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Use of adipose derived stem cells in Treacher Collins syndrome

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Abstract. – OBJECTIVE: Treacher Collins syndrome (TCS) is a rare congenital disorder of craniofacial development. TCS occurs with an incidence of 1:50,000, and more than 60% of TCS cases have no previous family history and arise as the result of de novo mutations. The high rate of de novo mutations, together with the extreme variability in the degree to which individuals can be affected, makes the provision of genetic counseling extremely complicated. Consequently, every case of TCS is unique and needs to be assessed individually. Patients with TCS frequently undergo multiple reconstructive surgeries from birth through adulthood, which rarely are fully corrective in the long-term. The nascent field of regenerative medicine offers the promise to improve some of these treatments. In particular, structural fat grafting (SFG) seems to be a good strategy not only to restore the normal volume and contour of the face, but also to provide a source of adipose-derived stem cells (ADSCs) with a multilineage differentiation potential. In this work, we present genetical analyses of ADSC affected by TCS.

MATERIALS AND METHODS: ADSCs from were analyzed for their stemness properties and shared many characteristics with those of a healthy subject. Screening of the genome of the TCS patient using array-Comparative Genomic Hybridization allowed us to identify some chromosomal imbalances that are probably associated with TCS.

RESULTS: We found that some alterations, involving the TIMELESS gene, were usually associated with embryonic stem cells.

CONCLUSIONS: With the aim to improve the final results, we need to consider combining knowledge of genetic alterations and expression profiles as a fundamental step before starting with surgical procedures.

Key Words:
Regenerative medicine, Structural fat grafting, Adipose-derived stem cells, Gene expression, array-CGH, Maxillofacial surgery.

Introduction

Treacher Collins syndrome (TCS) is a rare autosomal dominant craniofacial malformation affecting 1:50,000 newborns[1]. This syndrome occurs during the fifth to eighth week of fetal development and affects the proper formation of the first and second pharyngeal arches, leading to profound facial dysmorphis[2]. These pharyngeal arches are composed of mesenchymal cells derived from mesodermal and cranial neural crest cells, which give rise to a wide variety of facial structures including skeletal, muscular, and neural elements[2]. It is thought that most craniofacial disorders, such as those associated with TCS, arise due to defects in the formation, proliferation, migration, and/or differentiation of neural crest cells[3]. The resulting phenotype of TCS, which is often bilateral and relatively symmetric, includes hypoplasia of the facial bones, particularly the zygomatic complex and mandible, cleft palate, and middle and external ear defects that result in conductive deafness[4]. Considerable phenotypic variability exists among patients, with the most severe cases leading to perinatal death due to respiratory distress, and mild cases that escape clinical diagnosis. Despite this large spectrum of variability, penetrance is thought to be nearly complete[5].

The gene responsible for TCS has been mapped to chromosome 5q32-q33.1 and named Treacher Collins-Franceschetti syndrome 1 gene (TCOF1; OMIM *606847)[6]. More than 130 different mutations have been identified spanning all 26 exons of the gene and these are responsible for over 90% of TCS cases[1]. The majority of mutations in TCOF1 are predicted to result in truncated treacle proteins. Treacle is a nucleolar phosphoprotein implicated in ribosomal DNA gene transcrip-

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tion and preribosomal RNA processing[7]. Mice haploinsufficiency for Tcof1 exhibit diminished production of the mature 28S subunit in neuroepithelial cells and neural crest cells. This results in nucleolar stress activation of p53[8]. Increased p53 levels lead to G1 cell cycle arrest and specific apoptosis of neuroepithelial cells, which results in hypoplastic neural crest-derived structures of the craniofacial skeleton[1].

Molecular analysis of the *TCOF1* gene determined that 40% of newborns with TCS have inherited one mutated copy of *TCOF1*, whereas 60% of cases arise as the result of *de novo* mutations[9]. The extreme variability in the degree to which individuals can be affected, and the high rate of *de novo* mutations, make the provision of genetic counseling extremely complicated[10]. Furthermore, genetic screening for TCOF1 mutations during early gestation appears to be economically unviable, except in families with a known history of TCS[10]. Consequently, the majority of individuals with craniofacial abnormalities are detected during mid-to-late gestation through ultrasound screening[10]. Nevertheless, phenotypic diagnosis at this stage is extremely difficult, even with the most sophisticated ultrasonography available today[10]. Therefore, at present, multiple reconstructive surgeries remain the only available treatment option for TCS management[11]. Because of the complex interactions of the numerous and simultaneously affected tissues, care of individuals with TCS usually requires a multidisciplinary approach[11]. This approach may involve intervention from a number of healthcare professionals both pre and postoperatively, including craniofacial surgeons, orthodontists, ophthalmologists, otolaryngologists, and speech pathologists[11]. However, despite the multiple rounds of surgery that a TCS patient typically endures, they are rarely fully corrective in the long-term[11].

Regenerative medicine holds promise for new strategies to improve the treatment of this disorder. In this context, use of structural fat grafting (SFG) has increased in popularity in the last 10 years[12]. First introduced as a method to improve facial aesthetics, SFG has become an effective and reproducible technique to treat some craniofacial malformations[12]. Several advantages are rapidly making SFG the preferred approach for facial augmentation: it is autologous and completely biocompatible, with a minimal risk of infection, and grafts are available in sufficient quantities in most patients, are naturally integrated into the host tissue, and have a natural appearance and feel that is distinctly better than that of implants and most fillers[12,13]. In addition, long-term studies showed that these grafts can last for decades or longer[13]. Apart from these characteristics, SFG represents a delivery system of adipose-derived stem cells (ADSCs), which exhibit a multilineage differentiation potential and secrete angiogenic and antiapoptotic factors[14].

In this work, we performed gene expression analyses with real-time PCR in order to compare the stemness properties of ADSCs isolated from the TCS lipoaspirate and those of a healthy subject. Possible chromosomal imbalances related to the disorder were investigated by array-Comparative Genomic Hybridization (array-CGH) analysis of genomic DNA of the TCS patient. Deep comprehension of ADSC characteristics and their correlation with the genotype of the TCS patient could represent an innovative tool to improve the surgical treatment of maxillofacial deformities.

Materials and Methods

Ethics Statement

The Ethical Committee of Ferrara Hospital (Ferrara, Italy) approved the research protocol. Written informed consent was obtained from the subjects, in accordance with the Helsinki Declaration, before their inclusion in this study.

Surgical Treatment of the TCS Patient

Treatment of the TCS patient involved different surgical procedures over many years. The first step was SFG following Coleman's guidelines[13] into the periorbital and zigomatic area (25 cc in right area and 25 cc in left area). An upper eyelid myocutaneous flap was combined with lateral canthopexy. The second step was extensive orbital construction with a cranial bone graft. The third step was jumping genioplasty and mandibular distraction osteogenesis (advancement of 18 mm).

RNA Isolation

Total RNA was isolated using the RNeasy Lipid Tissue Kit (Qiagen, Hilden, Germany), including DNase digestion with the RNase-Free DNase Set (Qiagen, Hilden, Germany), from the high-density layer (HDL) of the centrifuged lipoaspirate of the TCS patient and a healthy subject. The RNA quality and concentrations of samples were determined using the NanoDrop™ ND-1000 (Thermo Scientific; Waltham, MA, USA).

RT² Profiler PCR Array

For first-strand cDNA synthesis, 500 ng of total RNA of each sample was reverse-transcribed with the RT² First Strand Kit (Qiagen, Hilden, Germany). Real-Time PCR was performed according to the user manual of the Human Mesenchymal Stem Cells RT² Profiler PCR array (SABiosciences; Frederick, MD, USA) using RT² SYBR Green ROX FAST Mastermix (SABiosciences; Frederick, MD, , USA). This array profiles the expression of 84 key genes involved in maintaining pluripotency and self-renewal status. Thermal cycling and fluorescence detection were performed using a Rotor-Gene Q 100 (Qiagen; Hilden, Germany). Data were analyzed using Excel-based PCR Array Data Analysis Templates (SABiosciences; Frederick, MD, USA). Results are reported in terms of the expression ratio of each target gene between the TCS sample and the control sample.

DNA Isolation

Total DNA was isolated from blood of the TCS patient using the DNeasy Blood & Tissue Kit (Qiagen; Hilden, Germany) following the manufacturer's protocol, using overnight incubation with proteinase K (Qiagen; Hilden, Germany). The DNA quality and concentrations of samples were determined using the NanoDrop™ ND-1000 (Thermo Scientific, Waltham, MA, USA).

Array-CGH

Array-CGH was performed using the Human Genome CGH Microarray (Agilent Technologies; Santa Clara, CA, USA) with a median probe spatial resolution of 44 Kb following the manufacturer's protocol. Briefly, 1 µg of DNA from blood of the TCS patient (TCS sample) and 1 µg of pooled sex-matched reference DNA (control sample) (Promega, Madison, WI, USA) were digested with AluI and RsaI for 2 h at 37°C. After inactivation of the enzymes at 65°C for 20 min, each digested sample was labeled by random priming for 2 h using Cy5-dUTP for sample DNA and Cy3-dUTP for control DNA. Labeled products were then column-purified [Microcon YM-30 filters, Millipore Corporation (Burlington, MA, USA)]. After probe denaturation and preannealing with Cot-1 DNA, hybridization was performed at 65°C with rotation for 24 h. At the end of the incubation, slides were washed and analyzed using the Agilent scanner (Agilent Technologies, Santa Clara, CA, USA). Data and graphics were elaborated using CGH Analytics software [v3.1, Agilent Technologies, (Santa Clara, CA, USA)].

Statistical Analysis

One-way analysis of variance (ANOVA) was used for data analyses. A repeated-measures ANOVA with a post-hoc analysis using Bonferroni's correction for multiple comparisons was performed. *t*-tests were used to determine significant differences ($p<0.05$). Repeatability was calculated as the standard deviation of the difference between measurements. All testing was performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

Results

Stemness Properties of ADSCs

To evaluate the stemness properties of ADSCs in the fat of the TCS patient, mRNA from the HDL was analyzed by real-time PCR. Figure 1 shows the gene expression of several mesenchymal stem cell (MSCs)-specific markers in ADSCs of the HDL of the TCS patient in comparison to those of a healthy subject. Expression of most of these genes did not significantly differ between the two populations of cells. Some genes were instead upregulated in ADSCs of the TCS patient, including genes encoding growth factors (*BDNF, FGF2, GDF15,* and *PDGFRB),* enzymes *(CASP3, ERBB2, and NT5E),* receptors *(FZD9* and *NOTCH1*), signaling molecules (*BMP7* and *SMAD4*), and other proteins (*INS, MCAM,* and *ZFP42*). Genes encoding the cytokine *CSF3* and the glycoproteins *ENG* and *PROM1* were downregulated.

Figure 2 shows expression profiling of genes involved in osteogenic (Figure 2a), chondrogenic (Figure 2b), and vasculogenic (figure 2c) differentiation in ADSCs isolated from the TCS patient's HDL of fat. Several genes were differentially expressed between cells of the TCS patient and those of a healthy subject. These genes belong to the bone morphogenetic protein (*BMP*) family and transforming growth factor β (*TGFβ*) superfamily (*BMP2, BMP6, BMP4, GDF5,* and *GDF7*), growth factors (*FGF10, HGF,* and *IGF1*), members of the integrin family (*ITGB1, ITGAX,* and *ITGAV*), transcription factors (i.e., *RUNX2* and *TBX5*), kinases (*PTK2* and *KDR*), and other proteins (*ABCAB1, ANPEP,* and *IL1B*).

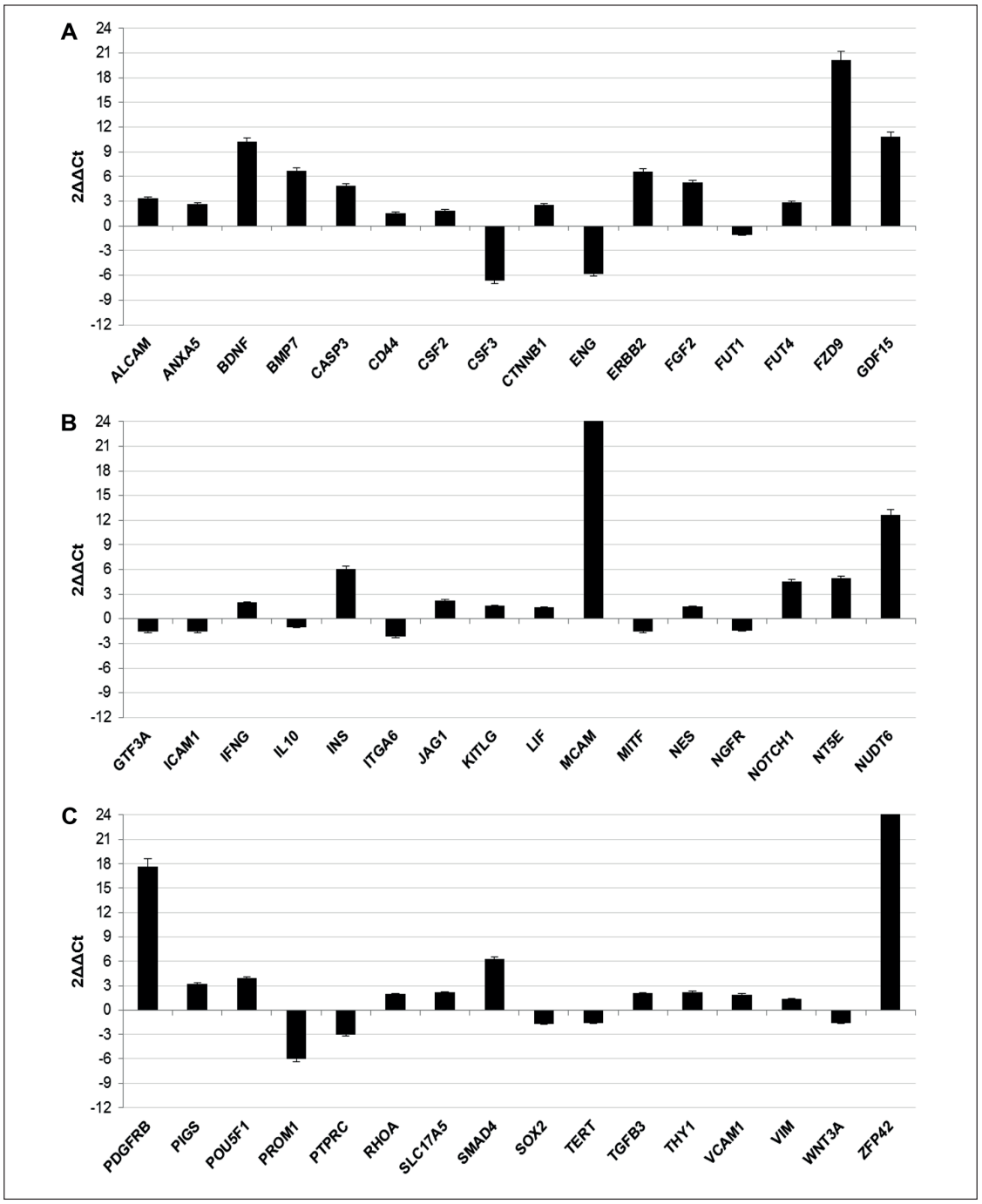


Figure 1. Gene expression profiling of MSCs-specific (**A-C**) markers in ADSCs isolated from the HDL of fat of the TCS patient compared to those of a healthy subject.

Analysis of Genomic Imbalances with Array-CGH

Array-CGH was used to rapidly screen the whole genome of the TCS patient in order to evaluate the presence of imbalanced regions. Table I and Figure 3 show a list of the genomic regions that were altered in the TCS patient.

Discussion

TCS is a rare congenital disorder of craniofacial development with bilateral midfacial zygomatic-orbital bony involvement[1]. This skeletal malformation is associated with hypoplasia of all the surrounding soft tissues layers[1]. Although

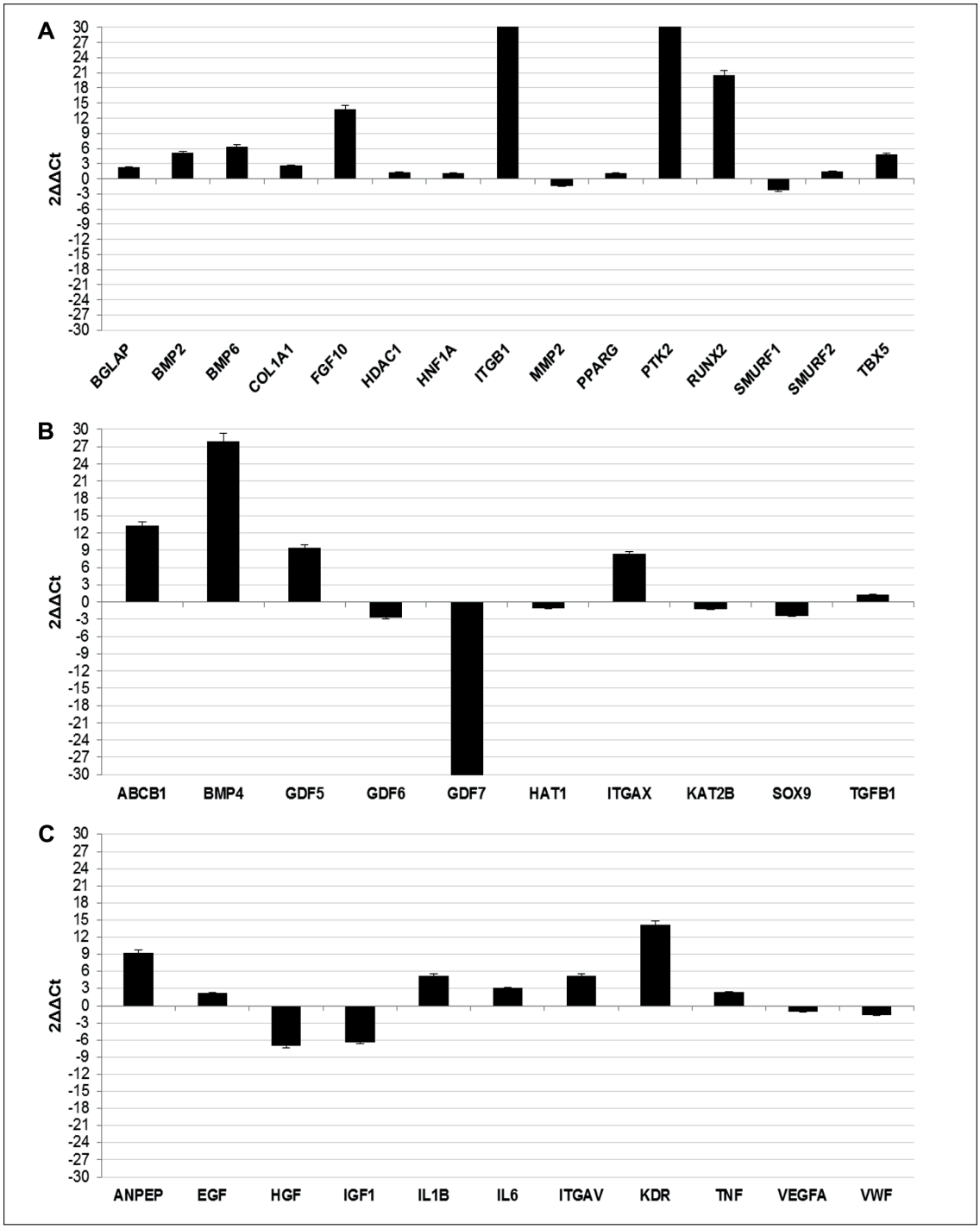


Figure 2. Gene expression profiling of (**A**) osteogenic, (**B**) chondrogenic, and (**C**) vasculogenic differentiation markers in ADSCs isolated from the HDL of fat of the TCS patient compared to those of a healthy subject.

skeletal reconstruction in theory corrects the projection of the orbits and zygomas, TCS patients continue to have a soft tissue deficit[1]. The superficial musculoaponeurotic system and periosteum are absent or extremely minimally represented in TCS patients; therefore, bone grafts, even if taken from the calvaria, undergo more resorption than in normal patients[16]. Fat grafting could increase the soft tissue facial volume, thereby supplying a valuable recipient bed for further osteoplasty[16].

Table I. Gene alterations Treacher Collins syndrome. For each chromosome, the table shows the type of chromosome abnormality (deletion or amplification), estimated size of the genomic alteration, and genes localized in the involved region and their full names.

Chromosome	Chromosome ab-normality	Estimated size kb	Gene	Gene full name
1	Deletion q24.2	24	*SAC*	Soluble adenyl cyclase
1	Deletion q32.1	25	*TMCC2*	Transmembrane and coiled coil domani family 2
4	Amplification p16.1	49	*SORCS2*	Sortin-related vsp10 doman containing receptor 2
8	Amplification p22	63	*MSR1*	Machrophage scavenger receptorl
1	Deletion q13	17	*MUS81*	MUS81 stucture-specific endonuclease subunit
			EFEMP2	Egf containing fibulin-like extracellular matrix protein 2
			CTSW	Cathepsin W
12	Deletionq13.12	24	*SPATS2*	Spermatogenesis associated, serine-rich 2
12	Deletion q13.3	40	*TIMELESS*	Timeless cicadian clock
			MIP	Major intrinsic protein of lens fiber
19	Amplification p13.2	36	*MORG1*	Mitogen-activated protein kinase organizer 1
			FBXW9	F-box and WD repeat domain containing 9
			TNPO2	Trasporting 2
x	Amplification q 28	72	*HSFX1*	Heat shock transcrition facto family, X linked 1

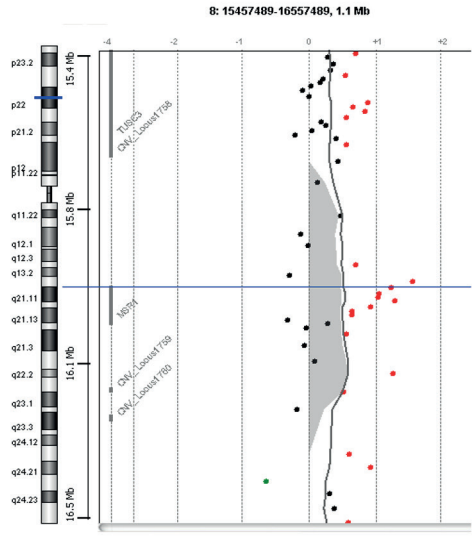
Recently, it was proposed that re-establishment of soft tissues is key in the early treatment phases of TCS[17].

In line with this hypothesis, autologous fat grafting has been used as a first-line approach in the management of Parry-Romberg disease, a type of progressive hemifacial atrophy, producing satisfying and long-lasting results[18]. SFG was used as the first surgical procedure to treat the young TCS patient[19,20]. It is speculated that SFG should be performed prior to reconstruction of bony structures because ADSCs present within the graft will stimulate subsequent bone repair by promoting osteoblast differentiation[19,20]. Moreover, the adipose tissue-derived stromal vascular fraction of the graft may favor early revascularization of bone grafts, promoting integration while minimizing bony resorption[19,20]. As confirmed by our recent work[14], ADSCs present in the HDL of injected fat highly express MSCs and endothelial markers, indicating a strong potential to use the cells in this layer[14]. In the present study, we compared the stemness properties of ADSCs between the TCS patient and a healthy subject. Interestingly, there was a strong correlation between the gene expression profiles of these two populations, indicating that autologous SFG is a good strategy to treat TCS patients[14]. Nevertheless, some genes were differentially expressed between the patient and the control sample (Figure 1 and Figure 2). Most of these belonged to the BMP family and TGFβ superfamily, such as *BMP2, BMP4, BMP6, BMP7, GDF5, GDF7,* and *GDF15*. Members of these families are mediators of a wide range of biological activities in the cranial neural crest, such as cell proliferation, differentiation, and extracellular matrix formation, suggesting that BMP and TGFβ proteins are fundamental for craniofacial development[21]. Recently, gain-of-function studies[22] indicated that elevated BMP4 activity in central neural crest results in dramatic changes in the facial skeleton. TGFβ/BMP signaling reportedly relies on SMAD4-dependent pathways to mediate epithelial-mesenchymal interactions that control craniofacial organogenesi[23]. Interestingly, SMAD4 was upregulated in the HDL of fat of the TCS patient. On the contrary, a significant downregulation was found for GDF7. In mice, this gene functions as an inductive signal from the roof plate required for the specification of neuronal identity in the dorsal spinal cord[24]. These data confirm that alterations occurring on neural crest cells are preserved during adult life in mesenchymal stem lineage[24]. Other molecules that regulate multiple developmental processes, including craniofacial development, are fibroblast growth factors (*FGF1-FGF10*)[24]. Several human diseases arise from dysregulated FGF signaling,

A

Chromosome	Chromosome abnormality	Estimated size (Kb)	Gene	Gene full name
1	Deletion q24.2	24	SAC	soluble adenylyl cyclase
1	Deletion q32.1	25	TMCC2	transmembrane and coiled-coil domain family 2
4	Amplification p16.1	49	SORCS2	sortilin-related VPS10 domain containing receptor 2
8	Amplification p22	63	MSR1	macrophage scavenger receptor 1
11	Deletion q13	17	MUS81	MUS81 structure-specific endonuclease subunit
			EFEMP2	EGF containing fibulin-like extracellular matrix protein 2
			CTSW	cathepsin W
12	Deletion q13.12	24	SPATS2	spermatogenesis associated, serine-rich 2
12	Deletion q13.3	40	TIMELESS	timeless circadian clock
			MIP	major intrinsic protein of lens fiber
19	Amplification p13.2	36	MORG1	mitogen-activated protein kinase organizer 1
			FBXW9	F-box and WD repeat domain containing 9
			TNPO2	transportin 2
X	Amplification q28	72	HSFX1	heat shock transcription factor family, X linked 1

B



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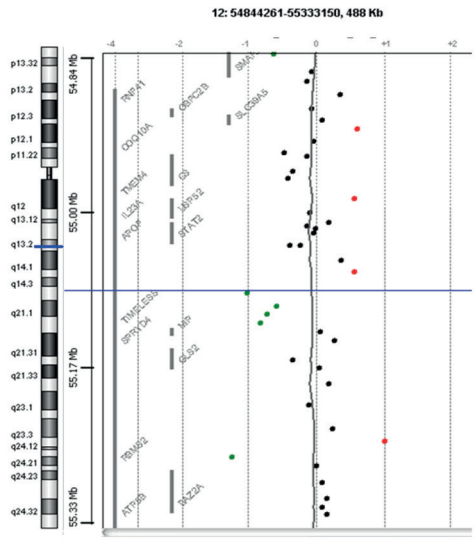

Figure 3. Array-CGH analysis of genomic DNA of the TCS patient. **A**, List of chromosomal alterations identified in the TCS patient with array-CGH. Representative array-CGH profile of (**B**) chromosome 8 and (**C**) chromosome 12, showing an amplified (red dots) and deleted (green dots) region, respectively.

such as craniosynostosis and skeletal dysplasia syndrome[25]. In our experiments, FGF2 and FGF10 were highly expressed in ADSCs isolated from the TCS patient. BMP and FGF signaling is also involved in the expression of RUNX2, the master transcription factor for osteogenesis[26]. Later, Choi et al[27] discovered that FGF2 is an earlier signal than BMP2 in the cranial intramembranous bone formation process, and that RUNX2 may mediate FGF2-induced BMP2 expression. The NUDT6 gene is positioned on the opposite strand to the FGF2 gene[28]. NUDT6 is an antisense gene of FGF2 in humans and rats and may regulate the stability of FGF2 through its 3' untranslated region of mRNA[28]. NUDT6 mRNA was upregulated in the TCS patient compared with the control sample[28]. Expression of NOTCH1 and FZD9 also significantly differed between the TCS patient and healthy subject[29]. NOTCH1 is a receptor of the Notch family, whose members play a role in a

variety of developmental processes by controlling cell fate decisions[29]. Gain of Notch signaling in neural crest cells reportedly produces severe craniofacial malformations including cleft face and palate, exencephaly, and micrognathia as a result of deficient cranial migration and a lack of differentiation of these cells[29]. FZD9 belongs to the Frizzled family of Wnt receptors[30]. FZD9 is expressed predominantly in brain and eye, and it is involved in tissue polarity and development. *ZFP42*, another gene found upregulated in ADSCs of the TCS patient, is critically important in maintaining proliferative state in MSCs, while simultaneously preventing differentiation[31]. Other genes upregulated in ADSCs isolated from the TCS patient were those related to cell-cell and cell-matrix interactions, such as integrins and *PTK2*, a member of the focal adhesion kinase family[32]. Interaction of cells with extracellular matrix is an essential event for differentiation, proliferation, and activity of osteoblasts[32]. In bone, binding of osteoblasts to bone matrix is required to determine specific activities of the cells and to synthesize bone matrix proteins[32].

Taken together, our results seem to corroborate the hypothesis that several signaling pathways, including TGFβ/BMP, FGF, Notch, and Wnt signaling, are involved in the development of craniofacial structures[30-32]. It is reasonable to assume that dysregulation of these complex pathways, as we observed in the gene expression experiments, is implicated in the onset of craniofacial diseases[30-32].

As stated above, due to the high variability of the resulting phenotype and the degree to which *de novo* mutations arise, every case of TCS is unique and needs to be assessed individually[30-32]. Array-CGH was performed on the blood of the TSC patient to evaluate the presence of imbalanced regions[2]. The main advantage of this technique is its ability to rapidly screen all chromosomes in a single test and to detect any DNA imbalances, including loss or gain of chromosome material, more precisely than conventional genetic analysis methods[2]. The array-CGH results demonstrated that the DNA of the TCS patient contained several amplified or deleted regions in which genes involved in different cellular processes, such as apoptosis, embryonic development, cell cycle, DNA damage control, cell proliferation, and differentiation, are localized (Figure 3). In particular, we found a deletion of 40 Kb in 12q13.3, which contains the *TIMELESS* gene (Figure 3). In mammals, *TIMELESS* controls the circadian rhythm and its expression exhibits a 24-hour oscillation[33]. Genetic studies[34] showed that *TIMELESS* is essential for early embryonic development, its homozygous knockout leads to early embryonic lethality in mice. A recent study[35] demonstrated that TIMELESS also plays a key role in the regulation of embryonic stem cell differentiation. One of the earliest steps in embryonic development is the formation of the proamniotic cavity, which involves the coordinated apoptosis of embryonic cells[35]. Embryonic cells lacking *TIMELESS* have reduced caspase activity and fail to undergo programmed cell death during cavitation and remain pluripotent[35]. These findings raise the possibility that circadian rhythms and early development are intimately linked[35]. *TIMELESS* also appears to be linked to the cell cycle and DNA damage control[36], as well as the gene *MUS81*[37]. In the DNA of the TCS patient, we found a deletion of 17 Kb in 11q13, where the *MUS81* gene is localized[37]. The endonuclease *MUS81* plays an important role in determining the rate of DNA replication and S-phase progression in human cells[37]. *MUS81* is important for DNA damage repair and genome integrity in murine and human cells[38]. In addition, we found an amplification in 4p16.1, where the gene *SORCS2* is localized[39]. A study[39-41] of murine embryos demonstrated that the receptor *SORCS2* is involved in embryonic development. Expression of this protein is high and transient in derivatives of all three germ layers[39]. In particular, high amounts of *SORCS2* protein are expressed in the facial mesenchyme, a derivative of the neural crest, up to 16.5 days of murine embryonic development[39].

Conclusions

The data we collected may represent a starting point for the identification of new strategies to treat TCS. We found that some alterations, usually associated to embryonic stem cells, are preserved in MSCs and conserved during adult life. Consequently, with the aim to improve the final results, we need to consider as a fundamental step combining knowledge of genetic alterations and expression profiles before starting with surgical procedures.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements
None.

Ethics Approval
The Ethical Committee of Ferrara Hospital (Ferrara, Italy) approved the research protocol. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Informed Consent
Written informed consent was obtained from the subjects before their inclusion in this study.

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Authors' Contributions
All the authors have made substantial contributions to the conception and design of the study, data acquisition, or data analysis and interpretation, drafting of the article or critically revising it for important intellectual content, final approval of the version to be submitted.

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