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Original

Dermal fibroblast transcriptome indicates contribution of WNT signaling pathways in the pathogenesis of Apert syndrome

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Cranial sutures are unossified connective tissue structures between the cranial bones, which allow expansion of these bones during development. Premature ossification of these structures is called craniosynostosis. Apert syndrome is a well-defined genetic syndrome, which is characterized by craniosynostosis and arises as a result of two missense mutations in Fibroblast Growth Factor Receptor, type 2 gene (*FGFR2*). In this study, differentially expressed genes in dermal fibroblasts from individuals with Apert syndrome and controls were investigated to identify important pathways in the pathogenesis of Apert syndrome. For this purpose, primary skin fibroblast cultures obtained from 3 individuals with Apert syndrome and 3 controls without craniosynostosis were compared by transcriptome microarray, GeneChip Human Genome U133 Plus 2.0. As a result, 181 genes were shown to be differentially expressed between experimental groups. Among these, 10 genes, which significantly differ in Apert syndrome fibroblasts compared to controls, were shown to be involved in a common interaction network and have common Gene ontology (GO) biological processes terms. *COL11A1*, *COMP*, *CPXM2*, *ITGA8*, *MGF* and *TNC* are differentially expressed genes that have GO terms associated with extracellular matrix (ECM) organization, while *FRZB*, *SFRP2* and *WNT2* are involved in WNT signaling pathway. Reorganization of ECM and changes in WNT signaling pathway show that Apert syndrome primary fibroblast cultures may have an increased potential for bone differentiation. The results of this study support craniosynostosis in Apert syndrome may be the result of fast and early differentiation of connective tissue along the sutures.

Key words: craniosynostosis, Apert syndrome, fibroblast growth factor receptor type 2, transcriptome, fibroblast.

Craniosynostosis is the premature ossification of sutures, which lies between neurocranial bones and is seen in about 1/1500 - 1/2500 newborns.[1-5] Cranial sutures, which are made up of connective tissue, ossifies with age and forms interlocked immovable joints connecting cranial bones.[6] Craniosynostosis may lead to major and potentially debilitating health problems such as increased intracranial pressure, vision and hearing loss due to compression of cranial nerves, and sleep apnea.[7]

Among the several causes of craniosynostosis, some arise primarily as the dysregulation of

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the cranial sutures, while some are secondary to the disturbance of cranial enlargement and development, such as brain injury and primary microcephaly syndromes.[3] Primary craniosynostosis can be either isolated or arise as part of a syndrome, which are associated with genetic disorders. More than 200 genetic disorders that include chromosomal abnormalities, copy number variations and single gene disorders have been associated with craniosynostosis.[8]

Apert syndrome (MIM101200) is a sporadic or autosomal dominantly inherited well-recognized disorder. It is characterized by primary craniosynostosis, other skeletal anomalies, osseous/cutaneous syndactyly, dysmorphic findings such as midface hypoplasia, as well as other less common systemic anomalies, such as congenital heart defects. Two missense mutations (p.Ser252Trp and p.Pro253Arg) in Fibroblast Growth Factor Receptor 2 gene (*FGFR2*), which result in ligand-dependent gain-of-function, are responsible for 98% of Apert syndrome in affected individuals.[2] Similarly, >90% of mutation prevalence for these mutations is observed in Turkish population.[9] Other *FGFR2* mutations and mutations in FGF/FGFR genes are also associated with craniosynostosis-related syndromes like Muenke syndrome, Crouzon syndrome and Pfeiffer syndrome, indicating the importance of fibroblast growth factor receptors (FGFRs) and its ligands, fibroblast growth factors (FGFs), in the genetic etiology of craniosynostosis.[2, 10]

The main role of FGFRs in cranial sutures is to oversee gene expression in sutural cells to regulate osteoblastic cell differentiation and stem cell proliferation.[11, 12] In this study, we investigated the transcriptome profiles of cultured primary dermal fibroblasts from individuals with Apert syndrome and controls to identify differentially expressed genes (DEGs) to search for pathways dysregulated in the pathogenesis of craniosynostosis.

Material and Methods

Participants

Three children with clinical findings of Apert syndrome and 3 children with no apparent genetic disorders, selected as the control group, with ages ranging between 2.5 and 10 years were enrolled in the study. Physical examination was performed in each individual to note the dysmorphic findings and exclude the presence of a skin disorder. Peripheral blood samples and gluteal skin biopsies gathered by punch biopsy of the skin under 2 mg/ml lidocaine anesthesia and were obtained from each individual with Apert syndrome, while abdominal skin biopsies were obtained from individuals with no apparent genetic disorders undergoing surgery for an unrelated intraabdominal disease. Informed consents were obtained from parents of all children for participating in the study. This study was reviewed and approved by the Non-Interventional Clinical Research Ethics Board of Hacettepe University (TBK 12/07).

FGFR2 Sequence Analysis

DNA was obtained from peripheral blood leukocytes in 3 children with Apert syndrome by ammonium acetate precipitation. Sanger DNA sequencing of Exon 7 of *FGFR2* (ENST00000358487) was performed by using BigDye Terminator Cycle Sequencing kit (Applied Biosystems, USA) and primers 5'-TGACAGCCCTCTGGACAAC-3' and 5'-TCATCCTCTCTCAACTCCAACA-3' in order to demonstrate the mutations responsible for Apert syndrome.[13]

Primary Dermal Fibroblast Cultures

Primary dermal fibroblasts were grown in DMEM containing 1% L-Glutamine, 1% Penicillin/streptomycin, 0.2% Gentamycin, 10% fetal bovine serum after careful removal of epidermal layer to avoid keratinocyte contamination. Penicillin, streptomycin, Gentamycin and fetal bovine serum were removed from culture media and cells were grown either in absence or presence of 2nM FGF2 for 48 hours — thus, forming 4 experimental groups (Apert syndrome 0pM (picomolars) FGF2, Apert syndrome 2000pM FGF2, Control 0pM FGF2, Control 2000pM FGF2). FGF2 concentration was used as determined in previous studies.[14, 15]

RNA isolation and Microarray for Transcriptome

Total RNA from primary dermal fibroblast cultures were isolated by using TRIzol (Sigma-Aldrich, Germany) as suggested by the manufacturer. GeneChip® Human Genome U133 Plus 2.0 Array (Affymetrix, USA) was utilized to determine transcription profiles of each sample according to protocols provided

by the manufacturer. Microarrays were scanned by GeneChip® 3000 Scanner (Affymetrix, USA). Affymetrix Expression Console using MAS 5.0 (Microarray Suite 5.0) protocol and R x64 v3.1.0 with *affy* and *limma* modules were used to compare gene expression levels between experimental groups.[16] Differentially expressed genes (DEGs) with greater than 1.5 times difference in logarithmic scale between groups and with a t-test value $p>0.05$ were considered statistically significant results. No further experiments were carried out to evaluate transcriptome microarray results. DEGs were evaluated for common gene ontology (GO) terms by using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins).[17]

Results

Clinical Findings and Mutation Analysis

Three female individuals with Apert syndrome (A1, A2, A3) were similarly affected and shared common features of the disorder (Table I). Individual A2 differed from others in having cleft lip and individual A3 had atrial septal defect. Individuals A1 and A3 were heterozygous for c.934C>G (p.Ser252Trp), while A2 was heterozygous for c.937C>G (p.Pro253Arg). On the other hand, three individuals in control group (C1, C2, C3) did not present with any dysmorphic features of the syndrome (Table I).

Transcriptome Analysis

In order to detect the possible effect of the substrate FGF2 on transcription in fibroblasts from Apert syndrome and control groups 4 experimental groups were formed (Apert syndrome 0pM FGF2, Apert syndrome 2000pM FGF2, Control 0pM FGF2, Control 2000pM FGF2). These experimental groups were compared in pairs and a total of 181 transcripts were identified as DEGs in at least one pair (Table II). STRING identified 90 associations between 71 of these genes which is statistically significant ($p = 2.58x10^{-11}$). These associations are clumped into 2 large clusters, containing 41 genes (Cluster I) and 14 genes (Cluster II) (Fig. 1).

DEGs detected in these comparisons are also enriched for GO biological processes (Table III). The significantly enriched terms are extracellular matrix (ECM) organization, cell adhesion and mesenchyme development. 33 of the 65 DEGs associated with these biological processes appear in a common interaction network in cluster I. Among these 10 are DEGs that arise when non-FGF2 treated Apert syndrome and control fibroblast trancriptomes are compared — *COL11A1*, *COMP*, *MGF*, *SFRP2*, *WNT2* are upregulated in Apert syndrome fibroblasts, while *CPXM2*, *FRZB*, *ITGA8*, *TBX5*, *TNC* are down regulated. *COL11A1*, *COMP*, *CPXM2*, *ITGA8*, *MGF*, *TNC* are involved in ECM organization according to associated GO terms, while *FRZB*, *SFRP2*, *WNT2* are components of the WNT signaling pathway.[18]

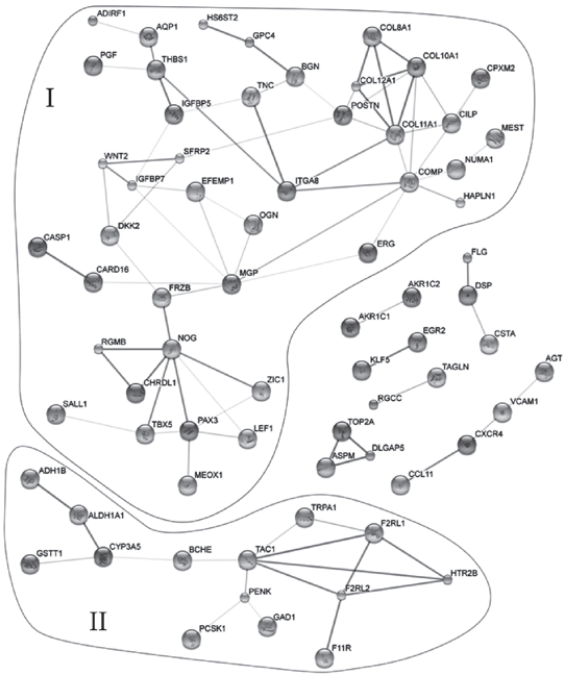

Fig. 1. Association of DEGs in STRING, showing 2 large association clusters.
*Note that the thickness of lines joining nodes of genes represents the strength of association.

Discussion

Apert syndrome is one of the oldest known syndromes associated with craniosyostosis, however the pathogenesis of this syndrome is still unclear. Missense mutations in *FGFR2* causes Apert syndrome and leads to increased ligand-dependent FGFR2 signaling activating intracellular signaling pathways that regulate gene expression.[19]

In this study, we focused on the DEGs in non-FGF2-treated fibroblasts from individuals with Apert syndrome and control groups, and found that 2 biological processes, ECM organization

Table I. Clinical Features of Individuals with Apert Syndrome (A1, A2, A3) and Those in The Control Group (C1, C23, C3).

Individuals	A1	A2	A3	C1	C2	C3
Age	8	7	2.5	6	7	10
Gender	F	F	F	F	F	F
Craniosynostosis	Coronal	Coronal	Coronal	-	-	-
Biparietal diameter	NFA	NFA	NFA	NFA	NFA	NFA
Height	NFA	NFA	NFA	NFA	NFA	NFA
Intellectual disability	-	Mild	-	-	-	-
Increased intracranial pressure	-	-	-	-	-	-
Proptosis	+	+	+	-	-	-
Hypertelorism	+	+	+	-	-	-
Visual loss	-	-	-	-	-	-
Hearing loss	-	-	-	-	-	-
Mid-facial hypoplasia	+	+	+	-	-	-
Dysmorphic nose	+	+	+	-	-	-
Cleft lip	-	+	-	-	-	-
Congenital heart disease	-	-	ASD	-	-	-
Hallux valgus	+	+	+	-	-	-
Broad thumbs	+	+	+	-	-	-
Brachy-syndactyly	+	+	+	-	-	-
Treatment	Cranioplasty, Syndactyly repair	Cranioplasty, Syndactyly repair	Cranioplasty, Syndactyly repair	-	-	-
FGFR2 Mutation	p.Ser252Trp	p.Pro253Arg	p.Ser252Trp			
Family History	-	-	-	-	-	-

F: female; M: male; NFA: normal for age; ASD: atrial septal defect

and mesenchyme differentiation, arise as the major pathways dysregulated. These pathways play a role in bone formation and osseous differentiation.

ECM related genes detected in this study include *COL11A1*, *COMP*, *CPXM2*, *ITGA8*, *MGF* and *TNC*. Among these genes *COL11A1* along with *COL10A1*, another upregulated transcript shown in this study, encodes for collagens expressed in skeletal tissues which is upregulated in differentiating chondroblasts and osteoblasts.[20, 21] Another ECM protein COMP, which is shown to be upregulated in this study, captures Bone Morphogenic Protein 2 (BMP2) and leads to a localized increase in its concentration, promoting bone differentiation.[22] In contrast to these 2 transcripts, downregulation of MGP is associated with abnormal tissue calcification in mice and men.[23,24] This transcript is upregulated in Apert syndrome fibroblasts which may compensate for increased tendency towards osseous differentiation.

FRZB, *SFRP2*, *WNT2*, which are 3 of the most pronounced mesenchyme differentiation related genes in this study, indicate changes in WNT pathway in fibroblasts obtained from individuals with Apert syndrome. This is a significant finding because upregulation of WNT signaling leads to craniosynostosis in mice.[25] A study in support of the importance of WNT2 and SFRP proteins shows that these genes are upregulated in coronal sutures of individuals with non-syndromic craniosynostosis.[26] In addition, the balance between WNT and FGF signaling pathways is important in normal limb

Table II. Paired Comparisons of 4 Experimental Groups to Identify DEGs.

Compared experimental groups	Increased expression	Decreased expression	Total
Apert Syndrome 0pM FGF2 – Control 0pM FGF2	32	28	60
Apert Syndrome 2000pM FGF2 – Control 2000pM FGF2	23	38	61
Apert Syndrome 2000pM FGF2 – Apert Syndrome 0pM FGF2	13	94	107
Control 2000pM FGF2 – Control 0pM FGF2	11	11	22

Table III. GO Terms for Biological Processes That 181 DEGs Are Enriched for Detected By STRING (DEGs In Cluster I Are Underlined).

GO Terms	p value	Genes
Extracellular Matrix Organization	$5.15x10^{-10}$	A2M, ADAMTS5, EGFL6, FKBP1A, LOXL2, NTN4, PLOD2, TLL2, F11R, AGT, VCAM1, BGN, COL8A1, COL10A1, COL11A1, COL12A1, COMP, EFEMP1, HAPLN1, ITGA8, POSTN, SFRP2, THBS1, TNC
Cell Adhesion	$1.06x10^{-11}$	AJAP1, EDIL3, EGFL6, EPHA3, LOXL2, MEGF10, NEDD9, NRXN3, OMG, PCDH10, PTPRD, SORBS2, WISP2, F11R, AGT, CSTA, DSP, CCL11, COL8A1, COL11A1, COL12A1, COMP, CPXM2, HAPLN1, IGFBP7, ITGA8, LEF1, MGP, POSTN, THBS1, TNC
Mesenchyme Development	$6.05x10^{-4}$	LOXL2, LOXL3, HTR2B, FRZB, LEF1, NOG, PAX3, THBS1, TBX5, WNT2

development.[27] A loss of balance between the 2 pathways as shown in dermal fibroblasts here may contribute to the pathogenesis of limb abnormalities in Apert syndrome. The emergence of changes in WNT pathway at gene regulation level is important as, to our knowledge, this has not been reported previously in transcriptome studies for Apert syndrome.[5, 15, 28-30]

The effect of FGF2 addition to the culture media for fibroblasts from individuals with Apert syndrome and control groups is hard to evaluate as minimal variation occurs with the addition of FGF2 (data not shown). However, it is interesting to note that FGF2 results in upregulation of *NOG* for which heterozygous loss-of-function mutations are associated with multiple synostoses syndrome type 1, while FGF2 addition does not significantly affect *NOG* expression in control fibroblasts, indicating the potential sensitivity of Apert syndrome fibroblasts.[31] In this study, only a single concentration of FGF is tested, and a more pronounced response in gene expression by higher concentrations of FGF cannot be excluded.

In conclusion, we have shown that Apert dermal fibroblasts have gene expression profiles indicative of changes in WNT signaling and reorganization of ECM which may suggest a tendency towards increased osseous differentiation. The results of this study must be tested in larger study groups and supported by functional analyses. Also, the biological significance of the results must be tested in *in vivo* experiments.

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