Characterization of the Larynx in Ephrin-B2 Knockout Mice

A Novel Animal Model for Laryngeal Clefts

Ryan E. Neilan, MD; Dongmei Shao, MD; Christopher Dravis, PhD; Mark Henkemeyer, PhD; Kenneth H. Lee, MD, PhD

Objective: To identify and classify laryngeal clefts in a novel mouse model.

Design: In vivo animal study.

Setting: Academic research laboratory.

Subjects: 129/CD1 mice with the ephrin-B2 gene disrupted by the β-galactosidase (lacZ) gene were humanely killed at embryonic day 18 (E18) and evaluated for the presence and characterization of a laryngeal cleft. Homozygous and heterozygous lacZ knockout mice as well as wild-type littermates were evaluated.

Main Outcome Measures: Microsurgical dissection of the oral cavity and pharynx allowed for a pseudoendoscopic view of the larynx to determine the presence or absence of a cleft. The specimens were also histologically sectioned and examined for characterization and classification of the cleft.

Results: A laryngeal cleft was identified in 12 of 27 ephrin-B2 homozygous lacZ knockout mice (44%). Laryngeal clefts were not identified in heterozygous ephrin-B2 knockout mice or in wild-type littermates.

Conclusions: Disruption of ephrin-B2 reverse signaling results in laryngeal clefts in lacZ knockout mice. This presents a novel mouse model in which future investigations into etiology of laryngeal clefts may be examined.

Laryngeal clefts are a rare congenital anomaly consisting of a defect in the posterior laryngotracheal wall. The reported incidence ranges from 0.0001% to 7.6%. Laryngeal clefts can present with a wide range of symptoms including cough, dyspnea, hoarse cry, stridor, cyanosis during feeding, and recurrent pulmonary infections. Diagnosis requires a high index of suspicion. The Benjamin-Inglis classification system categorizes these into types I to IV based on the severity of the anatomical abnormality, which is often correlated with symptom severity. In type I, the cleft is located above the vocal cord level, while in type II, the cleft extends down into the cricoid cartilage. In type III, the cleft extends completely through the cricoid cartilage and into the cervical trachea. In type IV, the cleft extends even further down the trachea into the thoracic cavity. Laryngeal clefts are associated with other congenital abnormalities in up to 68% of cases. Diagnosis is made with microlaryngoscopy and bronchoscopy and should include palpation and splaying of the arytenoids. The goal of management is the prevention of pulmonary complications related to aspiration and gastroesophageal reflux. Chronic aspiration can lead to chronic interstitial lung damage. Conservative management is the first-line treatment and includes thickened feeds, anti-reflux therapy, and upright positioning during feedings. Surgical intervention is reserved for those in whom conservative management fails and those with severe pulmonary symptoms; this may be undertaken through an endoscopic or open approach. The endoscopic approach, typically involving the use of carbon dioxide laser and 2-layer closure, has become the preferred method in all but the most severe clefts.

The embryological development of the foregut has yet to be fully elucidated.
A number of animal models have been developed in this endeavor, but none have demonstrated a laryngeal cleft. Ephrins are a family of membrane-bound molecules that bind to Eph receptors. Their interaction and resulting inhibitory signal have been shown to guide axons toward synaptic targets in both retinal and cochlear tissues. These proteins have also been shown to be involved in cardiovascular development, maintaining ionic homeostasis in semicircular canal development, as well as regulating midline fusion in urorectal development. We report and classify laryngeal clefts in a known mouse model in which the loss of ephrin-B2 signaling has disrupted normal midline fusion of the posterior laryngotracheal wall, leading to the development of upper aerodigestive anomalies.

**METHODS**

**EXPERIMENTAL ANIMALS**

The production of ephrin-B2 lacZ knockout mice have been previously described. All ephrin-B2 mutants were maintained on a mixed 129/CD1 background. Our study included the evaluation of homozygous and heterozygous knockout mice. At embryonic day 18 (E18), the mice were humanely killed by cardiac perfusion with 4% paraformaldehyde. The specimens were post-fixed with 4% paraformaldehyde overnight and then stored in phosphate-buffered saline until microdissection was performed. Wild-type littersmates were used as controls.

**GROSS PREPARATION OF SPECIMENS**

Microsurgical dissection was then performed with microforces under a Leica stereomicroscope (Leica Microsystems) to expose the supraglottis, glottis, and upper trachea. Once dissections were completed, the presence of a laryngotracheal cleft was determined by direct visualization under the stereomicroscope from a perspective analogous to the endoscope surgical view. Images were captured with a Nikon digital camera (Nikon Inc).

**HISTOLOGICAL ANALYSIS OF SPECIMENS**

The specimens were trimmed to exclude extraneous tissue and embedded in Tissue-Tek O.C.T. compound (optimal cutting temperature, polyethylene glycol– and polyvinyl alcohol–based embedding medium), frozen with dry ice, and sectioned with a Leica CM 3050S cryotome (Leica Microsystems) in the coronal plane with 14-µm sections and mounted on glass slides. The sections were stained with hematoxylin-eosin, coverslipped, and then viewed on an Olympus BX50 light microscope (Olympus Corporation) with a 40X objective (total magnification, ×40) and captured digitally with a Nikon DXM1200 camera for analysis. The degree of laryngotracheal cleft was determined by review of these sections.

**RESULTS**

On gross examination of 27 ephrin-B2 lacZ knockout homozygous mice, a laryngeal cleft was present in 12 (44%) (Figure 1). None of the 27 wild-type or heterozygous mice demonstrated a laryngeal cleft (Figure 2). The coronal sectioning of specimens was found to be the best orientation for grading the degree of cleft. Of the 12 homozygous specimens with laryngeal clefts evaluated in coronal sections, 6 displayed clefts that were rated type III or IV on the Benjamin-Inglis classification scale. (Table). Figure 3 shows a type III laryngeal cleft extending through the cricoid and down into the trachea itself.

**COMMENT**

This study demonstrates the presence of laryngeal clefts anatomically and histologically in mutant knockout mice with disruption of ephrin-B2 signaling. From histological analysis of coronal sections through the larynx and trachea of these animals, we have shown that loss of normal function of ephrin-B2 results in laryngeal clefts that are of either grade III or IV based on the Benjamin-Inglis classification. Our results demonstrate that we have identified a mouse model for laryngeal clefts.

By evaluating the gross specimens with stereomicroscopy, the presence or absence of a laryngeal cleft of type II or higher was clearly apparent. However, it is possible that a type I cleft may have been present but not identified by stereomicroscopic evaluation of the gross specimens. According to the Benjamin-Inglis classification, a type IV cleft is distinguished from a type III if the cleft extends inferiorly to involve the thoracic trachea. The methods used to prepare and perform the histological sections of the airway did not allow preservation of the surrounding structures. Thus this precluded our ability to
distinguish type III from type IV laryngeal clefts. As a result, from this analysis we can only conclude that all the clefts identified in our ephrin-B2 null mice were at least grade III.

Patients with laryngeal clefts often prove to be challenging to achieve successful surgical repair. In a review of 170 reported clefts, 19 patients required reoperation for recurrence. In addition, many of these patients required multiple procedures with different approaches, and a total of 48 procedures were performed on the 19 patients with recurrence. In addition, patients with milder type I and II clefts have been reported to have undergone 5 to 6 procedures and in some cases still remained open, with patients requiring revision in 11% to 50% of reported cases. On the basis of the proposed role of Eph-ephrin interaction in regulating midline fusion of the posterior larynx, if this dysfunction persists postnatally in certain patients with laryngeal clefts, this potentially could provide a molecular basis as to the high rate of persistent cleft after surgical repair in these patients. Further investigation with additional experiments could provide additional support for this hypothesis.

Ephs and ephrins have been studied for their roles in axon guidance in both the central and peripheral nervous systems. Patients with laryngeal clefts often have associated neurological disorders. The presence of neurological disorders in patients with laryngeal clefts has been reported as 17%, 20%, and as high as 32%. In light of this, the associated neurological anomalies further support the conclusion that disruption of Eph-ephrin interaction is a molecular mechanism accounting for the formation of laryngeal clefts.

Disruption of ephrin-Eph signaling has been shown to result in multiple midline fusion defects in mice. The mutant mice from the present study also demonstrated other defects including hypospadias, anorectal malformations such as a primitive cloaca, and cleft palates. In addition, patients with laryngeal clefts have also been found to have other associated midline defects. In a review of 25 pediatric patients who underwent surgical repair of a laryngeal cleft, 60% were found to have tracheomalacia; 28%, esophageal atresia; 16%, Opitz syndrome; 16%, hypospadias; 12%, anal stenosis; and 12%, VATER (vertebrae, anus, trachea, esophagus, and renal) syndrome. Thus, the laryngeal clefts seen in the present study likely represents one anatomical anomaly that is a part of multiple midline defects in mice with loss of normal ephrin-Eph signaling.

In conclusion, clinically laryngeal clefts present as an entity that is potentially difficult and complicated to repair. We have yet to gain a clear understanding of the ex-
act etiology of the embryological development of this anomaly. This study suggests a role for ephrin-B2 signaling in facilitating midline fusion of the posterior larynx, since the disruption of this function results in formation of a laryngeal cleft and provides a molecular basis for its development. This study also presents an animal model for laryngeal clefts that may prove to be useful for future investigations of the etiology and treatment of this disease.

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Correspondence: Kenneth H. Lee, MD, PhD, Department of Otolaryngology–Head and Neck Surgery, University of Texas Southwestern Medical Center, 3323 Harry Hines Blvd, Dallas, TX 75390-9035 (kenneth.lee@utsouthwestern.edu).

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REFERENCES