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Association of endothelin-β receptor (EDNRB) gene variants in anorectal malformations

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Abstract Animal models have demonstrated the role of genetic influences in anorectal malformations (ARM), although the pathogenetic mechanism remains uncertain. A body of collateral evidence points to possible connection with the endothelin-β receptor (EDNRB) gene and the endothelin system. This study investigates the EDNRB gene in patients with ARM.

Resected surgical specimens of terminal colonic tissue were obtained from 14 children (6 males and 8 females) undergoing surgery for ARM correction with ethical permission. DNA samples were screened for mutations in EDNRB. Polymerase chain reaction amplification of 7 exons of EDNRB was followed by heteroduplex single-strand conformation polymorphism analysis. Heteroduplex single-strand conformation polymorphism variants were validated with automated sequencing techniques on polymerase chain reaction products showing conformational variants in acrylamide gel.

All investigated patients with ARM showed mobility shift aberrations and polymorphisms in the EDNRB gene. These included one previously described polymorphism in exon 4 (831G/A) seen in association with Hirschsprung disease and 6 novel polymorphisms identified in exons 1 (178G/A), 2 (552C/T and 561C/T), and 3 (702C/T). No aberrant banding patterns were observed. The exon 1 (178 G/A) variation was identified in 2 (50%) of 4 low lesions compared with 1 (1%) of 84 control samples. The exon 3 (702C/T) single nucleotide polymorphism was present in 3 (60%) of 5 of the supralevator lesions being associated with exon 4 (831G/A). The patient with VATER associations including cardiac and limb anomalies had the 831G/A variation only.

Analysis revealed statistically significant differences for the polymorphism 178G/A ($P < .01$, χ^2 with Yates correction = 8.24) compared to controls.

Potential disease-related mutations were identified in South African patients with ARM, raising the question of its potential role in the pathogenesis of this condition.

Anorectal malformations (ARMs), represent a complex group of congenital anomalies resulting from the abnormal development of the hindgut, allantois, and müllerian duct, which occur in 1 of every 4000 to 5000 individuals [1,2]. It represents a common congenital cause of intestinal obstruction, the spectrum of which includes anal agenesis, rectal agenesis, and rectal atresia as well as complex abnormalities of the hindgut region; the level is determined by its relationship to the pelvic floor [3,4] and the fistula level [5].

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Although initially thought to play a minor or insignificant role in the development of ARM [6-8], a potential genetic association has been suggested because of the very early developmental disruption [9] and its recurrence in families [11]. This is supported by the fact that ARM occurs in the early critical period of organogenesis before the sixth or seventh week of gestation [9] and has been visualized on ultrasound as early as the 12th week [10]. Its recurrence in families (some demonstrating autosomal dominant inheritance patterns) was initially thought to be infrequent, but more recently the risk of familial recurrence appears to range between 8% and 20% [11-13]. There is also a clear association with other chromosomal abnormalities and syndromes [14-17] as well as other evidence to support a possible genetic hypothesis in ARM [18-22].

The etiologic mechanism of ARM is not yet clear; and the association with complex syndromes (eg, Currarino, VACTERL [VATER associations including cardiac and limb anomalies]) suggest a more complex pathogenesis with possible involvement of genes related to cell cycle function, DNA/RNA replication, ribosomal synthesis, neuronal differentiation, and intracellular/extracellular signal transduction and apoptosis. All of these may be of importance in the development of ARM. A more modern understanding of the link between genetic variations and downstream signaling pathways reopens the debate as to the pathogenesis of ARM. It is possible that genetic factors may play a more important role in certain forms of ARM (eg, anorectal stenosis) than others.

In addition to its key role in the enteric nervous system development, endothelins are involved in the development of smooth muscle [23]. Specific evidence supporting the involvement of the long arm of chromosome 13 (and thus the EDNRB gene) in ARM includes a number of associations with clinical syndromes such as Hirschsprung disease (HSCR) [15,24,25], Kaufman-McKusick [14], Pallister-Hall [15], sensorineural deafness (Townes-Brocks) [16], and Lowe [17] syndromes. This hypothesis is further supported by a reported association between ARMs and chromosome 13 (the "13q syndrome") [25]) as well as reports of EDNRB mutations in 60% of patients with penoscrotal transposition [26].

This study was therefore undertaken to explore the EDNRB gene by mutation analysis in patients with unrelated sporadic ARM in the diverse South African population.

1. Materials and methods

The study was approved by the ethics review committee of the University of Stellenbosch (reference no. 2001/C019).

2. Subjects

Classification of ARM was by the Pena classification [5]. Colonic tissue samples were obtained with informed consent from 14 unrelated South African patients diagnosed with ARM. Of these patients, 6 (43%) were males and 8 (57%) were females (ratio = 1.33). Three of these patients have low lesions (3 vestibular anus, 1 low rectovaginal fistula), 4 had high lesions (2 cloacae, 1 very high rectovaginal fistula, and 1 rectovesical fistula), and 5 had fistulae between the rectum and the lower genitourinary tract. Only 1 of the high lesions was associated with the VACTERL association of congenital abnormalities. The patient group was divided into the following groups: patients with perineal or vestibular fistulae, patients with cloacae or vesical fistulae, and patients with bulbar rectourethral fistulae. Apart from the cloacae where multiple abnormalities existed, 2 of these patients had associated genitourinary abnormalities (including double vagina plus renal abnormalities).

3. Methods

DNA was extracted from colonic tissue samples of 14 unrelated subjects with sporadic ARMs and 30 population-matched controls [27-29]. Polymerase chain reaction (PCR) amplification of the 7 exons of the *EDNRB* gene was performed using intronic primers [30]. The PCR products were subjected to heteroduplex single-strand conformation polymorphism analysis [31] and resolved on a 12% polyacrylamide gel with 7.5% urea at 4°C (350 V) for 18 hours. After electrophoresis, the gel was stained with ethidium bromide, and the DNA was visualized by ultraviolet light transillumination. DNA sequencing was performed on PCR products demonstrating mobility or conformational variants in the polyacrylamide gels using an ABI 3100 PRISM automated sequencer (Applied Biosystems, Foster City, CA).

4. Statistical analysis

Allele frequencies were estimated by allele counting, and differences between patient and control groups were tested for significance by the Fisher's Exact test and χ^2 test, where applicable. A probability value of smaller than .05 was regarded as statistically significant.

5. Results

Heteroduplex single-strand conformation polymorphism mutation analysis of the coding region of the EDNRB gene revealed several variations. These included 5 previously described polymorphisms (178G/A, 552C/T, 561C/T, 702C/T, and 831G/A) previously seen in association with HSCR in a previous study [32]. A novel variant (S196N) was identified in exon 2 that resulted in a guanine to alanine transition at amino acid 196 and resulted in a change serine to asparagine in the resultant protein. All these variants were observed only in the heterozygous state, except for the