ROCK1 is associated with non-syndromic cleft palate

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Running title: *ROCK1* role in non-syndromic cleft palate

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ABSTRACT

Background Craniofacial morphogenesis is the result of an intricate multistep network of tightly controlled spatial and temporal signalling that involves several molecules and transcription factors organised into highly coordinated pathways. Any alteration in even one step of this delicate process can lead to congenital malformations such as cleft palate. One of the first steps in embryonal orofacial development is the migration of cells from the neural crests to the branchial arches. Next, the cells have to proliferate, differentiate, move and connect to each other in order to correctly form the palate. Cell contraction, promoted by the interaction of non-muscle myosin II and actin A, is a crucial step in morphogenesis and is regulated by ROCK1 protein.

Methods A family-based association study was carried out in order to verify whether or not genetic variants of *ROCK1* were associated with non-syndromic cleft palate (nsCP). Two cohorts from Italy and Iran, a total of 189 nsCP cases, and their parents were enrolled.

Results The rs35996865-G allele was under-transmitted in cases of nsCP [$P = 0.006$, odds ratio (OR) = 0.63 (95% C.I. 0.45–0.88)].

Conclusion This investigation reveals for the first time data supporting a role for *ROCK1* in nsCP aetiology.

KEYWORDS

cleft palate, orofacial malformation, ROCK1, association analysis

INTRODUCTION

During human organogenesis, neural crest cells (NCCs), a population of cells with migratory capacity, migrate from the dorsal surface of the neural tube to different regions of the embryo, where they differentiate into a broad range of cell types.[1] In vertebrates, the cranial NCC-derived mesenchyme contributes considerably to the development of the face and pharyngeal arches.[2] Orofacial development is a complex and delicate process, and craniofacial morphogenesis and palate formation occur through a well-orchestrated series of actions - including growth, expansion and fusion of the prominences - which is tightly controlled by the spatial and temporal signalling network.[3] Correct formation of the secondary palate is conditioned by the ability of cells to interact with neighbouring cells and adapt their reaction to the changes occurring in the extracellular matrix leading to proliferation, migration, rearrangement, adhesion and fusion of the palatal shelves.[4,5]

Aberration in any step of morphogenesis could lead to inborn malformation. In particular, the lack of fusion of embryonic palatal shelves between the 6th and 8th weeks results in cleft palate, a condition that affects about 1 to 25 per 10,000 newborns.[6] Cleft palate is one of the commonest congenital defects. It may occur as a cleft in the palate alone (nsCP), affecting only the secondary palate involving the hard palate and/or soft palate, and sometimes being limited to one cleft uvula.[7,8] In other cases, cleft palate can arise together with cleft lip, potentially involving the alveolar process and the primary palate (cleft lip with or without cleft palate, CL/P).[9] Despite their apparent similarity, however, there is epidemiological evidence to support nsCP and CL/P being different malformations with distinct but potentially overlapping aetiology.[10]

A variety of signalling pathways are involved in palate development, and several mutations in developmental genes have been found to contribute to nsCP.[11-13] During morphogenetic movement, the cytoskeleton plays a crucial role in correctly allocating cells along the midline where the fusion of palatal shelves takes place.[14] Proliferation, migration and adhesion of cells in the branchial processes involves the myosin 9 gene (*MYH9*), one of the three class II myosins expressed in non-muscle cells to exert contractile force.[15,16] In a previous investigation, our group provided evidence supporting *MYH9* as a predisposing factor for CL/P.[17] Its involvement in orofacial clefts, including nsCP, has been confirmed in different populations.[18-20]

Crucial for the contractile ability expressed by cells is the interaction of activated non-muscle myosin II with actin.[21] Activation of myosin is brought about by Rho-associated coiled-coil-containing protein kinase 1 (ROCK1). Rho kinases can act both directly, through phosphorylation

of regulatory myosin II light chain, and indirectly, by inhibiting the activity of myosin phosphatase.[22]

Interestingly, transgenic mice embryos expressing a dominant-negative form of Rho kinase (Rock) developed severe NCC-related defects, including severe craniofacial clefting affecting the nose, palate and lip.[23] These mutant embryos showed disruption of the actin cytoskeleton, associated with NCC cell death in the craniofacial region. A recent investigation demonstrated that cell motility is crucial for normal palate development and that functional integrity of a contractility pathway, that includes both the *Myh9* gene product and its activator ROCK1, is needed.[14]

The two isoforms of the main downstream effectors of the Rho family, *ROCK1* and *ROCK2*, share 65% amino acid sequence homology, and both modulate cytoskeleton organization by phosphorylating different substrates, mainly myosin light chain and myosin phosphatase.[24] Nevertheless, they have distinct functions. In fact, while ROCK2 activity facilitates phagocytosis, ROCK1 is mainly responsible for stress fiber association and focal adhesion formation.[25] Since it is ROCK1, rather than ROCK2 that could play a role in the morphogenetic events that lead to the palate formation, we set out to conduct a family-based association study of *ROCK1* polymorphisms. A sample of 136 unrelated nsCP probands from Italy and their parents, as well as a second cohort of 53 triads from Iran were investigated in order to assess for genetic heterogeneity among populations of different ethnicities. This study represents the first attempt to evaluate a potential role for *ROCK1* in nsCP onset to date.

MATERIALS AND METHODS

Study subjects

This case-parent association study was performed on two cohorts: a set of 136 unrelated trios of Italian ancestry, and a set of 53 trios of Fars ethnicity (from the central region of Iran). Triads were defined by a nsCP proband, free of any other congenital anomalies, and his/her parents. Guidelines were shared by research teams to ensure that samples from the two groups were collected uniformly. A clinical team ascertained the non-syndromic status of all probands. Exclusion criteria were exposure to known risk factors like phenytoin and warfarin, and tobacco smoking or alcohol consumption during pregnancy. Positive family history of cleft was defined as having at least one relative (second, third, or fourth degree) affected by nsCP. The study was approved by the ethics committees of Ferrara Province (Comitato Etico Unico della Provincia di Ferrara) (Study n° 130795) and Sant'Orsola-Malpighi General Hospital (project n° 14020) in Italy, and the Ethics and Research Committee of Isfahan University of Medical Sciences (process n° 189087) in Iran. The study complied with the Helsinki Declaration's Ethical Principles for Medical Research Involving Human Subjects. Prior informed written consent was obtained from all participants.

Genotyping

Four tag single nucleotide polymorphisms (SNPs) were selected by the Tagger tool using Haploview v.4.2 software.[26] The four tagSNPs (Table 2) captured 100% of the *ROCK1* alleles, with a minor allele frequency (MAF) > 0.05 and a mean max $r^2 = 0.951$.

DNA was extracted from peripheral whole blood using the GenElute™ Blood Genomic DNA Kit (Sigma), and then quantified with a Nanodrop spectrophotometer (Thermo Fisher Scientific). Genotyping was performed using KASP™ genotyping technology (LGC group), a competitive allele-specific PCR, with the ABI PRISM 7500 thermalcycler (Applied Biosystems).

Statistical analyses

Statistical analysis to check for Hardy-Weinberg equilibrium in parents and family-based association was performed as before.[27] Briefly, the transmission disequilibrium test was performed using PLINK software v1.07 on a Windows PC.[28,29] The association level was quantified by means of the odds ratio (OR), i.e. the M1/M2 ratio, where M1 and M2 were the number of times the allele was transmitted and not transmitted from heterozygous parents, respectively.[30] Multiple testing correction was performed by the "set-based test" procedure of PLINK. The method, suited

for candidate gene studies, uses permutation to determine empirical *P* values of association between the disease and the sets of independent SNPs, which are recognized considering the observed levels of linkage disequilibrium between the SNPs.

RESULTS

The study included a total of 189 patient-parents triads of two different ethnicities. A description of the enrolled sample study was reported in Table 1.

Four tagSNPs at the *ROCK1* locus were investigated (Table 2). Genotype frequencies were distributed according to Hardy-Weinberg equilibrium law, and no Mendelian errors were detected. A family-based association study was performed to determine whether *ROCK1* polymorphisms were involved in nsCP. The analysis was performed for each population (Italian and Iranian samples), as well as on the entire data set, by the transmission disequilibrium test (Table 3). In the set pertaining to the entire sample, evidence of the association between rs35996865 alleles and the occurrence of nsCP emerged; indeed, the rs35996865-G allele was under-transmitted in nsCP cases [$P = 0.006$, odds ratio (OR) = 0.63 (95% C.I. 0.45-0.88)]. The multiple-test correction method adopted in the study indicates that this association is significant at a level of 0.05; indeed, the permutation set-based tests produced a corrected *P* of 0.013. Considering the two populations individually, the effect size appeared higher among Iranians [OR = 0.52 (95% C.I. 0.27–1.02)] than in Italians [OR = 0.70 (95% C.I. 0.48–1.03)], although the association between rs35996865 and nsCP was not significant in either the Iranian (*P* value = 0.052) or the Italian sample (*P* value = 0.069).

The haplotype association analysis was conducted with a combination of all four markers. Table 4 shows *ROCK1* haplotype association data for the sample as a whole. The haplotype rs288980-T|rs17202375-A|rs2127958-T|rs35996865-G was significantly under-transmitted to nsCP probands in both the entire sample (*P* value = 0.01) and the Italian group (*P* value = 0.018).

DISCUSSION

As *ROCK1*, which codes for a kinase that regulates myosin II activity and its interaction with actin filaments, is among the genes coding for proteins responsible for the reorganization of the actin cytoskeleton, cell shape regulation, attachment, and motility, we hypothesised that it may be implicated in the nsCP phenotype. Indeed, our association analysis supported the involvement of *ROCK1* in nsCP aetiology in the entire sample of 189 trios ($P = 0.006$). The association between nsCP and *ROCK1* was confirmed ($P = 0.013$) after corrections for multiple testing, and by haplotype association analysis, which detected significant under-transmission of a specific *ROCK1* haplotype in affected children ($P = 0.01$). The association analysis was repeated on the sample stratified by ethnicity, i.e. separating the 136 trios of Italian ancestry from the 53 trios of Fars ethnicity (Iran). Basing on the ORs obtained, the rs35996865-G allele showed association levels for the two populations similar to those observed for the whole sample. Because of the small sample size, however, the statistical power of the association was limited, yielding non-significant *P* values, albeit approaching the threshold level. That being said, a significant association with a specific haplotype (rs288980-T|rs17202375-A|rs2127958-T|rs35996865-G) was observed for the Italian population.

This investigation has yielded evidence of a significant association between *ROCK1* alleles and nsCP. Specifically, the rs35996865-G allele appeared to be under-transmitted in both Italian and Iranian populations. The associated SNP rs35996865 maps in the *ROCK1* promoter region, about 500 bp upstream of the transcription start site. However, it is unknown whether this polymorphism can alter the expression level of the investigated gene. Indeed, the SNP could be in linkage disequilibrium with another causative polymorphism, or a mutation that could affect either the promoter or coding sequence of *ROCK1*.

A genome-wide association analysis of a multiethnic sample, searching for common gene variants associated with nsCP risk, discovered a genome-wide significant association with a missense variant in the transcription factor *GRHL3*.[31] No association with polymorphisms in *ROCK1* locus was found in that investigation. However, genetic heterogeneity may account for different association results. Also of note, the same genome wide investigation failed to replicate a previously reported association with *FAF1*.[32] Nevertheless, the association data collected in this investigation support a role for *ROCK1* in nsCP aetiology. Cell motility appears to be determinant in normal palate development. Functional studies in mice indicate that cell extrusion from the medial epithelial seam appears a key step for palatal shelves fusion.[14] Particularly, at molecular

level, this process requires the activation of a contractility pathway that includes Rock1 and its downstream effector NMHCIIA, the *Myh9* gene product.[14] Considering that the product of *ROCK1*, by modulating the interaction between actin and non-muscle myosin, plays a role in the cell dynamics needed for palate development, the results of this investigation suggest the need to further explore the role of *ROCK1* in nsCP in independent sample studies.

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Conflict of interest

The authors declare no competing interests.

Author contributions

A Palmieri, L Scapoli and M Martinelli designed the study, as well as writing and revising the manuscript. PG Morselli, L Pannuto, N Nouri, F Carinci and D Lauritano collected biological samples and acquired clinical data. A Plamieri, F Cura and N Nouri performed the genetic analysis and screened the scientific literature. L Scapoli performed the statistical analysis. A Palmieri, L Scapoli and M Martinelli interpreted the data. M Carrozzo supervised the manuscript. All authors took part in manuscript preparation and gave final approval of the version to be submitted.

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TABLE 1 Characteristics of the two nsCP cohorts

	ITALIAN COHORT	IRANIAN COHORT
Sex		
Male	57	25
Female	79	28
Parental consanguinity		
YES	0	21
NO	136	32
History of familial cleft		
YES	8	8
NO	128	45
Type of cleft		
Hard, soft and uvula	73	27
Soft and uvula	61	23
Uvula	2	0
Submucous	0	3

TABLE 2 Gene polymorphisms and observed allele frequency

SNP ID	CHR[a]	BP[b]	Alleles[c]	MAF[d] ITALY	MAF[d] IRAN
rs288980	18	18609580	C/T	0.42	0.50
rs17202375	18	18621273	A/G	0.14	0.07
rs2127958	18	18653610	T/C	0.48	0.41
rs35996865	18	18692344	T/G	0.28	0.22

[a]chromosome

[b]chromosome position GRCh37/hg19

[c]major allele first

[d]minor allele frequency

TABLE 3 Family-based association analysis of ROCK1 polymorphisms in nsCP

ENTIRE SAMPLE	SNP ID	Alleles[a]	T[b]	U[c]	*P* value	OR (95% CI)
	rs288980	C/T	66	73	0.553	0.90 (0.65, 1.26)
	rs17202375	A/G	39	36	0.729	1.08 (0.69, 1.70)
	rs2127958	C/T	77	67	0.405	1.15 (0.83, 1.60)
	rs35996865	T/G	55	88	*0.006*	0.63 (0.45, 0.88)
ITALIAN COHORT	SNP ID	Alleles[a]	T[b]	U[c]	*P* value	OR (95% CI)
	rs288980	C/T	45	58	0.200	0.78 (0.53, 1.15)
	rs17202375	A/G	35	30	0.535	1.17 (0.72, 1.9)
	rs2127958	C/T	57	49	0.437	1.16 (0.79, 1.70)
	rs35996865	T/G	45	64	0.069	0.70 (0.48, 1.03)
IRANIAN COHORT	SNP ID	Alleles[a]	T[b]	U[c]	*P* value	OR (95% CI)
	rs288980	C/T	22	22	1.000	1.00 (0.55, 1.81)
	rs17202375	A/G	5	7	0.564	0.71 (0.23, 2.25)
	rs2127958	C/T	25	22	0.662	1.14 (0.64, 2.02)
	rs35996865	T/G	13	25	0.052	0.52 (0.27, 1.02)

[a]major allele first

[b]number of times the minor allele was transmitted from heterozygous parents

[c]number of times the minor allele was untransmitted from heterozygous parents

Italic font is used for *P* values < 0.05

TABLE 4 Haplotype association analysis at ROCK1

ENTIRE SAMPLE	HAPLOTYPE[a]	T[b]	U[c]	CHISQ	*P* value
	C|A|C|G	15.0	16.4	0.06	0.806
	T|A|T|G	34.2	59.3	6.71	*0.010*
	C|G|C|T	29.8	23.4	0.78	0.374
	C|A|C|T	63.3	54.2	0.70	0.403
	T|A|T|T	64.7	47.6	2.61	0.106
	C|A|T|T	28.6	35.6	0.76	0.382
ITALIAN COHORT	HAPLOTYPE[a]	T[b]	U[c]	CHISQ	*P* value
	C|A|C|G	13.7	10.0	0.59	0.444
	T|A|T|G	25.4	45.4	5.61	*0.018*
	C|G|C|T	26.0	18.8	1.14	0.287
	C|A|C|T	46.0	41.1	0.27	0.604
	T|A|T|T	45.2	36.6	0.91	0.340
	C|A|T|T	22.2	26.6	0.40	0.528
IRANIAN COHORT	HAPLOTYPE[a]	T[b]	U[c]	CHISQ	*P* value
	C|A|C|G	2.9	6.9	1.64	0.200
	T|A|T|G	9.1	14.1	1.08	0.300
	C|G|C|T	3.5	5.5	0.44	0.505
	C|A|C|T	21.6	13.6	1.80	0.180
	T|A|T|T	20.8	15.9	0.67	0.414
	C|A|T|T	9.1	10.0	0.05	0.828

[a]combination of alleles of the SNPs rs288980|rs17202375|rs2127958|rs35996865

[b]number of times the minor allele was transmitted from heterozygous parents

[c]number of times the minor allele was untransmitted from heterozygous parents

Italic font is used for *P* values < 0.05