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The Effect of *HOXB1* gene Expression in HCFP Patient using Real Time PCR Assay in Iranian Family

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ABSTRACT

Hereditary Congenital Facial Paresis (HCFP) is a rare syndrome of isolated facial nerve palsy causing facial asymmetry and ptosis. Many described cases showed an autosomal dominant pattern of inheritance. *HOXB1* is the first identified gene in HCFP. This study was aimed to evaluation the effect of a deletion mutation on *HOXB1* gene expression in Iranian HCFP patient using Real Time PCR. A large Iranian kindred with overall 5 affected individuals along with their unaffected siblings and parents were recruited. The candidate gene *HOXB1* was screened and analyzed. After RNA extraction from blood, cDNA were synthesized according to protocol and Real Time PCR carried out using SYBR®Premix Ex Taq™ II kit. *HOXB1* expression level was analyzed by ""CT method. The results of this study demonstrates that expression level of *HOXB1* gene in homo and hetero had similar expression. However, no differences expression level of *HOXB1* gene were found. Masked-like faces, bilateral facial palsy with variable sensorineural hearing loss, as well as low-set ears and some dysmorphic features were the most remarkable findings in the affected members of the family. Our findings expand the mutational spectrum of *HOXB1* involved in HCFP, due to the number of patient that had 7bp mutation did not show any expression change in *HOXB1* gene. Investigation of *HOXB1* gene expression in larger family might have different results.

Keywords: Hereditary congenital facial paresis, *HOXB1*, Real Time PCR, Iran**.**

INTRODUCTION

Hereditary congenital facial paresis (HCFP) due to congenital cranial dysinnervation disorders is characterized by isolated dysfunction of the facial nerve (Cranial Nerve VII). HCFP be the property of the congenital cranial dysinnervation disorders. HCFP is characterized by the isolated dysfunction of the seventh cranial nerve and in addition, strabismus, hearing loss, feeding difficulties and some recognizable dysmorphic features limited to the orofacial region can be detected (Vogel et al., 2016). Linkage analysis in large families with autosomal dominant HCFP revealed one locus mapped to chromosome 3q21-q22 (HCFP1; MIM 601471) and a second to 10q21.3-q22.1 (HCFP2; MIM 604185) and *HOXB1* (17q21; HCFP3; MIM 614744) (van der Zwaag et al., 2005; Webb et al., 2012). HCFP has genetic heterogeneity and *HOXB1* (17q21; HCFP3; MIM 614744) is the first identified gene (Sahin et al., 2016) and the only known causative gene for HCFP, encoding one of 39 homeodomain containing transcription factor of the *HOX* gene family under the *HOXL* subgroup of ANTP-class (Boncinelli et al., 1997), which regulates early developmental morphogenetic processes especially the anterior–posterior patterning of the developing embryo (Mallo and Alonso, 2013). The previously reported *HOXB1*

mutations change arginine 207 to another residue in the homeodomain and alter binding capacity of *HOXB1* for transcriptional co-regulators and DNA (Vogel et al., 2016). *HOXB1* has a characteristic helix-turnhelix DNA binding motif with three alpha helical regions (a1, a2, a3) where the specificity may be contemplated by heterodimerization with *PBX1* (Piper et al., 1999). NMR studies showed that the conserved hexapeptide of *HOXB1* (TFDWMK) stabilizes binding of *PBX1* and *HOXB1* to DNA (Slupsky et al., 2001).

To date, mutations in ten *HOX* genes have been found to cause different human disorders (Quinonez and Innis, 2014). Molecular modeling and in vitro functional analysis predicted the arginine-to-cysteine change at position 207 in the homeodomain to diminish binding of *HOXB1* to transcriptional co-regulators and DNA, thereby altering transcriptional activity of *HOXB1* (Webb et al., 2012).

The analysis of gene expression for mRNA sequences requires precise, sensitive, and reproducible measurements. Gene expression levels were commonly determined using northern blot analysis. However, this technique requires a large quantity of RNA and is timeconsuming (Dean et al., 2002). At present Real Time PCR is the most sensitive method for the detection of low abundance mRNAs, and can be used for different applications, such as clinical diagnostic, for the gene expression analysis of tissue-specific, and for plant studies (Gachon et al., 2004). Real Time PCR is typically referenced to an internal control gene. The conditions of the experiment should not influence the expression of this internal control gene (Schmittgen and Zakrajsek, 2000). However, many studies showed that internal standards, mainly reference genes used for the quantification of mRNA expression, could vary with the experimental conditions. A lot of reference genes are well described for the normalization of expression signals (Stürzenbaum and Kille, 2001). The most common are actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal genes, cyclophilin, 18s rRNA and elongation factor 1-a (ef1a) (Dean et al., 2002). Adenine phosphoribosyl transferase (aprt) and tubulin may also be used. Many studies on housekeeping gene expression mainly deal with human tissues, bacteria and viruses (Volkov et al., 2003). Consequently, choosing an internal control is too important to gene expression quantify (Langer et al., 2002). The use of GAPDH as the internal standard could be a valuable alternative to quantify genes of interest, keeping in mind that it could reduce the variations of expression (Nicot et al., 2005).

The aim of present study was to evaluation the effect of a deletion mutation on *HOXB1* gene expression in HCFP patient using Real Time PCR in a large Iranian family.

MATERIALS AND METHODS

Sample collection

36 blood samples were obtained from patients randomly. Clinical and pathological data of patients were collected. The experiment protocols and informed consent forms were approved by the Human Studies Committee at the Affiliated the Medical Science University.

RNA extraction and cDNA synthesis

Total RNA was extracted from blood samples using the RNX™-Plus solution (SinaClon, IRAN) according to the manufacturer's instructions, except for an extended 1-h treatment with DNase I. RNA was analyzed by Thermo Scientific NanoDrop™ 1000 Spectrophotometer to check its purity and concentration, and electrophoresd on 2% agarose gel to confirm its integrity. One microgram of RNA was used for complementary DNA (cDNA) synthesis by using random hexamer priming and PrimeScript™-RT reagent kit (TaKaRa, Japan). Synthesized cDNA was then checked spectrophotometrically to estimate its concentration.

Real Time PCR

All samples were carried out on a rotor gene 6000 corbett detection system and Real Time PCR using SYBR®Premix Ex Taq™ II kit (TaKaRa, Japan) according to the manufacturer's instructions. Thermal cycling conditions were an initial activation step for 5 min at 95 °C followed by 40 cycles at 95 °C for 15 s and 65 °C for 1 min. No template control (NTC) consisting of H2O was included in each run. Forward and reverse primers sequences have shown in table 1. Melting curve analysis was performed to verify specificity of PCR products. Besides, PCR products were electrophoresed on 2 % agarose

gel to verify product sizes and specificity. For Real Time PCR analysis, all samples were normalized to GAPDH. The mean value in each triplicate was used to calculate (”Ct = Ct mean lncRNAs-Ct mean GAPDH). Expression fold changes were calculated using $2^{-""Ct}$ methods. The qPCR assays were performed in triplicate and the data were presented as the mean ± standard error of the mean (SEM).

Statistical analysis

The pairwise fixed reallocation randomization test with 2000 iterations in the REST 2009 software was used to determine the significances. The level of statistical significance was set at P<0.05. Statistical analyses of demographic and clinical data were performed using SPSSv.15.0.1 (SPSS Inc., Chicago, IL). Chi-square and independent t tests were used for testing the relationship between categorical variables. Significance was defined as P<0.05.

RESULTS

General statistical information

Data have been analyzed based on the information taken from questionnaires, interviews, and clinical and laboratory tests.

Expression of *HOXB1* gene

The results of this study demonstrates that expression level of *HOXB1* gene in hemo and hetero had similar expression. 22 and 9 out of 36 were hetero and hemo respectively for this mutation. However, no differences expression level of *HOXB1* gene were found. Expression level of *HOXB1* gene have shown in figure 1.

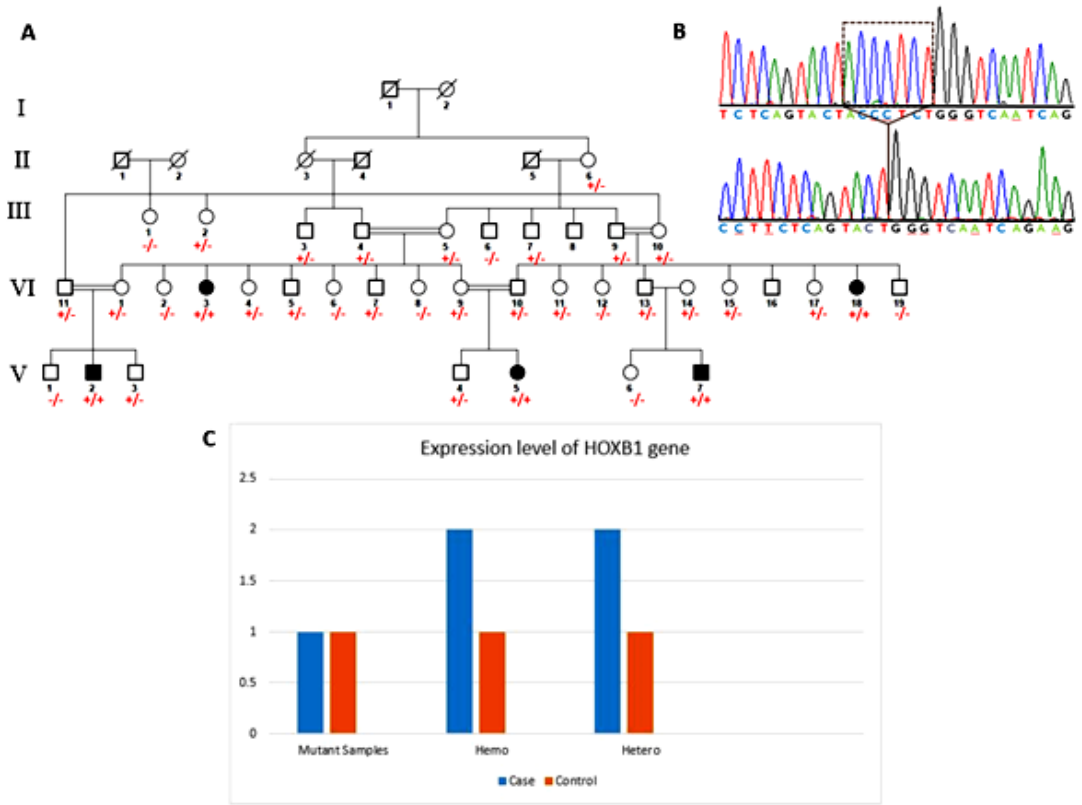

Fig. 1: A) Pedigree of the Iranian family with five individuals affected by HCFP (indicated as solid black symbols). Segregation of the LOF mutation with the family with mutant allele shown by ‘+’ and wild-type allele shown by ‘Š’. B) A section of Sanger sequencing Chromatograms for the homozygous mutation (below) and wild type (top) are depicted and demonstrate the insertion (in the box). C) Expression level of *HOXB1* gene

DISCUSSION

Facial paresis is a rare, hereditary congenital and nonprogressive. From 1995 till now only 3 cases of the disease have been reported in the world therefore there is no definition specific prevalence of this disease and its hereditary have been reported as autosomal recessive (Uyguner et al., 2015). Facial paresis due to the similarity of symptoms named Moebius syndrome. Three gene loci associated with HCFP have been reported HCFP1, HCFP2 and HCFP3 respectively (Alrashdi et al., 2010). *HOXB1* gene have been introduced the most likely gene related to HCFP. In 2015 a study on 56 family involved with HCFP indicated a mutation in *HOXB1* gene which had occurred in the same location (Miller, 2007) .

The first report was done by Goddard et al in 1996 showed a nonsense mutation in *HOXB1* gene in mice caused impaired development of facial nerve but no similar cases were reported in human (Goddard et al., 1996). In 1999 Piper et al investigated the existence of a binding motif in *HOXB1* gene (Piper et al., 1999). The role of *HOXB1* gene and its relationship with other developmental genes had been evaluated by Carolyn et al in 2001 (Slupsky et al., 2001). The results of Carolyn et al indicated that the structure of the protein product has been quite stable.

177 out of 272 families with MBS or HCFP previously reported by Webb et al. (2012), along with 95 samples (56 from Netherlands and 39 from Turkey), 3 cases were found to carry mutations in *HOXB1*, establishing the frequency of *HOXB1* mutations to be 1.1% in patients afflicted with congenital facial paralysis (Webb et al., 2012). Sahin et al. 2016 showed case reported has p.Arg230Trp mutation in the *HOXB1* gene which causes HCFP3 in a large family of Turkish origin. This mutation brings the total number of *HOXB1* mutations identified in HCFP3 to four within five families. This mutation lies in the DNA-binding homeodomain of *HOXB1* located in between 203 and 262 residues, which is highly conserved among several species (Sahin et al., 2016).

Michielse et al in 2006 using linkage analysis method find HCFP 3q21 in a Pakistani family and did not find result of on search for other candidate genes (Michielse et al., 2006). Another homozygous missense mutation in *HOXB1*, also affecting arginine 207 [c.620G>A/p. (Arg207His)] was found in a Turkish girl with bilateral facial weakness, left esotropia, left ptosis, and midface retrusion. Auditory brainstem response test revealed normal results and MRI scans showed no structural anomalies (MacKinnon et al., 2014). Webb et al. reported that another missense mutation in the critical homeodomain, would reduce binding of *HOXB1* to transcriptional co-regulators and DNA, hence altering transcriptional activity of *HOXB1* (Webb et al., 2012). Clinical features of all affected individuals including hearing loss, midface retrusion, lagophthalmos, oral dysfunction, swallowing difficulties, dysarthria, and speech delay suggested the diagnosis of HCFP (Vogel et al., 2016). The results of Vogel et al. 2016 in Germany indicated in a family with two consanguineous marriages and four individuals affected by HCFP over two generations, we identified a novel homozygous one-base-pair substitution in the *HOXB1* gene leading to introduction of a premature stop codon. This germline mutation most probably represents the first loss-of-function *HOXB1* allele associated with HCFP (Verzijl et al., 2003).

CONCLUSION

HOXB1 gene Identify was an effective step in HCFP. Accordingly comparative study of the disease using new techniques such as SNP array, NGS and Real Time PCR can be a milestone in the recognition of HCFP. Our findings expand the expression of *HOXB1* involved in HCFP, and consolidate the role of the gene in development of autosomal recessive type of HCFP. The number of patient that had 7bp mutation did not show any expression change in *HOXB1* gene. Investigation of *HOXB1* gene expression in larger family might have different results.

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