Two cases of Oto palate digital syndrome type II: Clinical features and a study of telomere length maintenance pathways.

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Abstract

Objective: To study the clinicopathological features of Oto palato digital syndrome type II (OPD-II) and telomere length maintenance pathway.

Design: Case-control study of OPD-II.

Setting: Different clinicopathological studies along with telomere length maintenance pathways were investigated.

Participants: Clinically diagnosed two OPD-II patients and four randomly selected age-matched normal individual.

Main outcome measures: Studied OstiumSecondum Atrial Septal Defects (osASD) by ECG, testes image by USG, subcortical dysrythmia by EEG, image of corpus callosum by MRI, 17 α oH progesterone level in blood. telomere length, expression of telomerase and telomere-associated genes of PBMC were also measured.

Results: Two children with OPD-II showed characteristic clinical symptoms such as cleft palate, broad forehead, facial dysmorphism, flat nasal bridge, low birth weight but no abnormality of the digits. The patient-1 exhibited osASD, bilateral undescended testes with hypospadiusand eventually developed seizure disorder in the follow up, ambiguous genitalia and high level of 17 α oH progesterone in blood. Interestingly, this patient showed shorter telomere length of PBMC – (~60%), low (<4%) expression of hTERT and hTERC compared with control group implicating that patient-1 had inactive telomerase. We observed de-regulated expression of all six shelterin proteins and two non-shelterin proteins such as BTBD12 and PARP-1, indicating the dysfunctional telomere of the patient-1. The patient-2 exhibits similar facial dysmorphism and absent corpus callosum as detected by MRI.

Conclusions: OPD-II patients showed osASD, subcortical dysrythmia, thin corpus callosum, undescended testes and higher 17 α oH progesterone level in blood. Patient-1 also showed short telomere, inactive telomerase and dysfunctional telomere.

Key words: Oto palato digital syndrome, OPD, Telomere length, Telomerase, TERT, TERC, shelterin, PARP-1, BTBD12

Introduction:

Otopalato digital syndrome (OPD) has been clinically characterized long back in 1960's with the distinctive featuresbut its molecular mechanism is still unknown.The abnormalities in OPD patients can be categorized into three major divisions - craniofacial, limbs and others. Most general craniofacial abnormalities are widely spaced eyes, distinctive forehead and faces, hearing impairment, cleft palate etc. and limb abnormalities include overlapping fingers, short broad thumb, polydactyly, small or absent fibula etc [1-3]. Abnormalities in others include microcephaly, pectusexcavatum, mental retardation; abnormal genitalia etc. and these could be associated with other congenital problems - called multiple congenital anomalies [4]. OPD is associated with mutation of FLNA gene which is located on chromosome Xq28 and it is involved in remodeling of cytoskeleton to effect in the cell shape and cell migration [5]. Mutations are observed in the exons 3, 4, and 5 of the *FLNA* gene for OPD type 1, whereas mutations are observed in regions of exons 3, 4, and 5, 11 and 29 of the *FLNA* gene for OPD type II [5]. Telomere length maintenance is urgently required in cells undergoing high rate of cell division during various stages of embryogenesis. In fact, telomerase activity and telomere length has been observed differential in various stages of gestation. As for example, telomerase activity has been detected during all the developmental stages of an embryo [6]. It has been found that fetal lung, liver, skin, muscle and adrenal to be positive of telomerase activity through 21 weeks of gestation, whereas telomerase activity was found to drop after 16 weeks of gestation in brain and kidney tissues [7]. So, proper embryonic development might be associated with the genes involved in telomerase and telomere maintenance pathway. As such there is no report of association of OPD with telomere biology. We have investigated telomere length and expression of few key genes involved in telomere length maintenance pathway. Telomerase activity or ALT pathway (where telomere-associated proteins are involved) is

required for maintaining telomere length and thereby chromosome integrity. De-regulation of either telomere maintenance pathway can cause various age-related diseases like dyskeratosiscongenita, Aplastic anemia, Fanconi anemia, Idiopathic pulmonary fibrosis, etc.[8−10]. Increasing number of reports regarding birth defects and congenital anomalies are coming into journals and causes of impaired embryogenesis and developmental defects are on search. Most of the cases of such developmental anomalies can lead to death or severely compromised life. Here, we present two patients of OPD-II with clinical data along with telomere length, expression of key proteins of telomerase and telomere-associated proteins.

Materials & Method
Chemicals & reagents
All the PCR reagents were procured from invitrogen or Thermo scientific.
Control individual group and patients
Blood samples were collected during out patients care unit of Calcutta National Medical College & Hospital, Kolkata, India strictly following the prescribed protocol of Institutional Ethical Committee. Since OPD is very rare case we obtained only two patients. The blood sample of patient-1 was collected at an age of 1 month and was under monitoring. We could not get blood sample of patient-2. Clinically confirmed 4 normal individual of age group 0-1 year was treated as age-matched control group and telomere length and all gene expression of patient was compared with mean±s.d of control group.

RNA isolation from whole blood and cDNA preparation for gene expression studies by Real time PCR
Total RNA were isolated from whole blood using Blood RNA isolation kit (Invitrogen; life technologies) following manufacturer's protocol. To avoid DNA contamination RNA was treated with RNase free DNase for 1hr at 37^0C and purified. cDNA was prepared from 1µg of RNA from all samples using RevertAid reverse transcriptase (Thermo Scientific) and random hexamer (Farmentas; Thermo Scientific). The quantitative PCR analysis was done in 7900HT

Real-Time PCR System (Applied Biosystem; Life Technologies) using Taqman assay primers [BTBD12 (Hs00536164_m1); PARP-1 (Hs00242302_m1); TPP1 (Hs00166099_m1); TERF2 (Hs00194619_m1); TIN2 (Hs01554309_g1); POT1 (Hs00209984_m1); hTERT (Hs00972650_m1); hTERC (Hs03454202_s1)] and SYBR green primers TERF1, RAP1 [11]. The taqman reaction were performed under standard assay programme (95 °C for 10 min and then 40 cycles of 95 °C for 15 s, followed by annealing and extension at 60 °C for 1 min) and the SYBR green reaction was done as per Wang et al. 2014 [11]. The threshold fluorescence signal was set up manually and the corresponding Ct values were determined. Then the expression levels were analysed using 18s rRNA as endogenous control by $2^{-\Delta\Delta Ct}$ method.

Extraction of PBMC from Whole blood

PBMC were extracted from the whole blood cells using ficoll (Invitrogen) following authors manual. The extracted PBMC were kept at -80°C in PBS for further downstream experiments.

Telomere length measurement by real time PCR

Relative Telomere length was measured using genomic DNA from the isolated PBMC following the protocol of [12] using a qPCR technique. The relative T/S ratio (also represent relative telomere length) was calculated using albumin as an endogenous control.

Results
Clinical diagnosis OPD

We have studied 2 patients with common characteristic clinical features of OPD such as broad forehead, flat nasal bridge and cleft palate etc. They showed some other additional features as given below.

Patient-1

Baby was born with ambiguous genitalia, low birth weight and facial dysmorphism. On examination, there were bilateral undescended testes with hypospadias (Fig 1A & B). Baby had

cleft palate and broad forehead and flat nasal bridge but no abnormality of the digits (atypical otopalato variant). Echocardiography showed ostiumsecondumASD. USG showed both the testes in lumber region. It showed elevated 17 αoH progesterone level in blood (>20ng/ml) on day 3 with a normal range being 0.7-3.5ng/ml from 5-30 days of life (data not shown). EEG showed generalized subcortical dysrythmia consistent with seizure disorder as given in Fig 2.

Patient-2:

This is an 18 month old male child with similar facial features with that of the previous case. There was broad forehead, hypertelorism, flat nasal bridge and high arched palate. The patient showed global neurodevelopmental delay and there were no other anomaly related to cardiovascular, genitourinary and respiratory systems. MRI brain showed thin corpus callosum as shown by white arrowhead in Fig 4. The patient died within 2 months of follow up and we could not collect blood for molecular study in this case. As per clinical phenotype this patient was also diagnosed to be another case of OPD-II.

Telomere length and expression of telomerase and telomere-associated genes

Telomere length of the patient-1 is about 60% of age-matched control group as shown in Fig 5a. To search the reason(s) behind telomere attrition, we monitored expression of two key telomerase genes such as hTERT and hTERC; six shelterin proteins such as TRF1, TRF2, TIN2, POT1, TPP1, RAP1; and two non-shelterin protein BTBD12 and PARP-1. Notably, we see hTERT and hTERC expression reduces to below 4% of the control group as shown in Fig 5b, implicating completely loss of telomerase activity. Expression of TPP1 and TRF1 reduces to almost zero, RAP1 reduces to almost 40%, expression of rest of the shelterin and non-shelterin protein increases up to 2.5 fold or more compared with control group. This data implicates telomere destabilization in the patient.

Fig 1. Patient-1 at age 12 days shows dysmorphicfacies (a) and ambiguous genitalia (b). The same patient at the age of 2 years (c).

Fig 1a

Fig 1b

Fig 1c

Fig 2. EEG (electroencephalogram) of Patient-1 showing generalized cortical dysrythmia (black arrowheads).
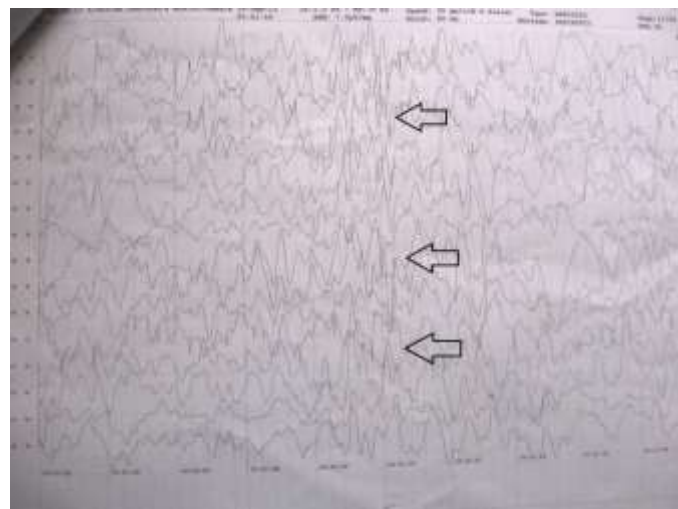

Fig 3. Patient-2 with broad forehead, flat nasal bridge and cleft palate with normal digits

Fig 4. MRI of brain of Patient-2 shows thinned out corpus callosum in sagital T1w view as shown by white arrowhead

Fig 5. Telomere length and expression of telomerase and telomere-associated genes in PBMC obtained from patient. (a) T/S ratio or relative telomere length of patient compared with

control group. (b) Expression of telomere-associated genes and telomerase genes in patients compared with control group.
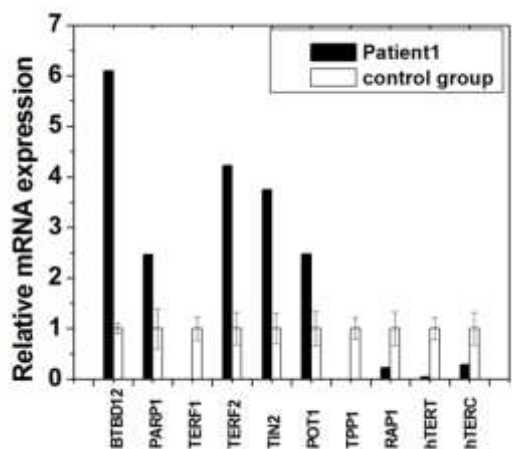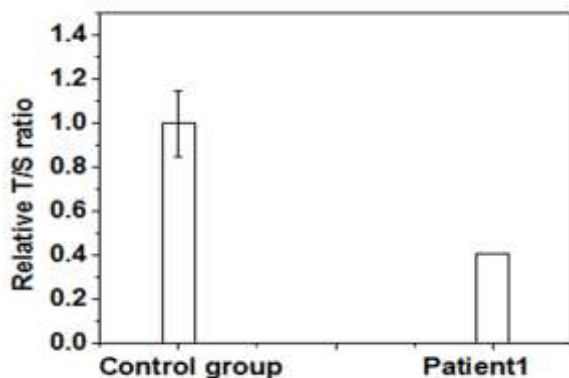

Fig 5b

Fig 5a

Discussion

Oto palato digital syndrome type II (OPD-II) is X linked with features of postnatal growth deficiency, late closure of anterior fontanel, ocular hypertelorism, antimongoloidslunt of palpebral fissures and cleft palate. Limbs usually remain flexed with overlapping fingers, short broad thumb and great toes, polydactyly and variable syndactyly of hands and feet [13]. Other features include mental retardation, microcephaly and posterior fossa brain abnormalities [13]. Notably, hypoplastic corpus calosum is truly an unusual finding in OPD-II as found in patient-2. This disorder is X linked with mild manifestations such as broad face, antimongoloidslunt of palpabral fissures, and cleft palate or bifid uvula in heterozygote females. OPD-I & II, frontometaphyseal dysplasia and Melnick-needles syndrome are allelic conditions all caused by mutations in FLNA gene [4].

We got opportunity to study telomere maintenance in blood sample from one patient only which shows about 40% reduction of telomere length along with significant reduction of expression of hTERC and hTERT subunit of telomerase in comparison with normal age-matched control group. So, the patient has inactive telomerase because these two subunits are essential for telomerase activity [14]. So, telomere length maintenance by telomerase is not working in OPD patient. Telomere can also be maintained by telomerase-independent pathway called ALT pathway where a number of telomere-associated proteins are involved. Shelterin proteins play crucial role for stabilization of telomere native structure [15–18]. Three shelterin such as POT1, TRF2 & TIN2 are up-regulated whereas rest three shelterin such as TPP1, TRF1 & RAP1 are down-regulated in our OPD patient. Such de-regulation of telomere-associated proteins may cause destabilization of native telomere capping structure leading to compromised ALT pathway [19,20]. So, both the pathways of replenishment of telomere erosion - telomerase-mediated and ALT pathway, is impaired in OPD patient. Probably, that's why we see shorter telomere in patient. Moreover, hTERT and hTERC can play pivotal role in embryogenesis, modulate expression of several genes independent of telomerase integrity or telomere localization [21–25]. So, their role independent of telomere length maintenance in proper embryonic development cannot be ruled out.

We have seen PARP-1 is over-expressed in patient. Apart from repair activity, PARP-1 can modulate expression of several genes via interacting with several transcription factors like NF-κB, B-MYB, Oct-1 [26–28]. Recently, we observed that expression of p53 can be modulated by PARP-1 [29]. Moreover, PARP-1 has also been reported to be involved in transcriptional regulation of SMAD3 [30], sex determining region Y-Box-2 [31] and E-cadherin [32], most of which are important factors for implantation of embryo. Joshi et al. 2014 [33] demonstrated the role of PARP-1 in embryo implantation by preparing the uterus for it. Thus role of PARP-1 in embryonic development in our study cannot be ignored. BTBD12, which is mammalian ortholog of Yeast BTBD12 has also been found to have role in mammalian gametogenesis by coordinating the repair process [34]. Besides it is a key TRF2-

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interacting partner and has role in telomere stabilization, DNA recombination and repair [35]. However, up-regulation of BTBD12 in our studies is not really known to us.

In conclusion, our study demonstrated that OPD-II showed osASD, undescended testes, deformed genitalia, subcortical dysrythmia, thin corpus callosum, high 17 αoH progesterone level in blood along with characteristic craniofacial symptoms. It seems that OPD-II may be linked with telomere length maintenance pathway but molecular mechanism is not clear to us. However, molecular studies of telomere from only one patient are insufficient to establish the association of OPD-II with telomeropathy. A systematic study of telomere homeostasis in a large number of such kinds of patients is needed to establish link between OPD and telomere maintenance pathway.

- **WHAT IS ALREADY KNOWN?**

OPD-I and OPD-II are associated with mutation in the various exons of the *FLNA* gene along with characteristic craniofacial manifestation like widely spaced eyes, distinctive forehead and faces, hearing impairment, deformed genitalia, cleft palate, etc. OPD may also be associated with osASD, undescended testes, subcortical dysrythmia, thin corpus callosum etc.

- **WHAT THIS STUDY ADDS?**

This study adds that OPD-II patient has shorter telomere, inactive telomerase and de-regulated telomere associated proteins implicating compromised telomere maintenance pathway.

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