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Mutational analysis of *NOG* in esophageal atresia and tracheoesophageal fistula patients

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Abstract

Purpose—The *NOG* protein is a secretory antagonist of bone morphogenetic proteins (BMPs). *Nog*−/− mouse embryos demonstrate proximal esophageal atresia (EA) and distal tracheoesophageal fistula (TEF) compatible with the most common configuration of EA/TEF observed in humans. Four microdeletions that span the *NOG* locus at 17q22 have been described in human patients having EA/ TEF. We investigated the incidence of point mutations in the coding region of the *NOG* gene in human EA/TEF.

Methods—DNA was collected from 50 patients previously treated for EA/TEF. PCR was used to amplify the coding region of *NOG*. To detect single nucleotide polymorphisms (SNPs), amplicons were subjected to temperature gradient capillary electrophoresis (TGCE). Candidate SNPs were directly sequenced.

Results—TGCE analysis revealed a SNP in the coding region of *NOG* in 1 of 50 patients (2%). DNA sequencing revealed a synonymous SNP at position 468 (C–T) of the *NOG* coding region.

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Conflict of interest The authors declare that they have no conflicts of interest.

Conclusion—SNPs in the coding region of the *NOG* gene are identified infrequently in human cases of EA/TEF. Further investigation of SNPs in the promoter region of *NOG* is warranted, as is the effect of synonymous SNPs on *NOG* mRNA stability.

Keywords

Esophageal atresia; Tracheoesophageal fistula; *NOG*; Noggin

Introduction

Esophageal atresia (EA) and tracheoesophageal fistula (TEF) are the most common congenital disorders of foregut development and occur in approximately 1 in 3,500 live births [1]. This spectrum of anomalies is thought to arise from failure of the lung bud and foregut to separate during embryogenesis, resulting in EA with the variable presence and location of a fistula between the airway and gut. The most common configuration of EA/TEF is characterized by a proximal blind esophagus and a distal TEF and occurs in 85% of patients (Fig. 1b) [2].

Approximately 50% of EA/TEF cases are associated with additional developmental anomalies. Several single gene mutations have been identified that are associated with syndromic EA/TEF, including mutations in *SOX2*, *CHD7*, *MYCN*, *GLI3*, *FANCA*, and *MID1* [3–8]. The VACTERL association (vertebral defects, anal atresia, cardiac defects, TEF, EA, renal, and limb defects) encompasses those cases of EA/TEF that are observed in the context of other predictable developmental anomalies, but for which a unifying genetic cause has not been identified [9]. Mutations in the forkhead box gene FOXF1 have recently been identified in patients with EA/TEF, other VACTERL features, and alveolar capillary dysplasia [10]. Consequently, the remaining half of EA/TEF is non-syndromic and occurs in isolation. Less is known about the genetic events that lead to isolated EA/TEF.

Several animal models of EA/TEF have been developed that greatly inform our understanding of mammalian foregut development. The *Nog*−/− mouse embryo phenotype most closely resembles the configuration typical of human disease (Fig. 1a) [11]. In this model, Foxa1 (endoderm marker) immunohistochemical labeling of e11.5 *Nog*−/− embryos clearly reveals a proximal blind esophagus with distal TEF (Fig. 1a) [11]. The phenotype can be rescued by simultaneous knockout of BMP7, illustrating the critical interplay of NOG and BMP signaling in foregut development [11].

The *Noggin* (*NOG*) gene encodes a secreted protein initially identified as an antagonist of bone morphogenetic proteins (BMPs) in *Xenopus* [12]. It was subsequently found that *NOG* directly binds to BMPs and inhibits their signaling during vertebrate development. In humans, *NOG* is 1890 bp, located at 17q22, and contains a single 696 bp exon. Mutations in human *NOG* cause proximal symphalangism (SYM1; OMIM 185800) and multiple synostoses syndrome (SYNS1; OMIM 186500), two disorders affecting skeletal development [13, 14]. The phenotypes of these diseases are also conserved in mice lacking *NOG* function; *Nog*−/− mice are embryonic lethal and possess multiple synostoses of the axial and appendicular skeleton [15]. Several missense and nonsense mutations that are predicted to result in either amino acid substitutions or truncations of the NOG protein have

been indentified in SYM1 and SYNS1 patients [13, 16, 17]. No mutations within the *NOG* gene have been documented in human patients having foregut malformations to date, although several cases of EA/TEF and symphalangism having chromosomal deletions that span the NOG locus have been reported (Table 1) [18–24].

Taken together, the chromosomal deletions that span the *NOG* locus in EA/TEF patients and the striking similarity of the *Nog*−/− mouse phenotype to the most common form of human EA/TEF strongly suggest a link between EA/TEF and *NOG* mutations in humans. We hypothesized that mutations in the coding region of *NOG* are linked to the human form of EA/TEF.

Methods

Study cohort

To explore the presence and frequency of point mutations in the coding region of *NOG*, we conducted a mutational analysis of this gene in humans having EA/TEF. After obtaining institutional IRB approval for human subjects research (IRB# 030042), letters soliciting participation in our study were mailed to guardians of 87 consecutive cases of EA/TEF, and 50 patients were enrolled in the study. After obtaining informed consent, 3 mL of blood were collected from each patient by peripheral venipuncture. A blood sample from an unaffected sibling was collected to isolate control (wild-type) DNA.

DNA extraction and polymerase chain reaction (PCR)

DNA was isolated from blood samples of the 50 EA/TEF patients and the control subject using a QIAamp spin column mini kit (Qiagen, Valencia, CA). Prior to utilizing the control DNA, the *NOG* coding region was amplified by PCR as described below. Control amplicons were directly sequenced and confirmed to match the wild-type *NOG* coding sequence using the Basic Local Alignment Search Tool (BLAST; NCBI; Bethesda, MD). The *NOG* gene has a single exon and the protein coding region consists of 698 nucleotides. The ATG translational start codon begins at nucleotide 812. The TAG stop codon ends at 1510. The *NOG* coding region was amplified by PCR using a proofreading polymerase (9:1 mixture AmpliTaq Gold to Pfu-Turbo) (Agilent Technologies, Santa Clara, CA and Applied Biosystems Foster City, CA) with the following primers: NOG (forward): 5'-GGACGCGGGACGAAGC AGCAG-3'; NOG (reverse): 5'-GAGGATCAAGTGTCCG GGTGC-3'.

PCR conditions were as follows: 94°C for 4 min; 35 cycles of (94°C for 30 s, 64°C for 60 s, 72°C for 60 s); 72°C for 10 min. The 765-bp PCR product was evaluated and confirmed by electrophoresis.

Mutational analysis of amplicons derived from genomic DNA of EA/TEF patients

Amplicons were evaluated for candidate single nucleotide polymorphisms (SNPs) using temperature gradient capillary electrophoresis (TGCE) analysis, as previously described [25]. Briefly, in preparation for TGCE analysis, PCR fragments were denatured for 3 min at 95°C and annealed in a thermal cycler via a stepwise reduction in temperature as follows:

decreased from 95 to 80°C at 3°C/ min; decreased from 80 to 55°C at 1°C/min; held at 55°C for 20 min; decreased from 55 to 45°C at 1°C/min; and decreased from 45 to 25°C at 2°C/ min. The Center for Molecular Neuroscience Neurogenomics Core at our institution performed TGCE analysis using a Spectru-Medix (State College, PA) REVEAL TGCE apparatus SCE9610 in a 96-well format. This technology involves combination of test DNA to control DNA. In the event of identical sequences, a homoduplex with a uniform melting temperature is created. In the event of sequence variation such as a single nucleotide polymorphism (SNP), a heteroduplex between mismatched test and control DNA forms, resulting in a downshifted melting temperature. A temperature gradient is applied to specimens that cover all possible melting temperatures for the analyzed specimens, therefore separating heteroduplexes from homoduplexes. Melt curves from homoduplexes have a uniform contour, while those from heteroduplexes have multiple peaks and represent candidate SNPs.

Samples that resulted in heteroduplex formation and therefore contained candidate single nucleotide polymorphisms were subjected to direct DNA sequencing.

Results

NOG mutational analysis

REVEAL analysis indicated heteroduplex formation in 3 of the 96 wells analyzed, representing 3.125% of the overall study sample (Fig. 2). cDNA in each of these wells originated from the same patient and represented the detection of a candidate SNP in the coding region of the *NOG* gene. This patient was the only of 50 analyzed to possess a SNP in the *NOG* coding domain (2% of the overall study population). The remaining 93 wells contained homoduplexes and therefore did not represent candidate SNPs. Direct sequencing of the three samples containing heteroduplexes resulted in detection of a SNP at position 468 of the *NOG* coding region (C–T), which happens also to code for pro-line, the naturally occurring amino acid at this location in the NOG protein. This finding is in contradistinction to a point mutation in which the amino acid sequence of the resulting protein would change.

Description of study cohort

Data describing our study cohort are reported (Table 2). Of note, 42% of patients in this study had syndromic EA/TEF associated with additional congenital anomalies. The patient in whom this synonymous SNP was detected had a proximal EA with distal TEF, the type most common in humans (Fig. 1b). The patient did not have any associated congenital anomalies.

Discussion

Our analysis detected a synonymous SNP at position 468 in the coding region of the human *NOG* gene in 1 of 50 patients treated at our institution for EA/TEF. Because this synonymous SNP does not alter the amino acid sequence of the NOG protein, it is unlikely to have functional significance in the pathogenesis of EA/TEF due to degeneracy, or redundancy, of the genetic code. However, evidence is emerging that synonymous SNPs can indeed alter mRNA splicing and stability [26]. Alternative splicing is not implicated in this

index case because the *NOG* gene contains no introns and therefore does not have splice variants. The identified SNP could conceivably affect *NOG* mRNA stability, which is a potential area for future investigation.

In our index case, the codon CCC is altered to CCT, both of which code for the amino acid proline. Therefore, it is unlikely that point mutations in the coding region of the human *NOG* gene contribute frequently to the development of EA/TEF.

While our genetic screen for point mutations in the human *NOG* gene revealed only the above-mentioned synonymous SNP, our study is limited by the sample size of 50 patients and by examination of only the coding region of *NOG*. We did not screen for SNPs in the promoter region of the gene. SNPs located in the promoter region of genes have been shown to modulate transcription factor binding and DNA unwinding, both of which result in functional consequence [26]. It is possible that the *NOG* gene could be dysregulated in EA/TEF due to suppressed promoter activity. A recent review of NOG mutations catalogues 17 detected polymorphisms in the *NOG* gene, 5 of which are in the coding region, among asymptomatic patients [27]. The most common polymorphism in the coding region of *NOG* (G–A at base pair 582 of the coding region) is found in 21% of individuals, while allele frequencies for the remaining polymorphisms have not been determined [27]. The SNP we detected in this analysis has not been previously reported.

Nevertheless, our study is the first reported attempt to explore mutations in the *NOG* coding region in patients having EA/TEF. Previous reports have documented both missense and nonsense mutations in the *NOG* coding region in patients with proximal symphalangism and multiple synostoses syndrome, however, these patients did not also possess EA/TEF [13, 16, 17]. Interestingly, chromosomal microdeletions that span the NOG locus have been reported to cause both symphalangism and EA/TEF (Table 1). The most recent microdeletion reported by Puusepp et al. identified by array-based comparative genomic hybridization significantly narrowed this region of interest to a 5.9-Mb region in 17q22–q23.2. Other candidate genes in this region include the retinoid receptor RARa and also TBX4, however, no mutational analysis of these genes has been conducted in patients with EA/TEF [24]. While we did not detect a significant proportion of patients with point mutations in the *NOG* coding region, our results may redirect future investigation to the *NOG* promoter region or to associated players in BMP signaling.

The role of environmental factors on *NOG* and BMP signaling during the period of organogenesis is another intriguing area for future investigation. A litany of maternal exposures including methimazole, statins, alcohol, smoking, maternal phenylketonuria, infectious disease, agricultural profession, and diethylstilbestrol have been implicated anecdotally or epidemiologically in the pathogenesis of EA/TEF, however, these observations have not been consistently confirmed in multiple studies nor have their potential mechanisms been elucidated [28]. Circulating maternal BMP7 is confirmed to cross the placenta in experimental animal models and is available until e14 of mouse gestation [29]. Perhaps the role of circulating and transplacental migration of maternal BMPs in organogenesis and specifically in the pathogenesis of EA/TEF warrants investigation as well.

Applying lessons learned from basic science and genetic animal models, our study represents an important investigation into mechanisms influencing congenital anomalies in human patients. Our innovative approach serves as a paradigm to clarify the clinical significance of animal findings in human disease. Although mutations in the coding region of the *NOG* gene do not appear to be significantly implicated in the pathogenesis of human EA/ TEF, the animal data strongly support a role for this protein and its binding partners (BMPs) in the development of this anomaly. Further study of the antagonistic signaling interface between NOG and BMP7 is certainly justified in the EA/TEF population.

Ethical standards statement

All human subjects' research outlined in this manuscript was conducted with prior approval from the Vanderbilt University Institutional Review Board and therefore in compliance with the standards outlined in the 1964 Declaration of Helsinki. All parents or guardians of human subjects in this study gave informed consent prior to their inclusion in this study.

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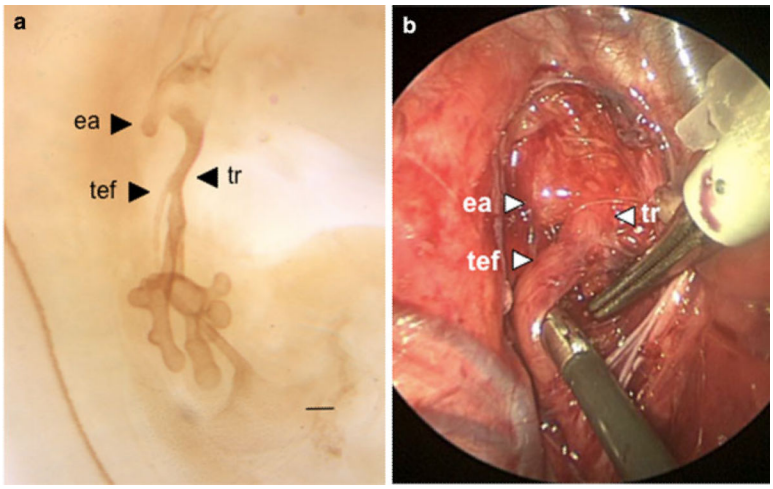

Fig. 1.

Similarities in mouse (**a**) and human (**b**) forms of proximal esophageal atresia (ea) with distal tracheoesophageal fistula (tef) provided rationale for this study. **a** *Nog*−/− mouse phenotype exhibits proximal esophageal atresia (ea) and distal tracheoesophageal fistula (tef). *Scale bar* 20 μm. *b* Operative photo shows similar anomalous anatomy of EA/TEF in humans. *ea* esophageal atresia, *tr* trachea, *tef* tracheoesophageal fistula

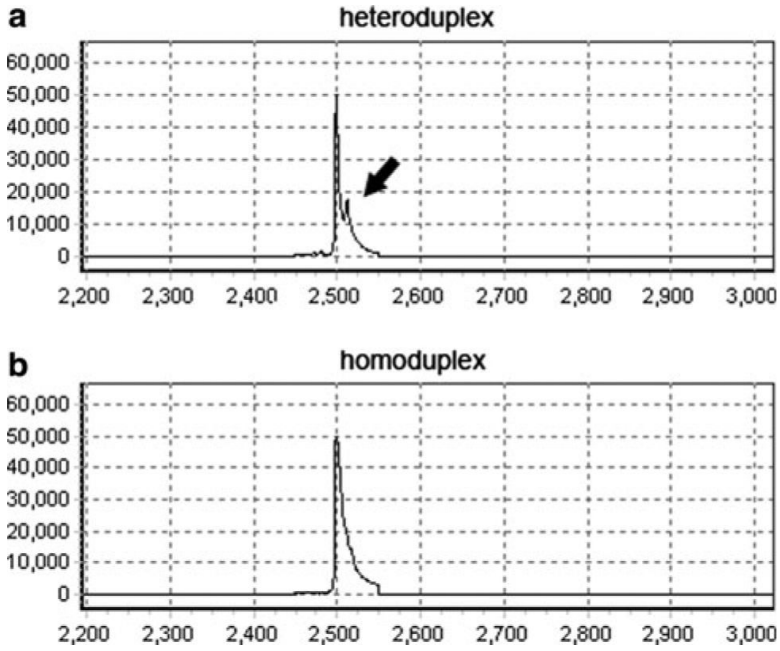

Fig. 2.

Screening for candidate SNPs in *NOG* gene of EA/TEF patients. **a** Temperature gradient capillary electrophoresis (TGCE) analysis of hybridized patient and control DNA reveals a second peak in the melt curve (*arrowhead*). This additional peak represents heteroduplex formation and therefore a candidate single nucleotide polymorphism (SNP). *b* A uniform melt curve indicates homoduplex formation, or identical sequence, between patient and control DNA

Table 1

Reported chromosomal deletions that span the NOG locus (17q22) in patients with EA/TEF and symphalangism

	Park et al. [19]	Dallapiccola et al. [20]	Khalifa et al. [18]	Mickelson et al. [23]	Levin et al. [22]	Marsh et al. [21]	Puusepp et al. [24]
Karyotype	46, XX, del(17) (q21.3q23)	46,XY, del(17) (q21.3q24.2)	46, XY, del(17) (q22q23.3)	46, XY, del(17) (q23.1q23.3)	46, XY, del(17) (q23.2q24.3)	46, XY, del(17) (q22q23.3)	46, XY, del(17) (q22q23.2)
EA/TEF	+	+	–	–	–	+	+
Symphalangism	+	+	+	+	–	+	+

Table 2

Description of study cohort

	Number (%)
Gender	
Male	25 (50)
Female	25 (50)
Type of EA/TEF	
A (EA alone, no TEF)	1 (2)
B (EA with proximal TEF)	2 (4)
C (EA with distal TEF)	40 (80)
D (EA with proximal and distal TEF)	0 (0)
E (No EA, H-type TEF)	7 (14)
Associated anomalies	21 (42)
Vertebral	5 (10)
Anogenital	7 (14)
Cardiac	16 (32)
Renal	11 (22)
Limb	14 (28)