lifetime exposure to such factors would be in agreement with the higher penetrance in HDGC (80%) [Pharoah et al., 2001], as compared to NS CL/P (47%). Germline, pathogenic variants in *CDH1* could determine the resultant phenotype (NS CL/P or gastric cancer) under the influence of the following factors: time (early development or later in life), tissue (craniofacial or gastric structures), and exposure to environmental factors. In addition, given the prevalence of *CDH1* pathogenic variants found in this study, we believe that the NS CL/P-associated *CDH1* mutations are currently underrepresented, and future research should focus on their identification.

In summary, our results indicate a consistent role of rare, loss-of-function, moderately penetrant *CDH1* variants in NS CL/P etiology. To better comprehend the mechanisms linking *CDH1* to NS CL/P, as well as the risk of gastric cancer among NS CL/P individuals with mutations in *CDH1*, further studies are needed. Finally, *CDH1* testing in NS CL/P familial cases should be discussed for genetic counseling purposes.

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Fig 1. *In vitro* functional characterization of *CDH1* missense variants p.Asp254Asn (D254N) and p.Arg784His (R784H). (A) Protein expression levels of CHO cells transfected with the WT E-cadherin, the variants D254N and R784H and the empty vector (Mock). α-Tubulin was used as a loading control. Band intensity was quantified and normalized against WT E-cadherin-expressing cells. The graph shows the average ± SE of E-cadherin protein level in five independent experiments. (B) Immunocytochemistry showing E-cadherin subcellular localization (green staining). Nuclei counterstained with DAPI (blue). Scale bar represents 100µm. (C) Cell-cell adhesive properties assessed by slow aggregation assays. (D) Invasive ability of cells expressing WT or the D254N and R784H variants. The graph shows the number of invasive cells ± SE of five independent experiments. Cells expressing a known invasive mutation associated with gastric cancer (Q16H), used as a positive control, showed higher invasive potential than the studied mutations.

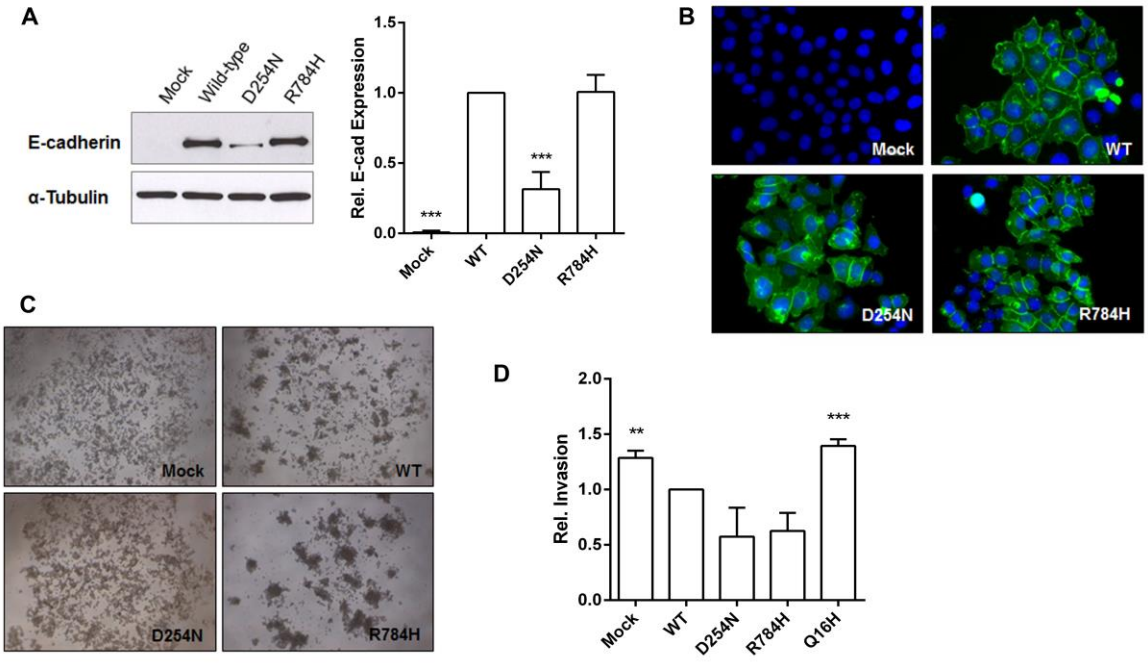

Fig. 2. Scheme of CDH1 protein showing the distribution of *CDH1* germline mutations (missense x nonsense / splice site / frameshift) associated with gastric cancer [Corso et al., 2012], cleft lip /palate and both phenotypes. Circles depict the proportion of mutations associated with each phenotype along the protein domains (signal, precursor, extracellular, transmembrane [TM] and cytoplasmic).