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Copy number variants of Ras/MAPK pathway genes in patients with isolated cryptorchidism

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SUMMARY

Cryptorchidism is the most common congenital disorder in boys, but the cause for most cases remains unknown. Patients with Noonan Syndrome are characterized by a typical face, growth retardation, congenital heart defects, learning disabilities and cryptorchidism. Copy number variations of Ras/MAPK pathway genes are unusual in patients with several clinical features of Noonan Syndrome; however, they have not been studied in patients with only one feature of this condition, such as cryptorchidism. Our aim was to determine whether patients with isolated cryptorchidism exhibit Ras/MAPK pathway gene copy number variations (CNVs). Fifty-nine patients with isolated cryptorchidism and negative for mutations in genes associated with Noonan Syndrome were recruited. Determination of Ras/MAPK pathway gene CNVs was performed by Comparative Genome Hybridization array. A CNV was identified in two individuals, a ~175 kb microduplication at 3p25.2, partially including *RAF1*. A similar *RAF1* microduplication has been observed in a patient with testicular aplasia. This suggests that some patients with isolated cryptorchidism may harbor Ras/MAPK pathway gene CNVs.

INTRODUCTION

Noonan Syndrome (NS, MIM: 163950) is caused by germline mutations in genes that encode components of the Ras/mitogen-activated protein kinase (MAPK) pathway (Ras/MAPK) (Rauen, 2013). This pathway is an essential mediator that transmits extracellular stimuli from cell surface receptors to the cytoplasm and nuclei, controlling cell cycle and differentiation. Signal transmission through the Ras/MAPK pathway is initiated by binding of growth factors, hormones or cytokines to their receptor tyrosine kinases, which triggers receptor dimerization and autophosphorilation at tyrosine residues. Phosphotyrosines are docking sites for an adaptor protein designated GRB2, which recruits and activates SOS1. In turn, this guanine exchange factor catalyzes the dissociation of GDP from the GDP-bound Ras (KRAS, HRAS or NRAS) inactive form, favoring Ras binding to GTP. Activated GTP-bound Ras interacts with RAF1 or BRAF, favoring their catalytic activation. These kinases phosphorylate and activate the kinases MEK (MEK1 and/or 2). Upon activation, MEK phosphorylates regulatory residues of the dual specificity kinases ERK (ERK1 and/or 2). ERK exerts its function on nuclear and cytosolic substrates that participate in cell cycle progression, differentiation and the control of cellular growth (Takai *et al.*, 2001). In addition to the backbone components of the Ras/MAPK pathway, various proteins regulate signaling through this cascade. One of them is the cytoplasmic phosphatase SHP2 that promotes the activation of the pathway (Matozaki *et al.*, 2009). Missregulation of the Ras/MAPK signaling pathway has important consequences, both during the prenatal and postnatal stages of life (Tidyman & Rauen, 2009). Activating mutations in genes that encode (i) the phosphatase SHP2 (*PTPN11* - MIM: 176876) (Tartaglia *et al.*, 2001); (ii) the guanine exchange factor SOS1 (MIM: 182530) (Tartaglia *et al.*, 2007); (iii) the serine/treonine kinases RAF1 (MIM: 164760) (Razzaque *et al.*, 2007) and BRAF (MIM: 164757) (Sarkozy *et al.*, 2009); (iv) the small GTPases KRAS (MIM: 190070) (Schubbert *et al.*, 2006) and NRAS (MIM: 164790) (Cirstea *et al.*, 2010) and (v) the dual specificity kinases MAP2K1(MIM: 176872)

and MAP2K2 (MIM: 176872) (Nava *et al.*, 2007); have been documented in patients with NS.

Patients with NS present with a characteristic facies, growth retardation, congenital heart defects, developmental delay, learning disabilities, and cryptorchidism. Undescended testes are reported in 60–77% of boys with NS (van der Burgt, 2007; Jorge *et al.*, 2009). Furthermore, analysis of the Cryptorchidism Gene Atlas discloses a strong genomic association between cryptorchidism and Ras/MAPK pathway genes (Cannistraci *et al.*, 2013).

The prevalence of NS is estimated to be 1 : 1000–2500 live births (van der Burgt, 2007), but this may be an underestimate due to patients with oligosymptomatic forms of the syndrome (Rauen *et al.*, 2010). Therefore, some of these patients may not be recognized during their life.

Since most mutations detected in NS result in a hyperactivated Ras/MAPK pathway (Tartaglia *et al.*, 2006) the possibility of increased gene dosage as its genetic etiology had been explored in different cohorts. Nevertheless, very few CNVs have been reported to date in NS (Shchelochkov *et al.*, 2008; Graham *et al.*, 2009; Chen *et al.*, 2014a; Nowaczyk *et al.*, 2014; Lissewski *et al.*, 2015). However, a comprehensive investigation of possible Ras/MAPK pathway gene dosage imbalance in patients with isolated cryptorchidism has not been performed to date. Our aim was to determine whether monosymptomatic patients who present with undescended testes, may exhibit molecular alterations in genes of the Ras/MAPK pathway.

MATERIALS AND METHODS

Study subjects

This study, as well as the Informed Consent for DNA extraction, was approved by the Ethics Committees of the University of Chile and the Central Metropolitan Health Service in Santiago, Chile.

Sixty-five patients between 1 and 15 years old with unilateral or bilateral undescended testes who had undergone orchidopexy were invited to participate. After surgery, each patient was examined by a clinical geneticist and an endocrinologist. Six patients who presented known causes of cryptorchidism such as chromosomal or syndromic forms, disorders of sex development (DSD) or other anatomic anomalies (persistent Müllerian duct syndrome, micropenis or hypospadias) were excluded. Finally, fifty-nine patients were included in this study. No mutations in nine known genes causing NS (*PTPN11*, *SOS1*, *RAF1*, *KRAS*, *BRAF*, *NRAS*, *MAP2K*, *MAP2K2* and *RIT1*) were detected by High Resolution Melting screening followed by sequencing (Rodriguez *et al.*, 2015, unpublished data). Height, weight and body mass index (BMI) *Z* score were calculated using the 2000 CDC charts (Table S1). Birth length and weight *Z* score were calculated according to Chilean birth weight and length references (Milad *et al.*, 2010).

Hormone determination

Serum testosterone was determined by competitive specific binding RIA. Serum LH and FSH were measured by immunoradiometric assays. All kits were supplied by Diagnostic System Laboratories (Webster, TX, USA). Serum AMH was assayed using the AMH/MIS ELISA kit (Immunotech- Beckman, Marseilles, France) and serum inhibin B was measured using specific two-site ELISAs (Diagnostic Systems Labs, Webster, TX, USA).

Comparative genomic hybridization array (aCGH)

Peripheral blood samples were obtained from patients after informed consent and, whenever possible, from relevant family members for DNA preparation. A customized oligonucleotide array including nine genes involved in NS (*PTPN11*, *SOS1*, *RAF1*, *BRAF*, *KRAS*, *HRAS*, *NRAS*, *MAP2K1* and *MAP2K2*) was designed. Features were selected from Agilent's eArray probe library in a custom high-resolution format of 8 × 60K (Agilent Technologies, Santa Clara, CA, USA; https://earray.chem.agilent.com/earray). The median probe spacing within targeted genes was 250 bp. In addition, the flanking regions of these genes (500 kb upstream and downstream) were also enriched on this array, with a median probe spacing of 1.03 kb. Arrays were performed as recommended by the manufacturer (Agilent Technologies, Santa Clara, CA, USA). Briefly, DNAs from the sample and a sex-matched reference (Promega, Madison, WI, USA) were double-digested with RsaI and AluI. After inactivation of the enzymes, samples and references were labeled using Cy5-dUTP for the patient's DNA and Cy3-dUTP for the reference's DNA. Labeled products were column-purified. Hybridization was performed at 65 °C with rotation for 24 h. After two washing steps, the array was scanned with an Agilent DNA microarray scanner and the extraction of microarray TIFF images was performed using the FEATURE EXTRACTION software (v9.1 Agilent Technologies).

Analysis and visualization of the data were performed using AGILENT CYTOGENOMICS v3.0.1.1 software. Comprehensive description of the statistical algorithms is available in the user's manual provided by Agilent Technologies. Analysis of CNVs detected was performed with DECIPHER v9.10 software (Firth *et al.*, 2009), to determine genes included, CNVs breakpoints and overlapping CNVs previously reported in the DECIPHER database (https://decipher.sanger.ac.uk/).

Quantitative PCR

To confirm aCGH results and to determine whether the CNVs were de novo or inherited from the parents, quantitative PCR (qPCR) was performed using genomic DNA. Primers for exons 7 and 14 of *RAF1*, and β-*actin* (reference) were designed using Primerquest® on line software (IDT, USA) (primer sequences available in Table S2). Primers sequences for β_2-*microglobulin*, a second reference gene, were as previously reported (Vaughn *et al.*, 2008). Efficiency of designed primers was determined through standard curves (15, 7.5, 3.75 and 1.88 ng) using the following formula $E = 10^{-1/slope}$ (Rassmussen, 2001). Specificity was determined by melting curve analysis. An efficiency of ~2 and absence of non-specific products was required for each pair of primer. Each sample was run in triplicate. Fifteen nanograms of genomic DNA was amplified in a 10 μL volume containing 1 × HOT FIREPol® Eva Green® HRM Mix (Solis BioDyne, Tartu, Estonia) and 0.5 μM of each primer. Amplification was performed on a Eco™ Real-Time PCR System (Illumina, San Diego, CA, USA) with a preincubation of 95 °C for 15 min followed by 40 cycles of denaturation at 95 °C for 20 sec, annealing at 60 °C for 20 sec, and extension at 72 °C for 20 sec. The dosage ratio for each amplicon was calculated through relative quantification using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

RESULTS

Molecular findings

Molecular analysis of the 59 patients with isolated cryptorchidism revealed a ~175 kb microduplication at 3p25.2 (hg19, chr3:12630854-12806345), which includes *RAF1* (MIM: 164760) and *TMEM40* (MIM: 612048), in patients P-13 and P-76 (Fig. 1A, B). Further analysis of aCGH results, through DECIPHER v9.10 (Firth *et al.*, 2009), showed that *TMEM40*, which encodes a transmembrane protein of unknown function, is completely duplicated. Meanwhile, the duplication includes 11 of the 16 coding exons of *RAF1* (Fig. 1C). These findings were confirmed by qPCR of *RAF1* exons 7 and 14 (Fig. 1D). These qPCR analyses were also applied to determine CNVs segregation. The microduplication detected in patient P-13 was detected in his mother, but not in his father. Further analysis of P-13 family revealed the same duplication in a distant relative, in a four year old boy with isolated bilateral cryptorchidism (individual III.1 in Fig. 2A). The *RAF1* microduplication observed in patient P-76 was not detected in his mother, his sister or his paternal half-brother (Fig. 2B). The DNA of the father of patient P-76 was not available for study (deceased).

CNVs positive patients

Patient P-13

This patient (Fig. 3A,B) was born to non-consanguineous healthy parents after a term pregnancy, with a birth weight

Figure 1 *RAF1* microduplications (3p25.2) detected by customized aCGH. (A, B) aCGH shows a ~175 kb microduplication at 3p25.2 (hg19, chr3:12630854-12806345) in patients P-13 and P-76, respectively. (C) Microduplication (shadow region) is shown with the DECIPHER v9.10 (Human GRCh37/hg19 Assembly), below is shown the *RAF1* exon-intron structure (minus strand) where rectangles and lines represent exons and introns, respectively; and the dotted line shows that the duplication extends and includes intron 12. The arrows indicate the localization of qPCR primers (exon 14 grey arrows and exon 7 black arrows). Microduplications reported at DECIPHER copy number variant database are shown (Patients 332401 and 615). (D) Quantitative PCR for exons 14 and 7 in patients P-76 and P-13, confirmed the aCGH results. Statistics: Mann-Whitney test (four to six independent experiments) where $*p \leq 0.05$ and ns: non-significant values with regard to Ctrl. [Colour figure can be viewed at wileyonlinelibrary.com].

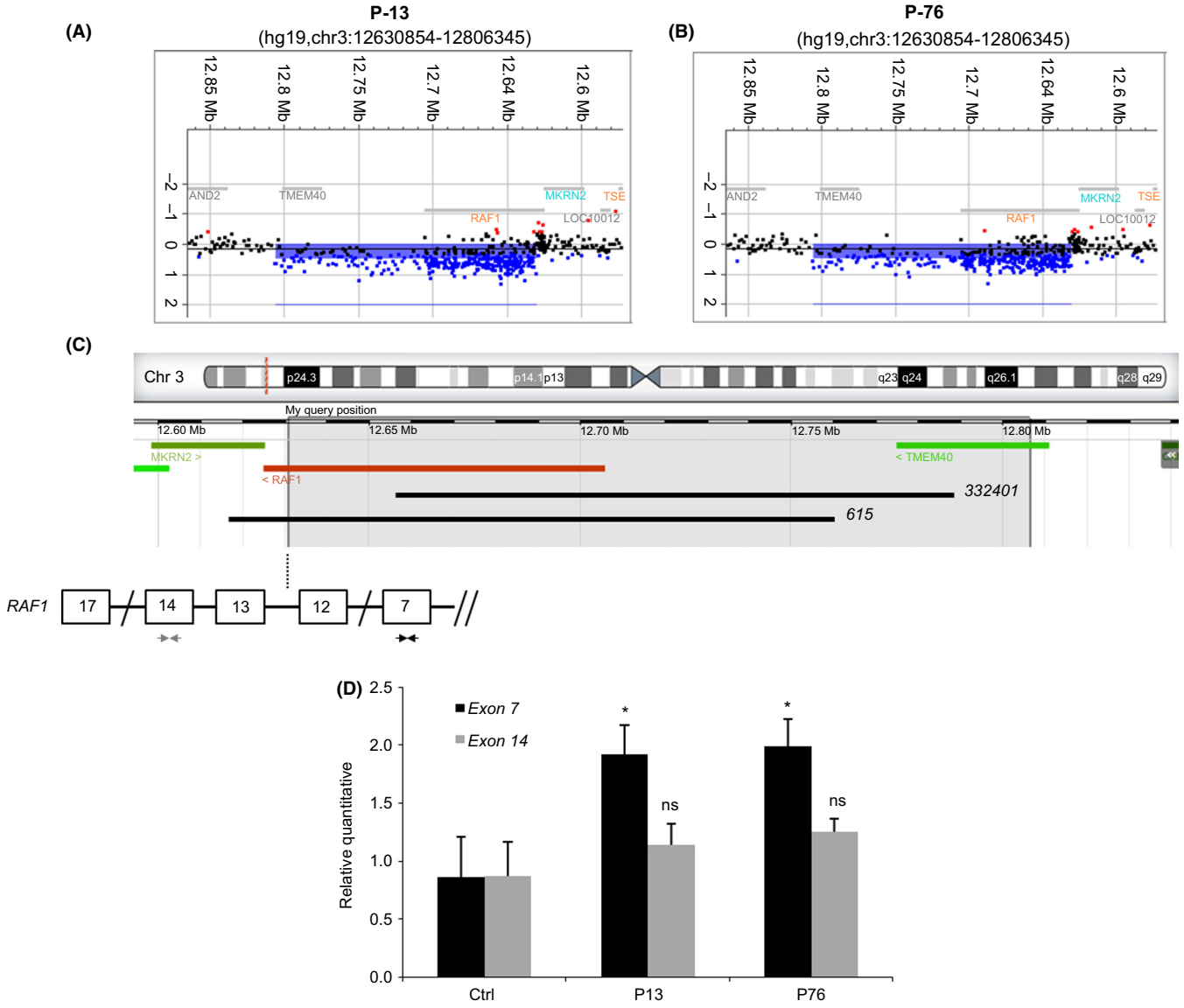
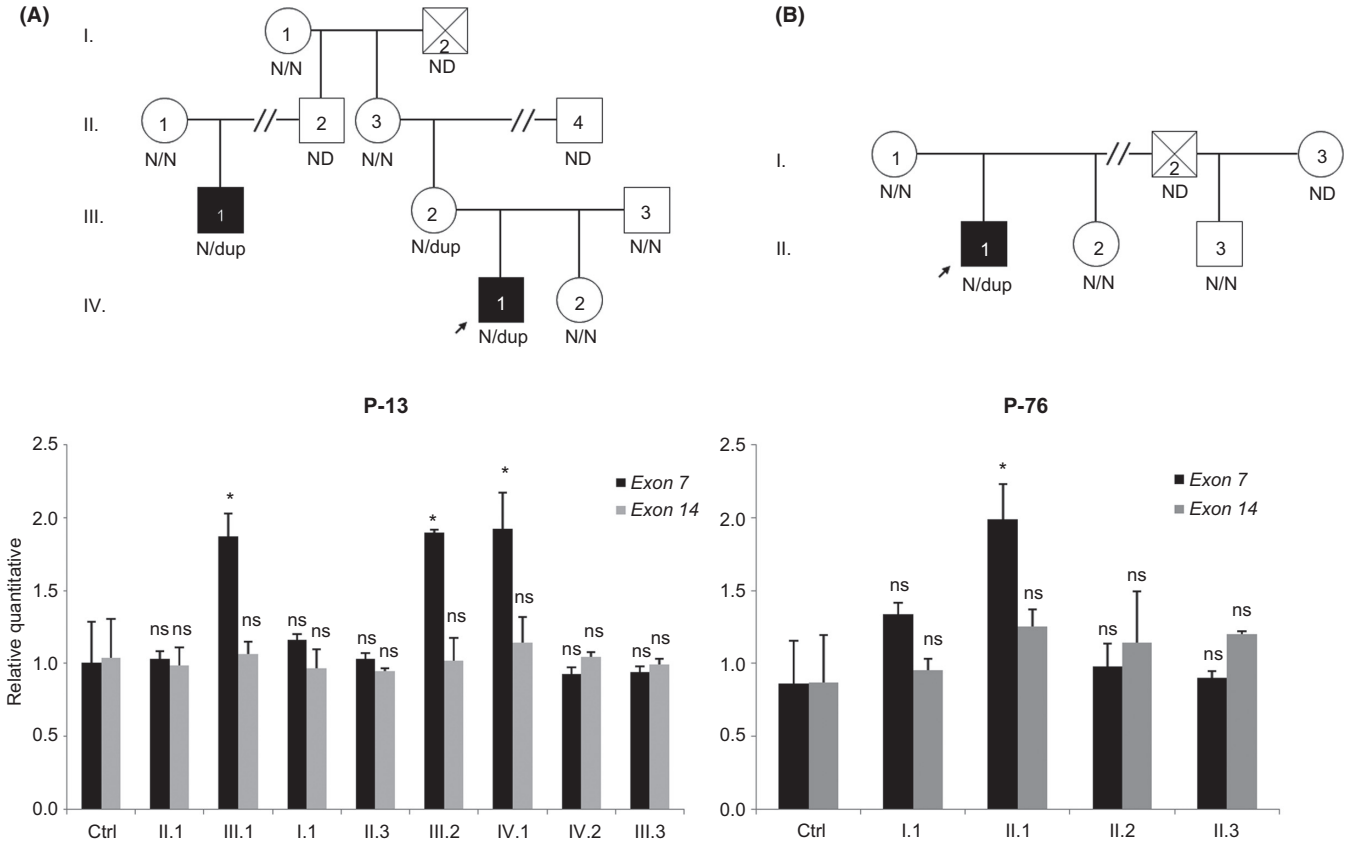


Figure 2 Determination of *RAF1* microduplication segregation by qPCR. Pedigree and qPCR results for *RAF1* exons 14 and 7 in families of patients P-13 (A) and P-76 (B). Statistics: Mann-Whitney test (four to six independent experiments) where $*p \leq 0.05$ and ns: non-significant values with regard to Ctrl. Arrows indicate index patients; dup: duplication; N: normal; ND: no determined.



of 3.7 kg (+0.68 SD), and a birth length of 52 cm (+1.6 SD). His developmental milestones were normal, but he grew quite rapidly and had a height of 123 cm (+3.7 SD) and a weight of 34 kg (+4.9 SD) at 4.5 years of age. He has relatively tall parents, with a median parental height of 177 cm. His armspan/height and SS/SI ratios were normal (1 and 1.2, respectively). Hormonal levels were normal (testosterone, LH, FSH and AMH), except for inhibin b which was lower (32.8 pg/mL) than the age-matched reference values (153 ± 91 pg/mL) (Rey *et al.*, 2005). The patient underwent a first orchidopexy at age 4.5 years (right testicle at lower inguinal canal), and a second orchidopexy in the same testicle at age 6.5 years. There was no paternal history of cryptorchidism, but a maternal distant relative had undescended testes.

Patient P-76

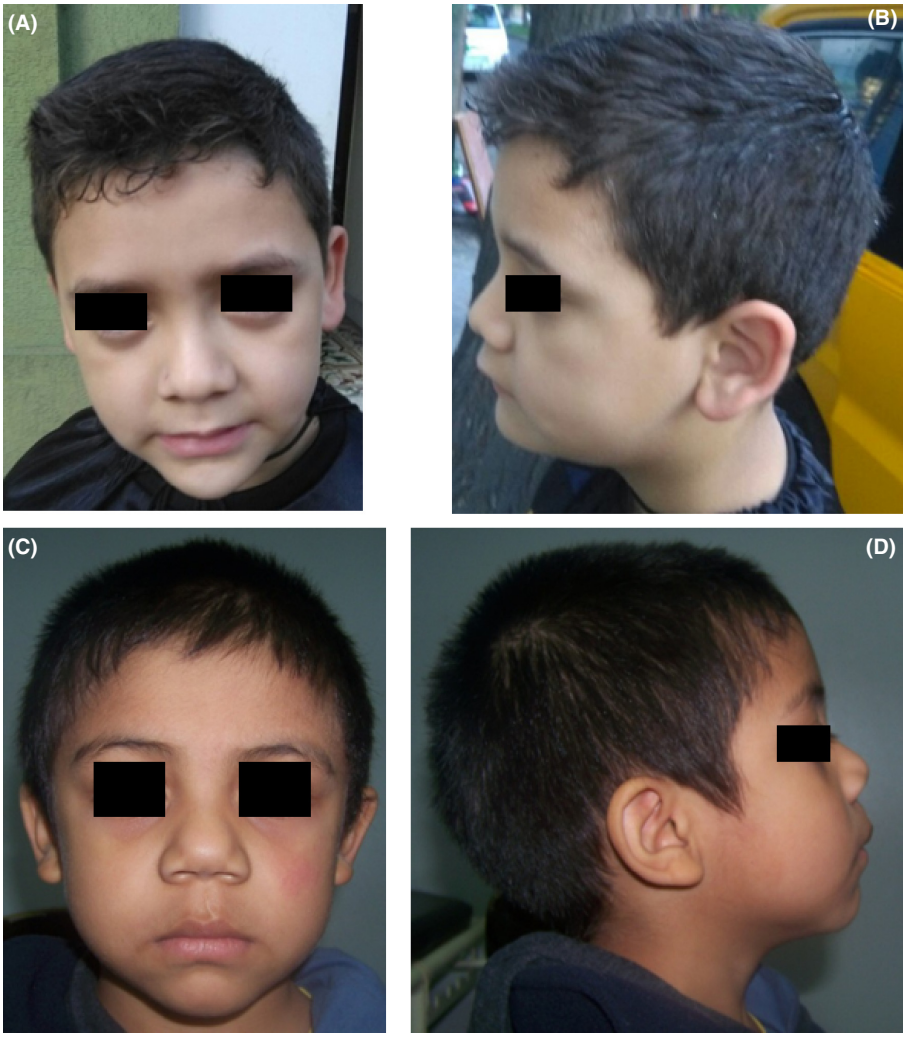
The index patient (Fig. 3C,D) was the first child of healthy non-consanguineous parents. The patient was delivered at 38 weeks, small for gestational age (SGA) with a birth weight of 2.4 kg (−2.15 SD), and birth length of 46 cm (−2.33 SD). He had unilateral cryptorchidism, which was surgically corrected at the age of 5 years and 11 months. His linear growth was normal, and his serum testosterone, LH, FSH and AMH levels were appropriate for age. His serum inhibin b, however, was lower (47 pg/mL) than the reference values (153 ± 91 pg/mL) (Rey *et al.*, 2005).

DISCUSSION

Cryptorchidism, the most common genitourinary defect of the new born male, may be associated with infertility/subfertility and testicular malignancy later in life (Hutson *et al.*, 2013). The etiology of cryptorchidism remains largely unknown, nevertheless, available data suggest the participation of genetic, maternal, and environmental factors (Kollin & Ritzén, 2014). Different approaches to identify possible genetics factors have shown that variants in *INSL3* (insulin-like 3) (Lim *et al.*, 2001; Ferlin *et al.*, 2009; Mamoulakis *et al.*, 2014); *RXFP2* (relaxin/insulin-like family peptide receptor 2) (Ferlin *et al.*, 2009) and *AR* (androgen receptor) (Aschim *et al.*, 2004) are associated with a small percentage of isolated cryptorchidism cases.

A recent report by Wang *et al.* (2016) described a genome wide genotyping analysis performed in 800 non-syndromic patients with cryptorchidism and 2688 controls, in an attempt to identify structural variations. To our knowledge, this is the most thorough CNV analysis of non-syndromic patients with cryptorchidism, but no structural variations were detected. However, the SNP arrays employed may have had insufficient coverage for Ras/MAPK pathway genes to detect smalls CNVs which may contribute to disease risk. Our study utilized a significantly higher resolution custom aCGH with an average probe distance of 250 bp for the Ras/MAPK pathway genes *PTPN11*, *SOS1*, *RAF1*, *KRAS*, *NRAS*, *HRAS*, *BRAF*, *MAP2K1* and *MAP2K2*; which enabled the detection of two CNVs.

Figure 3 Facial photographs of patients. Patients P-13 (A, B) and P-76 (C, D) showing normal phenotype without NS facial features. [Colour figure can be viewed at wileyonlinelibrary.com].



This study represents the first CNV analysis of Ras/MAPK pathway genes in a cohort of patients with isolated cryptorchidism. We observed a CNV in *RAF1* in 2 out of 59 Chilean patients who underwent orchidopexy to correct cryptorchidism. Analysis of DECIPHER copy number variant database looking for *RAF1* partial duplications, similar to that found in our patients, shows one microduplication (Fig. 1C) in a female patient (Patient: 332401) without information regarding her phenotype, nor information about cryptorchidism history in her family. In addition, a similar microduplication, which affects all *RAF1* coding exons, was observed in a patient (Patient: 615) with testicular aplasia and micropenis (Krepischi-Santos *et al.*, 2006). On the other hand, genotype analysis of 512 patients with Tetralogy of Fallot (TOF) revealed a similar microduplication to that observed in the present study, in a 15 year old girl who presented isolated TOF (Greenway *et al.*, 2009). A larger duplication (250 kb) which includes *RAF1*, *TSEN2*, *MKRN2*, and *TMEM40* genes was also identified in a 15 year-old male with TOF and some NS-like features (Luo *et al.*, 2012).

The microduplication detected in patient P-13 was shared by his mother and a maternal distant relative with bilateral cryptorchidism. Even though analysis of P-13 pedigree suggests that the detected duplication is associated with cryptorchidism, they may have arisen independently. Meanwhile, the microduplication found in patient P-76 was not detected in his mother, who reported no family history of cryptorchidism. Unfortunately, the DNA from the father of P-76 was unavailable (deceased), but no family history of cryptorchidism was known.

Patient P-76 was small for gestational age, a condition that may be associated with cryptorchidism. However, in this case, the testicle remained undescended until orchidopexy was performed at the age of almost 6 years. Most SGA children undergo descent of their testes by 1 year of age, at which time they have no higher prevalence of cryptorchidism compared to normal birth weight children (Berkowitz *et al.*, 1993; Preikša *et al.*, 2005).

Recently, improvements in genetic analysis technologies such as whole exome sequencing, have allowed the identification of further genes in NS, such as *RIT1,* a member of the Ras subfamily of small GTPases (Aoki *et al.*, 2016). A number of studies (Aoki *et al.*, 2013; Bertola *et al.*, 2014; Chen *et al.*, 2014b) have shown that the frequency of *RIT1* mutations is approximately 5% in patients with NS, similar to the frequency reported for *RAF1* mutations in these patients (Aoki *et al.*, 2016). It is noteworthy that the frequency of cryptorchidism in those patients was 70% (Bertola *et al.*, 2014), similar to that found in NS patients with

RAF1 mutations (63%) (Kobayashi *et al.*, 2010). It is possible that some of the 57 patients without any CNV detected in this study harbor *RIT1* CNVs. On the other hand, recent evidence from breeding studies of a cryptorchid rat strain (Barthold *et al.*, 2016); and from association studies in both pigs (Elansary *et al.*, 2015) and man (Barthold *et al.*, 2015) have suggested that cryptorchidism is inherited as a polygenic trait, whit different variants contributing to susceptibility. Therefore, we suggest that the *RAF1* microduplication detected in this study represents one of these variants.

Cannistraci *et al.*, created an accessible database (http://www.integratomicstime.com/cryptorchidism/) which integrates information from all cryptorchidism associated genomic loci reported in the literature and classified these loci in the corresponding biological pathway (Cannistraci *et al.*, 2013). Analysis of this Cryptorchidism Gene Atlas discloses a strong genomic association between cryptorchidism and the Ras/MAPK pathway and also a significant association between cryptorchidism and hypertrophic cardiomyopathy (HCM). Approximately 20% of NS patients have HCM (Nishikawa *et al.*, 1996), of these 95% have a *RAF1* mutation which results in increased kinase activity (Pandit *et al.*, 2007; Razzaque *et al.*, 2007). On the other hand, breeding studies in cryptorchid rat strains have shown an altered expression of genes that participate in muscle development, and in the contraction of tissues that participate in testicular descent (Barthold *et al.*, 2016). There is evidence which indicate that the Ras/MAPK pathway participates in muscle cell differentiation both with negative (Yang *et al.*, 2006; Yokoyama *et al.*, 2007) and positive effects (Li & Johnson, 2006; Cho *et al.*, 2007). Consequently, it is possible to speculate that a deregulation of Ras/MAPK pathway, through alteration of one of its pathway member (*RAF1*), could have effects over tissues involved in testicular descent. Indeed, reduced RAF1 expression was observed in cryptorchid testes biopsies (Hadziselimovic *et al.*, 2010).

In summary, our study reveals that 2/59 (3.4%) of patients with isolated cryptorchidism have a *RAF1* microduplication. Thus, we suggest that some patients with isolated cryptorchidism may present CNVs affecting a Ras/MAPK pathway gene. Further studies, such as analysis of *RAF1* gene expression (mRNA or/and protein) in related tissues, such as testicular biopsies or gubernaculum, would provide valuable evidence to support this hypothesis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

F.R. wrote the paper, coordinates patients' recruitment, direct qPCR design and assays and contributed with the conception of the study. C.V. participates in patients' recruitment and selection. F.G. standardized and performed qPCR assays. N.U. performed clinical evaluation, result interpretation and contributed to manuscript preparation. M.I.H. and P.G. performed clinical evaluation. S.C. performed orchidopexy. R.M-A., M.P-B and K.E.H. designed and performed aCGH; analyzed aCGH results and contributed to manuscript preparation. M.T.L. performed orchidopexy and participates in patients' recruitment. F.C. contributed with the conception of the study and revision of the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Clinical characteristics of patients.
Table S2. Primers designed for the qPCR confirmation.

