



Salivary *FOXP2* expression and oral feeding success in premature infants

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Abstract The objective of the study is to determine whether salivary *FOXP2* gene expression levels at the initiation of oral feeding attempts are predictive of oral feeding success in the premature newborn. In this prospective study, saliva samples from 21 premature infants (13 males; birth gestational age [GA]: 30–34 wk) were collected around the initiation of oral feeding trials. Total RNA was extracted and underwent reverse transcription-quantitative polymerase chain reaction amplification for *FOXP2*. Oral feeding success was denoted by the days required to attain full oral feeds. A linear regression model, controlling for sex, birth GA, and weight at salivary collection, revealed that *FOXP2* expression was significantly associated with oral feeding success ($P = 0.002$). The higher the expression level of *FOXP2*, the shorter the duration to feed. Salivary *FOXP2* expression levels are significantly associated with oral feeding success in the preterm infant. *FOXP2* may serve as a novel and informative biomarker to noninvasively assess infant feeding skills to reduce morbidities and length of stay.

[Supplemental material is available for this article.]

INTRODUCTION

The development of successful oral feeding skills and normal speech emergence is dependent on many of the same-shared muscle groups and cranial nerves required for proper oromotor planning and execution. Although research has begun to identify gene targets that could help elucidate the biological complexities associated with oral feeding in the newborn (Maron et al. 2015), there have been no studies examining expression levels of genes known to be involved in speech impairments in infants with feeding difficulties.

The forkhead box protein 2 (*FOXP2*) was the first gene to be implicated in a developmental disorder of speech and language (Lai et al. 2001). Identified as the “speech and language gene” by Drs Simon Fisher and Anthony Monaco following molecular studies of 15 individuals in the “KE family” who suffered from speech–language delays (Hurst et al. 1990), *FOXP2* is now known to play an essential role in normal speech development. Located on Chromosome 7 (Fisher et al. 1998; Lai et al. 2000, 2001), *FOXP2* is not only implicated in speech–language delays but also has a function in regulating a large number of downstream target genes associated with common forms of language impairment (Vernes et al. 2008).

Based on the shared developmental oromotor skills required for both successful feeding and speech, we speculate that *FOXP2* also plays a critical role in oral feeding success in the newborn. Neurons that express *FOXP2* are found in deep cortical layers, the basal ganglia, parts of the thalamus, and the Purkinje cells of the cerebellum (Ferland et al. 2003; Lai et al. 2003; Liegeois et al. 2003; Teramitsu et al. 2004; Spiteri et al. 2007; Takahashi et al. 2008; Campbell et al. 2009; Enard et al. 2009; Reimers-Kipping et al. 2011). In the mammalian

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brain, these areas belong to a distributed network of circuits that are involved in motor coordination, learning, and acquisition of sensorimotor skills, all essential developmental components for oral feeding (Ullman 2001; Watkins et al. 2002; Liegeois et al. 2003; Haesler et al. 2007; Ackermann 2008; Groszer et al. 2008; Campbell et al. 2009; Enard et al. 2009). Examining *FOXP2* expression levels in the at-risk preterm newborn may elucidate an additional functional role of the gene and further our understanding of the developmental complexity of oral feeding (MacDermot et al. 2005; Fisher 2007).

RESULTS

Enrolled infants had an average birth gestational age (GA) of 32.58 wk (± 1.03), average birth weight of 1882.95 g (± 271.16 g), and an average postmenstrual age (PMA) of 33.58 wk (± 0.57) when the saliva sample was attained. Pertinent demographic information on all enrolled subjects may be found in Table 1.

Total RNA was extracted and underwent reverse transcription–quantitative polymerase chain reaction (RT-qPCR) amplification for *FOXP2*, along with two reference genes

Table 1. Participant characteristics

Subject number	Sex	Birth GA (wk)	Birth weight (g)	PMA at salivary sample	Weight at salivary sample	PMA at full PO
Subject 1	Male	33.3	2562	34.1	2390	35.1
Subject 2 ^a	Male	33.5	2442	35.0	2315	34.3
Subject 3	Male	32.2	1910	32.5	1794	36.1
Subject 4 ^a	Male	33.3	1985	33.5	1984	33.4
Subject 5	Female	32.2	1919	32.5	1764	34.1
Subject 6	Male	31.3	1769	33.5	1883	37.0
Subject 7	Male	31.3	1600	33.5	1647	36.2
Subject 8	Female	31.2	1780	33.1	1754	35.3
Subject 9	Male	33.1	1600	33.3	1514	34.6
Subject 10	Male	33.1	1905	33.3	1820	34.5
Subject 11	Male	31.6	2025	33.3	1956	34.1
Subject 12 ^a	Female	33.4	1632	34.0	1495	33.6
Subject 13	Female	30.6	1587	33.1	1592	34.6
Subject 14	Female	33.6	1965	34.1	1855	34.3
Subject 15	Female	31.0	1539	33.6	1629	37.5
Subject 16	Male	32.6	2052	34.1	2088	35.2
Subject 17	Male	33.5	1830	34.0	1840	34.3
Subject 18	Female	32.5	1735	33.3	1723	35.3
Subject 19	Female	33.5	1590	33.6	1531	34.1
Subject 20	Male	33.5	2105	33.6	2096	34.1
Subject 21	Male	34.0	2010	34.2	2022	34.3
Average	13 males; 8 females	32.58	1882.95	33.58	1842.47	34.85
SD		1.03	271.16	0.57	246.76	1.072

GA, gestational age; PMA, postmenstrual age.

^aThe infant attained full PO feed, or full oral administration of feed, prior to the salivary sample. This was because these infants received minimal nasogastric supplementation and attained full PO relatively soon after birth (<5 d).

(*GAPDH*, *YWHAZ*) for normalization. One sample failed to amplify both reference genes and was excluded from the analysis. Of the remaining 21 samples that met quality control standards, all had amplifiable *FOXP2*. There was no amplification of gene targets in the negative control wells. Using the same sample, we assessed the impact of multiplexing on gene amplification. There was no statistically significant difference in amplification for any gene (*FOXP2*, *GAPDH*, *YWHAZ*) between singleplex and multiplex formatting. Singleplex amplification of *FOXP2* occurred at threshold cycle (C_t) 35.3 and multiplex at C_t 34.82 (*GAPDH*); and at C_t 34.67 (*YWHAZ*), singleplex amplification *GAPDH* was C_t 27.15 and multiplex at C_t 28.66; singleplex amplification of *YWHAZ* occurred at C_t 26.36 and multiplex at C_t 26.17.

The linear regression model, controlling for sex, birth GA, and weight at salivary sample, revealed that *FOXP2* was significantly ($P = 0.002$) associated with oral feeding success. Within this model, the *FOXP2* standardized coefficient β , a measure of how strong a predictor variable influences the dependent variable, was also significant ($P = 0.049$) with a 95% confidence interval of 0.011–4.91; please see Supplemental Table 1 for each participant's ΔC_t number. Although a relatively weak correlation (Pearson correlation = 0.057) remains between *FOXP2* expression levels and oral feeding success without the model, the emerging data indicate that as *FOXP2* expression increases, days to attain full oral feed decreases.

To determine whether salivary *FOXP2* gene expression differs between preterm males and females, the mean ΔC_t values of *FOXP2* for male and female participants were compared. The average ΔC_t value for female participants was $6.95 (\pm 1.04)$. The average ΔC_t value for male participants was $7.38 (\pm 1.39)$. A one-way ANOVA revealed there to be no significant differences in *FOXP2* expression level based on sex ($P = 0.460$). In addition, there was no difference in days to full oral feeding between the males (8.38 ± 7.11) and females (12.12 ± 11.28) ($P = 0.360$).

DISCUSSION

To our knowledge, this is the first study to noninvasively and prospectively examine *FOXP2* gene expression in preterm infants learning to feed and is the first to demonstrate an association with *FOXP2* gene expression levels and feeding success. Historically, the *FOXP2* gene has been retrospectively examined only when there is a mutation present and/or speech–language delays have already been manifested (Hurst et al. 1990; Lai et al. 2001, 2003; Liegeois et al. 2003; MacDermot et al. 2005; Fisher and Scharff 2009; Bowers et al. 2013). To date, *FOXP2* gene expression levels have not been measured prospectively to assess how variations in expression relate to behaviors, specifically the ability to orally feed.

Feeding is a complex biological behavior that involves many pathways and mechanisms. The goal of this study was to examine whether salivary gene expression of *FOXP2* is a novel and noninvasive way to predict these behaviors in newborns. Our data reveal that a higher *FOXP2* expression level resulted in less time (days) required for the infant to reach full oral feeds. Interestingly, previous reports have shown that *FOXP2* is differentially expressed based on sex. Bowers et al. (2013) examined the amount of *FOXP2* protein in the left cortical hemisphere in children and found that 4-yr-old boys had significantly lower *FOXP2* expression levels than aged-matched girls (Bowers et al. 2013). This research suggests that *FOXP2* expression levels vary among different populations, such as males and/or preterm infants. In the current study, a similar pattern was evident with males having a higher ΔC_t and therefore a lower *FOXP2* expression level than females at the same PMA. More research needs to be completed with a larger sample size equal in sex distribution to examine this difference in more detail.

The identification of informative biomarkers of oral feeding maturity holds great potential to improve neonatal clinical care. It is estimated that between 40% and 70% of premature

infants experience feeding difficulties (Rudolph and Link 2002), and that these difficulties likely stem from poor oromotor coordination and immature sucking patterns (Bu'Lock et al. 1990; Tamura et al. 1996; Lau et al. 2000; Estep and Barlow 2007; Medoff-Cooper et al. 2009). Currently, assessment of sucking and feeding in the NICU (neonatal intensive-care unit) is subjective, and failure to detect delays can result in choking, hypoxia, and aspiration, which may ultimately result in feeding aversions (Mizuno and Ueda 2005; Lau 2006). Feeding aversions are difficult to treat and often lead to prolonged hospitalization and poor outcomes such as failure to thrive and poor growth. In fact, if left untreated, feeding problems may persist well into early childhood and manifest as long-term feeding disabilities, requiring management by pediatric gastroenterologists (Jadcherla 2006; Jadcherla et al. 2007, 2008). Premature infants make up >40% of patients followed in feeding disorder clinics (Lau 2006). Because of the immense amount of feeding issues in this population, researchers have focused their efforts on the development of more objective assessments to implement targeted therapeutic strategies aimed at improving sucking, feeding, and weight gain in pre-term infants (Barlow et al. 2008; Poore et al. 2008; Maron et al. 2012; Zimmerman and Barlow 2012; Zimmerman et al. 2013). More recently, gene expression salivary biomarkers have been identified to predict neonatal feeding maturity (Maron et al. 2012, 2015). However, none of the previously identified biomarkers focuses solely on oromotor function and development. Rather, their biological functions include sensory integration, hypothalamic regulation of feeding, hunger signaling, and palate development.

Examining neonatal *FOXP2* expression levels has great potential not only for predicting oral feeding but also for predicting subsequent speech development. The behavioral tasks of oral feeding and speech production share a considerable amount of neural resources and muscle systems. Although this study is the first to examine *FOXP2* expression levels and feeding success, the link between mutations in the *FOXP2* gene and speech production is clear. For example, heterozygous mutations of the *FOXP2* gene in humans cause severe speech–language delays (Lai et al. 2001, 2003; Liegeois et al. 2003; MacDermot et al. 2005; Fisher and Scharff 2009)—specifically verbal apraxia (dyspraxia), a speech disorder of motor planning. Additionally, mutations to the *FOXP2* gene hinder vocalizations and motor abilities, as is evidenced by the functional knockdown of *Foxp2* in young zebra finches, which leads to incomplete and inaccurate vocal imitations during song learning (Middleton and Strick 2000; Fisher and Scharff 2009). Furthermore, knockout mice with mutations in one copy of *Foxp2* have reduced vocalizations; whereas a mutation in two copies of the gene causes impaired vocalizations, as well as lung and brain development (Shu et al. 2007).

Limitations to this proof of principle study include a small sample size derived from a single-center NICU and quantification of *FOXP2* from a single moment of time in an infant's development. Future multicenter studies will need to be conducted in order to validate our findings across a diverse group of newborns and to further explore *FOXP2* gene ontogeny in the at-risk newborn. Nevertheless, these data are the first to identify a potential novel role of *FOXP2* in the human that may serve as an important adjunct to clinical decision-making to improve care.

CONCLUSIONS

When sex, birth GA, and weight at salivary sample were controlled for, a higher *FOXP2* expression level resulted in less time (days) required for the infant to reach full oral feeds. Overall, these findings provide preliminary data to support the hypothesis that *FOXP2* plays a critical role in neonatal oral feeding emergence and has the potential to be used as a clinically relevant and objective measure to identify infants that are at-risk for feeding difficulties.

Identifying these at-risk infants before they begin to feed orally may result in interventions that could ultimately improve health outcomes and decrease the length of stay.

METHODS

Participants

Twenty-one premature infants born between 30 and 34 wk GA with a birth weight >1500 g were recruited for this study. Exclusion criteria included major chromosomal anomalies, intraventricular hemorrhage (>grade II), necrotizing enterocolitis, excessive drug/alcohol use, data missing regarding the date to full oral feeds, and/or the need for prolonged respiratory support (i.e., high-flow nasal cannula, continuous positive airway pressure, or ventilator support) that would have delayed oral feeding trials.

Salivary Collection, mRNA Extraction, and FOXP2 Gene Expression Analysis

Saliva samples were taken at the closest approximate time (within the 32–34-wk period) that an infant commenced oral feeding attempts. There were a few infants (see those indicated by an ^a in Table 1) that attained full oral feeds before the acquisition of the salivary sample. Two of these infants were capable of full oral feeds from birth, and the other took 5 d to attain full oral feeds, only briefly requiring nasogastric support before the attainment of a sample. All infants in the Tufts Medical Center NICU are subjectively assessed for oral feeding readiness based on the cue-based feeding algorithm described by Ludwig and Waitzman (2007). Salivary samples were collected and processed with previously described techniques in order to simulate routine bedside care of the neonates (Dietz et al. 2012; Maron et al. 2015). Briefly, saliva was collected with a 1-mL syringe attached to low wall suction. The neonate's oropharynx was gently suctioned (<1 min) and saliva was immediately stabilized in 500 μ L of RNeasy Protect Saliva (QIAGEN). This stabilizing agent halts gene expression changes, inhibits microbial overgrowth, and destroys ubiquitous RNases. Two salivary samples were collected from a single time point. One sample was analyzed for FOXP2 expression; the other sample was stored in a biobank for subsequent validation studies. Once collected, samples were stored for a minimum of 48 h at 4°C before total RNA extraction with the use of the RNeasy Protect Saliva Mini Kit (QIAGEN). On-column DNase digestion was performed on all samples to eliminate DNA genomic contamination. Samples were stored at –80°C pending analysis.

Multiplex RT-qPCR

Every attempt was made to adhere to minimum information for quantitative real-time PCR experiments (MIQE) guidelines established in 2009 to ensure proper and accurate reporting of RT-qPCR data (Bustin et al. 2009). Relative quantitative gene expression differences of salivary FOXP2 were assessed on the Applied Biosystems QuantStudio 7 Flex real-time PCR instrument with the use of two reference genes (GAPDH and YWHAZ) previously shown by the Maron laboratory to maintain their gene expression across PMAs (Maron et al. 2012). Inventoried stock sequences of reference and target genes were provided by Life Technologies: GAPDH (Hs03929097), YWHAZ (Hs03044281), and FOXP2 (Hs00362818_m1). For each salivary sample, FOXP2 was run in duplicate, multiplexed one time each with the two reference genes with the Path-ID Multiplex One-Step RT-PCR Kit (Life Technologies). To ensure that multiplexing did not interfere with gene amplification, each gene was also run in singleplex one time with the use of a single sample. Negative controls with nuclease-free water were run on each plate to ensure that there was no primer–primer amplification that may have skewed the data. The RT-qPCR cycle profile was as follows:

reverse transcription, 48°C × 15 min; activation of DNA polymerase, 95°C × 10 min; 40 cycles of PCR, denaturing 95°C × 15 sec followed by annealing/extension at 60°C × 1 min. If a sample failed to amplify both reference genes, it was deemed to be of insufficient quality and was not considered in the analysis. The ΔC_t method was used for relative gene expression quantification in all samples (Livak and Schmittgen 2001). The geometric mean of the C_t values of both reference genes were used to calculate ΔC_t s with the following equation:

$$\Delta C_t = (\text{Mean } C_t \text{ FOXP2} - [\sqrt{(C_t \text{ GAPDH} \times C_t \text{ YWHAZ})}]).$$

Sex and Oral Feeding Data Collection

The infant's sex was obtained from their NICU admission medical note. Daily oral feed percentage was extracted from the nursing care notes and calculated across the eight daily feeds for all infants in the study. Total number of days required to reach full oral feeding was determined by subtracting PMA on the day of the first oral feeding attempt from the PMA on the day the nasogastric tube was successfully removed. Infants who never required nasogastric tubes to sustain oral feeds were considered to have the developmental maturity of a successful oral feeder from birth. These infants achieved a full oral intake of ≥ 140 cc/kg/day within the first 7 d of life.

Statistical Analyses

A linear regression model was completed to examine the association between oral feeding and FOXP2 gene expression with birth GA, sex, and weight at salivary sample entered as covariates into the model as they are known factors to influence feeding development (Maron et al. 2015).

ADDITIONAL INFORMATION

Ethics Statement

The Tufts Medical Center Institutional Review Board approved this study and parental consent was attained prior to the start of the study.

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Competing Interest Statement

The authors have declared no competing interest.

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