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Regular paper

Expression of RUNX2 and its signaling partners TCF7, FGFR1/2 in cleidocranial dysplasia

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RUNX2 is a member of the PEBP2/CBF transcription factors family controlling the expression of genes whose products are essential for bone formation. Mutations in the *RUNX2* gene may be associated with cleidocranial dysplasia (CCD), a rare skeletal disease characterized by stature aberrations, delayed closure of the cranial sutures, hypoplastic or aplastic clavicles, and multiple dental abnormalities. As RUNX2 is involved in many signaling pathways, we hypothesize that CCD may be associated with their changes. We determined the expression of *RUNX2* and its signaling partners *TCF7*, involved in canonical Wnt signaling, and fibroblast growth factor receptors, *FGFR1* and *FGFR2* in periodontum of CCD patients and control individuals. We did not observe any differences between the level of *RUNX2, TCF7* and *FGFR1/2* mRNA, determined by real-time PCR, in CDD patients and controls. Therefore, RUNX2 signaling pathways with their partners TCF7 and FGFR1/2 may not be involved in CCD pathogenesis.

Key words: RUNX2, Wnt signaling, TCF7, fibroblast growth factor signaling, FGFR1; FGFR2

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INTRODUCTION

The human *RUNX2* gene (also designated *Cbfa1, PEBP2A1, Osf2* or *AML3*) is a member of the mammalian *Runx* (runt-related transcription factor x) gene family, including also *Run2* and *Run3*. All of these genes encode transcription factors homologous to the *Drosophila* runt protein whose products form heterodimers with transcriptional co-activator core-binding factor β, Cbfβ, binding the same DNA consensus sequence 5'-PyGPyG-GTPy-3' with runt-homologous DNA binding domains (Jonason *et al*., 2009). RUNX2 is essential for proper tooth morphogenesis and it interacts with Runx3 to regulate maturation and proliferation of chondrocytes (D'Souza *et al*., 1999; Wuelling & Vortkamp, 2011). *RUNX2* transcription can be initiated either from an upstream promoter or a promoter within the third intron and its primary transcript may be alternatively spliced, which results in three known *RUNX2* transcripts. Moreover, several post-translational modifications can be imposed on the polypeptide product of *RUNX2* expression (Shui *et al*., 2003). Multiple signaling pathways are involved in the regulation of the *RUNX2* gene expression at multiple levels. These include growth hormones, cytokines and hormones, playing an important role in signal transduction regulating spatial and temporal expression of *RUNX2* (Komori, 2011; Nishimura *et al*., 2012). Aberrant expression of *RUNX2* is important, because RUNX2 activity should be tightly regulated temporally and spatially to properly control the rate of bone formation (Nishimura *et al*., 2012). It is also a key target of mechanical stimulation in human periodontal ligament osteoblastic cells (Ziros *et al*., 2002). In general, the role of RUNX2 in tooth formation seems to be of a special significance because of its essential function for the later stages of this process and differentiation of mesenchymal cells to osteoblasts (Ducy *et al*., 1997).

An important role of *RUNX2* in osteoblast differentiation is underlined by a network of protein-protein interactions with essential role of RUNX, SP7, FGF and Wnt signaling (Komori, 2011).

Mutations in the *RUNX2* gene may play an important role in the pathogenesis of cleidocranial dysplasia (CCD, OMIM 119600, cleidocranial dysostosis), a skeletal disorder associated with dysplatic clavicles, patent sutures and multiple supernumerary teeth (Jensen & Kreiborg, 1990; Lee *et al*., 1997; Bergwitz *et al*., 2001). Disturbed pattern of dentition is probably the most pronounced hallmark of CCD. The absence of functional RUNX2 may result in the inhibition of the development of molars and incisors at the late bud and bell stage, respectively (D'Souza *et al*., 1999; Aberg *et al*., 2004). However, besides a direct action of RUNX2 on the pattering of dentition, this protein is involved in a complex network of interactions with many signaling proteins and transcriptional factors (Komori, 2011). This interaction involves the Wnt/beta-catenin, TGF-beta/BMP, FGF, Notch and Hedgehog signaling pathways, and RUNX2, Osterix, ATF4, TAZ, and NFATc1 transcriptional factors, but they represent only part of all proteins involved (Deng *et al*., 2008). Therefore, in assessing the connection between disturbed *RUNX2* expression and pathological phenotypic trait, such as CCD, all these interactions should be taken into account. Due to high number of proteins involved in these interactions it is rather difficult to study them in a single cycle of experiments. In this work we investigated the level of mRNA expression of *RUNX2* and three genes interacting with it — *TCF7, FGFR1* and *FGFR2,* in teeth of CCD patients and controls.

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Abbreviations: ACTB, β-actin; CCD, cleidocranial dysplasia; FGFR1, fibroblast growth factor receptor 1; FGFR2, fibroblast growth factor receptor 2; FGFs, fibroblast growth factors; RT-qPCR, real time reverse transcription quantitative polymerase chain reaction; Runx, runt-related transcription factor x; TCF7, transcription factor 7

The *TCF7* (Transcription factor 7 (T-Cell Specific, HMG-Box) also known as *Tcf1*) gene is located at 5q31.1 and encodes a transcriptional activator that plays an important role in lymphocyte differentiation. It is a Wnt/β-catenin target gene and may play a role in cementoblast/osteoblast differentiation (Silverio *et al*., 2012). Wnt signaling may increase the expression of RUNX2 through binding of TCF7 to its promoter (Gaur *et al*., 2005). On the other hand, TCF7 was induced by RUNX2 in the process of chondrocyte maturation and proliferation (Mikasa *et al*., 2012). No information on association of this gene with CCD is available.

Fibroblast growth factors (FGFs) are a family of 22 protein ligands acting in paracrine or endocrine fashion (Goetz & Mohamadi, 2013). They are bound by their receptors, encoded by the *FGFR1-4* genes, which dimerize upon binding and signal through their tyrosine kinase activity. FGF signaling is important for development and tissue homeostasis. Mutations in the *FGFR1* and *FGFR2* genes play a role in the pathogenesis of craniosynostosis and other skeletal growth disorders (Morriss-Kay & Wilkie, 2005). FGF/FGFR signaling stimulates expression, transcriptional and DNA-binding activity of RUNX2 (Kim *et al*., 2003; Lu *et al*., 2012) and is important in controlling of self-renewal and differentiation of dental epithelial stem cells (Chang *et al*., 2013). The FGF signaling is essential in mammalian tooth development (Li *et al*., 2014). No direct association on FGF signaling and CCD has been reported although a putative functional relationship between type 2 craniosynthosis and CCD was suggested (Rice *et al*., 2003).

Therefore, RUNX2 may be involved in mutual regulation of TCF7 and FGFR1/FGFR2 in the process of osteoblast differentiation in tooth development (Fig. 1). It is possible that this network may be disturbed in pathologies associated with tooth development, including CCD. We speculate that the level of the expression of genes encoding these proteins may be associated with CCD. To test this hypothesis we checked the expression of the *RUNX2, TCF7, FGFR1* and *FGFR2* genes in teeth of CCD patients and controls.

MATERIALS AND METHODS

Patients and ethics. This study included 12 CCD patients (7 males and 5 females, age range 17–22) and 15 controls who were enrolled at the Department of Developmental Dentistry and Department of Orthodontics, Medical University of Lodz, Lodz, Poland in 2012–2014. The diagnosis of CCD was determined on the basis of clinical signs of the disease and supported by mutational analysis at the Department of Clinical Genetics, University of Lodz, Lodz, Poland. The only treatment the patients had undergone was extraction of supernumerary teeth. Control individuals were age- and sex-matched, they did not show signs of CCD and had tooth extraction performed for variousreasons, most often due to pain.

This study was approved by the Bioethics Committee of the Medical University of Lodz, Lodz, Poland (permit no. RNN/151/11/KE) and each patient gave an informed consent, which in the case of minors was signed by their parents. All individuals were personally interviewed and 3 CDD cases were considered as hereditary, while the rest — as sporadic.

Reagents. RNeasy Mini Kit was obtained from Qiagen (Valencia, CA, USA), Maxima First Strand cDNA Synthesis Kit for RT-qPCR was purchased from Thermo Scientific (Northpoint Parkway, FL, USA), Tris, EDTA were obtained from Sigma (St. Louis, MO, USA). All other reagents were of the highest purity available.

RNA isolation. Total RNA was isolated with RNeasy Mini Kit from periodontum obtained by mechanical scraping from teeth extracted from 12 CCD patients and 15 control individuals without CCD. All samples were obtained following informed consent for genetic testing, but the tooth extraction itself was a routine procedure

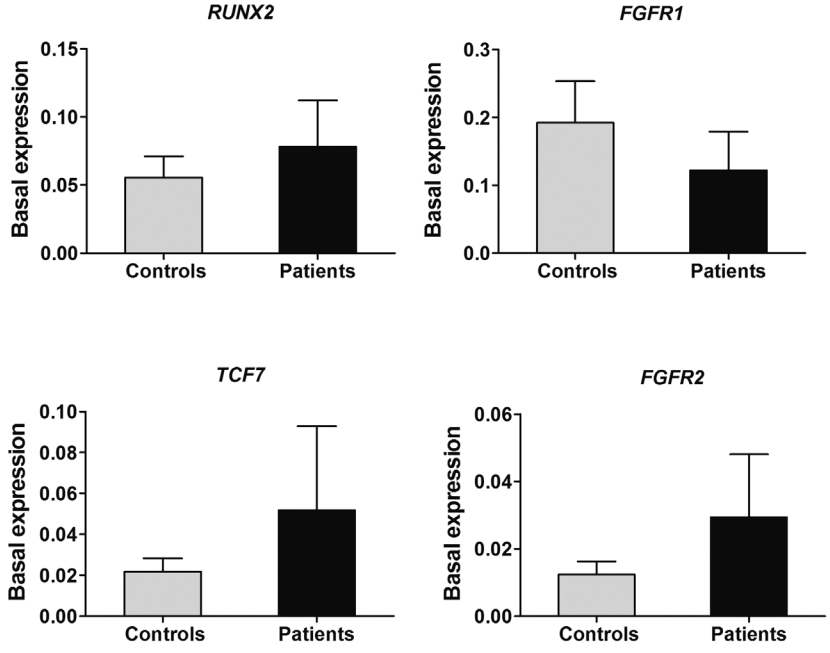

Figure 1. Relative mean mRNA expression of the *RUNX2, TCF7* and *FGFR1/2* genes in patients with cleidocranial dysplasia (n=12) and controls (n = 15).
Expression was calculated according to the $2^{-\Delta C_T}$ formula with human β-actin as a reference gene. Each measurement was performed in duplicate, error bars denote S.E.M.

followed for clinical reasons. RNA samples were stored in the TE buffer (5 mM Tris/HCl, 0.1 mM EDTA, pH 8.5) at –20°C until use.

Gene expression and data analysis. cDNA was synthesized with Maxima First Strand cDNA Synthesis Kit. The TaqMan Gene Expression Assay was employed to analyze the expression of the *RUNX2* (probe ID Hs00231692), *TCF7* (Hs00175273), *FGFR1* (Hs00915142) and *FGFR2* (Hs01552918) genes in a thermal cycler CFX96™ Real-Time PCR Detection System (BIO-RAD Laboratories, Hercules, CA, USA). The gene for human β-actin (*ACTB*) was used as an internal control for all real time reverse transcription quantitative polymerase chain reactions (RT-qPCRs). The thermal conditions were: 10 min of polymerase activation at 95°C, followed by 40 cycles of 30 s denaturation at 95°C and 60 s annealing/extension at 60°C. Each sample was run in duplicate. Negative controls containing no DNA were included in each RT-qPCR run. The cycle threshold (C_T) values were calculated automatically by CFX96 Real-Time PCR Detection System software and the $2–\Delta C_T$ method was used to calculate relative basal expression of mRNA of each gene normalized to the average mRNA, i.e. ΔC_T was obtained by the subtraction of C_T of *ACTB* mRNA from C_T for each gene (Schmittgen & Livak, 2008). Data were analyzed by the Student's *t*-test for CCD patients and controls.

RESULTS AND DISCUSSION

We did not observe any difference ($p>0.05$) between the expression level of the *RUNX2, TCF7, FGFR1* and *FGRFR2* genes in CCD patients and controls (Fig. 1).

The *RUNX2* gene plays an important role in the tooth formation and pattering through a complex network of signaling, including many protein-protein interactions. Our knowledge about all relationships within this network is far from completeness, which impedes molecular view of pathologies associated with impaired function of RUNX2. So far, CCD is the only abnormality associated with mutations in the *RUNX2* gene. However, molecular mechanism on the pathway from mutated *RUNX2* to the CCD phenotype is largely unknown. Moreover, about 30% of all CCD cases are not associated with *RUNX2* mutations (Baumert *et al*., 2005; Zhang *et al*., 2010). Some information on this mechanism may be provided by comparing the level of expression of *RUNX2* itself and genes whose products directly interact with it. In the present work we analyzed the expression of three such genes, *TCF7, FGFR1* and *FGFR2,* which were not studied in CCD so far. Our results indicated that the signaling pathways of RUNX2 along with these three proteins may not be involved in CCD pathogenesis.

It was shown that a TCF regulatory element responsive to canonical WNT signaling is located in the *RUNX2* promoter and TCF7 along with β-catenin is recruited to *RUNX2* (Gaur *et al*., 2005). It was also shown in that report that coexpression of TCF7 with proteins of canonical WNT pathways resulted in overexpression of *Runx2* in mouse. The conclusion from the data obtained in that research was that TCF7 with WNT signaling, activated the expression of *Runx2* to control osteoblast differentiation and skeletal development. Therefore, disturbance in this signaling pathway may lead to disturbed osteogenesis, resulting also in anomalous tooth formation. However, this abnormality may not involve that observed in CCD, as our data suggest.

FGF/FGFR signaling may regulate RUNX2 expression and activity in several ways, including activation of protein kinase C (PKC) (Kim *et al*., 2003). RUNX2 has multiple phosphorylation sites and its phosphorylation may be associated with both inhibition and activation (Franceschi *et al*., 2003; Wang *et al*., 2004; Xiao *et al*., 2000). It was shown that FGFR2 upregulated the transactivating activity of exogenously overexpressed RUNX2, but inhibitors of PKC hampered this overexpression. Therefore, although PKC signaling may increase expression of *RUNX2*, which is usually associated with increase in the activity of RUNX2, this signaling pathway may also stimulate transactivation functions of RUNX2 by other mechanism(s) (Kim *et al*., 2006). FGF2 may mediate transactivation of RUNX2 by phosphorylation of its Ser247 residue. It was shown that RUNX2 did not exert direct effects on the promoter of the FGFR2 gene, but it inhibited the stimulatory effect of Twist1 on this promoter (Lu *et al*., 2012).

Our patient population was relatively small and so, our results have limited significance and this research should be continued to draw more robust conclusions. However, CCD is a rare disease (incidence about 1:200 000) and our results suggest that the interaction of RUNX2, with TCF7 and TGFR1,2 may not be involved in its pathogenesis, which is an important information in the context of contribution of RUNX2 to the etiology of this disease and its involvement in many signaling pathways.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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REFERENCES

Aberg T, Cavender A, Gaikwad JS, Bronckers AL, Wang X, Waltimo-Siren J, Thesleff I, D'Souza RN (2004) Phenotypic changes in dentition of Runx2 homozygote-null mutant mice. *J Histochem Cytochem* **52**: 131–139.

Baumert U, Golan I, Redlich M, Aknin JJ, Muessig D (2005) Cleidocranial dysplasia: molecular genetic analysis and phenotypic-based description of a Middle European patient group. *Am J Med Genet A* **139A**: 78–85.

Bergwitz C, Prochnau A, Mayr B, Kramer FJ, Rittierodt M, Berten HL, Hausamen JE, Brabant G (2001) Identification of novel CBFA1/RUNX2 mutations causingcleidocranial dysplasia. *J Inherit Metab Dis* **24**: 648–656.

Chang JY, Wang C, Liu J, Huang Y, Jin C, Yang C, Hai B, Liu F, D'Souza RN, McKeehan WL, Wang F (2013) Fibroblast growth factor signaling is essential for self-renewal of dental epithelial stem cells. *J Biol Chem* **288**: 28952–28961.

Deng ZL, Sharff KA, Tang N, Song WX, Luo J, Luo X, Chen J, Bennett E, Reid R, Manning D, Xue A, Montag AG, Luu HH, Haydon RC, He TC (2008) Regulation of osteogenic differentiation during skeletal development. *Front Biosci* **13**: 2001–2021.

D'Souza RN, Aberg T, Gaikwad J, Cavender A, Owen M, Karsenty G, Thesleff I (1999) Cbfa1 is required for epithelial-mesenchymal interactions regulating tooth development in mice. *Development* **126**: 2911–2920.

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G (1997) Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* **89**: 747–754.

Franceschi RT, Xiao G, Jiang D, Gopalakrishnan R, Yang S, Reith E (2003) Multiple signaling pathways converge on the Cbfa1/Runx2 transcription factor to regulate osteoblast differentiation. *Connect Tissue Res* **44**: 109–116.

Gaur T, Lengner CJ, Hovhannisyan H, Bhat RA, Bodine PV, Komm BS, Javed A, van Wijnen AJ, Stein JL, Stein GS, Lian JB (2005) Ca-

nonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. *J Biol Chem* **280**: 33132–33140.
Goetz R, Mohamadi M (2013) Exploring mechanisms of FGF signalling through the lens of structural biology. *Nat Rev Mol Cell Biol* **14**: 166–180.
Jensen BL, Kreiborg S (1990) Development of the dentition in cleidocranial dysplasia. *J Oral Pathol Med* **19**: 89–93.
Jonason JH, Xiao G, Zhang M, Xing L, Chen D (2009) Post-translational Regulation of Runx2 in Bone and Cartilage. *J Dent Res* **88**: 693–703.
Kim HJ, Kim JH, Bae SC, Choi JY, Kim HJ, Ryoo HM (2003) The protein kinase C pathway plays a central role in the fibroblast growth factor-stimulated expression and transactivation activity of Runx2. *J Biol Chem* **278**: 319–326.
Kim BG, Kim HJ, Park HJ, Kim YJ, Yoon WJ, Lee SJ, Ryoo HM, Cho JY (2006) Runx2 phosphorylation induced by fibroblast growth factor-2/protein kinase C pathways. *Proteomics* **6**: 1166–1174.
Komori T (2011) Signaling networks in RUNX2-dependent bone development. *J Cell Biochem* **112**: 750–755.
Lee B, Thirunavukkarasu K, Zhou L, Pastore L, Baldini A, Hecht J, Geoffroy V, Ducy P, Karsenty G (1997) Missense mutations abolishing DNA binding of the osteoblast-specific transcription factor OSF2/CBFA1 in cleidocranial dysplasia. *Nat Genet* **16**: 307–310.
Li CY, Prochazka J, Goodwin AF, Klein OD (2014) Fibroblast growth factor signaling in mammalian tooth development. *Odontology* **102**: 1–13.
Lu Y, Li Y, Cavender AC, Wang S, Mansukhani A, D'Souza RN (2012) Molecular studies on the roles of Runx2 and Twist1 in regulating FGF signaling. *Dev Dyn* **241**: 1708–1715.
Mikasa M, Rokutanda S, Komori H, Ito K, Tsang YS, Date Y, Yoshida CA, Komori T (2011) Regulation of Tcf7 by Runx2 in chondrocyte maturation and proliferation. *J Bone Miner Metab* **29**: 291–299.
Morriss-Kay GM, Wilkie AO (2005) Growth of the normal skull vault and its alteration in craniosynostosis: insights from human genetics and experimental studies. *J Anat* **207**: 637–653.
Nishimura R, Hata K, Matsubara T, Wakabayashi M, Yoneda T (2012) Regulation of bone and cartilage development by network between BMP signalling and transcription factors *J Biochem* **151**: 247–254.
Rice DP, Rice R, Thesleff I (2003) Molecular mechanisms in calvarial bone and suture development, and their relation to craniosynostosis. *Eur J Orthod* **25:** 139–148.
Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* **3**: 1101–1118.
Shui C, Spelsberg TC, Riggs BL, KhoslaS (2003) Changes in Runx2/Cbfa1 expression and activity during osteoblastic differentiation of human bone marrow stromal cells. *J Bone Miner Res* **18**: 213–221.
Silvério KG, Davidson KC, James RG, Adams AM, Foster BL, Nociti FH Jr, Somerman MJ, Moon RT (2012) Wnt/β-catenin pathway regulates bone morphogenetic protein (BMP2)-mediated differentiation of dental follicle cells. *J Periodontal Res* **47**: 309–19.
Wang X, Manner PA, Horner A, Shum L, Tuan RS, Nuckolls GH (2004) Regulation of MMP-13 expression by RUNX2 and FGF2 in osteoarthritic cartilage. *Osteoarthritis Cartilage* **12**: 963–973.
Wuelling M, Vortkamp A (2011) Chondrocyte proliferation and differentiation. *Endocr Dev* **21**: 1–11.
Xiao G, Jiang D, Thomas P, Benson MD, Guan K, Karsenty G, Franceschi RT (2000) MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. *J Biol Chem* **275***:* 4453–4459.
Zhang C, Zheng S, Wang Y, Zhao Y, Zhu J, Ge L (2010) Mutational analysis of RUNX2 gene in Chinese patients with cleidocranial dysplasia. *Mutagenesis* **25**: 589–594.
Ziros PG, Gil AP, Georgakopoulos T, Habeos I, Kletsas D & Basdra EK, Papavassiliou AG (2002) The bone-specific transcriptional regulator Cbfa1 is a target of mechanical signals in osteoblastic cells. *J Biol Chem* **277**: 23934–23941.