Published in final edited form as:

J Pediatr Surg. 2009 February ; 44(2): 307–311. doi:10.1016/j.jpedsurg.2008.10.077.

A Pediatric Surgeon Retools in Genetics and Genomics to Study Congenital Diaphragmatic Hernia

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Abstract

Prof Donahoe outlines her career long interest in Congenital Diaphragmatic Hernia and how this has led to her current investigations into it's aetiology and possible treatment using gene probes.

Keywords

Congenital Diaphragmatic Hernia; CHD; aCGH; SNP; CNV; LRP2; bioinformatics

The Forshall lectureship, for me, is an esteemed honor and a personal fulfillment, since I had the opportunity to train as both registrar and senior registrar at the Alder Hey Children's Hospital in the surgical unit which Isabella Forshall founded. She had the foresight, energy, and perseverance, to establish an infrastructure which led to the regionalization of neonatal care. With its success in improving the outcome of newborn congenital anomalies, Alder Hey thus became a model for the United Kingdom (UK) and for the rest of the world. She organized training for house officers, registrars, and senior registrars who came from all over the world to train in this system of high quality surgical management of pediatric disorders which was emulated nationally and internationally, as we carried home her lessons. Ms. Forshall was recognized for her accomplishments by being selected as the second president of the British Association of Pediatric Surgery. Her textbook, Neonatal Surgery, became the bible of surgical technique and illustrated that her concept of regionalization resulted in improved outcomes for newborn infants cared for in this system.

I came to Liverpool after Ms. Forshall's retirement to Alder Hey as a registrar for Mr. Rickham's service, and then served as senior registrar, where I had the pleasure of working with himself and his colleagues, Richard Cooke, Roger Cudmore, Irene Irving, and Herbert Johnston, for all of whom I had enormous respect and who all taught me a great deal that has forever affected my practice as a pediatric surgeon over the ensuing 35 years. So, it is with warm gratitude to Ms. Forshall and for her legacy that I dedicate this lecture in her memory.

As I was nearing the end of my tenure as the Chief of the Pediatric Surgical Services at the Massachusetts General Hospital for Children, we became increasingly aware of the breadth and depth of new tools and strategies becoming available to define genetic changes related to disease, beyond the few Mendelian diseases that could be discovered by linkage analysis of large families. We posited that most of the congenital anomalies that we cared for surgically

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could be attributable to genetic changes. One gene/one anomaly was not expected as a paradigm. Rather, it was anticipated that the multiple genetic etiologies would be found which would elucidate a pathway in which functional mutation of genes, i.e., ligand, receptor, signal transduction pathway, and transduced downstream genes, could produce the same phenotype, as has been shown in knockout mice produced by homologous recombination [1].

Strategies to Study the Genetics of CDH

After receiving an National Institute of Health (NIH) grant we set about to determine the genetics of Congenital Diaphragmatic Hernia (CDH) as a starting point to understand the genetics of all congenital anomalies. Personally, this required retooling in genetics and genomics which has been both challenging and rewarding. This NIH grant established a collaboration between all the pediatric surgeons at The Massachusetts General Hospital for Children (MGH) and at Children's Hospital, Boston, as well as senior geneticists, cytogeneticists, and study coordinators from each institution. We were joined in this effort by a number of research Fellows from the MGH and Children's Hospital and a team of collaborators in biochemistry, molecular genetics, and developmental biology, as well as geneticists from The Broad Institute who are attempting to determine the contributions of genetics to complex diseases, such as diabetes, hypertension, autism, schizophrenia, and inflammatory bowel diseases, to name but a few. More recently, this effort has been enhanced by computational biologists. We designed an Oracle-based database which meets strict confidentiality requirements for both clinical and research results, and over the past six years have enrolled 350 patients at both institutions and from families with multiple numbers of affected children, in the same or in different generations (multiplex families), from around the world. We chose CDH because it is one of the more common of all major congenital anomalies, occurring at approximately 1 in 2500 live births [2]. Although mortality has improved with the institution of good perinatal intensive care consisting of high frequency ventilation, extracorporeal membrane oxygenation (ECMO) [3,4], and most importantly improved gentle ventilation strategies [5], the morbidity remains high. Such high mortality resulted in small families; therefore, classical linkage studies were not feasible. Most importantly, we felt that with even as little as a 10% improvement in lung hypoplasia, we could then substantially increase survival and reduce morbidity, and thus enhance the quality of life of these babies [1,6].

Evidence for Heterogeneous Genetic Causation in CDH

There is strong evidence for genetic causation or predisposition. For example, it has been known for many years that 10% of CDH patients have associated chromosomal aberrations large enough to be detected by karyotyping. The recent addition of new molecular cytogenetic analyses such as array-based Comparative Genomic Hybridization (aCGH) [7,8,9], a method of detecting much smaller copy number variations (CNVs) [10,11] throughout the genome, has given us a clearer picture of the potential diversity of genomic changes that can lead to CDH. Genes in these regions are now being studied intensively for their relationship to the CDH phenotype, with the hypothesis that understanding the interaction between these genes will reveal cellular and molecular pathways leading to CDH. The heterogeneity of genetic components may be explained by the fact that the same phenotype can occur whether the defect is in a ligand, receptor, signaling transduction molecule, transcription factors, epigenetic modulators of gene expression, or from changes resulting in the up- or down-regulation of downstream genes or those even further removed in subsequent pathways, that they in turn regulate.

Candidate genes for CDH have been generated in a number of ways:

1. Genes inactivated by homologous recombination or in gene mutation screens, such as those produced by the teratogen ENU, produced a CDH phenotype from such genes as SLIT3 [12], ROBO1, the Slit receptor [13], Coup-TFII [14], WT1 [15], and Fog2 [16].
2. Genes in the retinoic acid pathway which have been shown in experimental models of vitamin A deficiency [17,18], and by the use of the toxicant, nitrofen, to be important in lung [19,20] and diaphragm development [21,22,23,24].
3. Genes in chromosomal regions delineated by karyotyping and array CGH, such as the 15q26 interval [7] which contains genes such as COUPTFII (NR2F2), and 1q41–42 [9] which contains the DISP1 gene, so important for sonic hedgehog (SHH) release [25].
4. Genes in mice in which expression varies at the time of branching morphogenesis or alveolization, such as Shh [26,27], and its downstream transcription factors Gli1 and Gli2 [28], and FoxF1 [29].
5. Genes in the Wnt pathway [27].
6. Migration molecules such as SLIT and ROBO [12,13].
7. Transcription factors that interact with FOG-2 [30], such as GATA-4 [31], GATA-5, GATA-6, and COUPTFII. All are also important in the retinoic acid pathway [19].
8. Autosomal recessive genes in syndromic patients, such as LRP2 [1] and STRA-6 [32], both found by homozygosity mapping and both receptors for retinoic acid binding protein, will be important to resequence in isolated cases.

Array-based Comparative Genomic Hybridization (aCGH); the Importance of Copy Number Variations (CNVs) in Congenital Anomalies such as CDH

Small structural variations in the genome, or Copy Number Variations (CNVs), including deletions, duplications, insertions, and rearrangements have recently been found in large numbers throughout the human genome [10,11] by array CGH, with ever-increasing resolution. Such CNVs are now appreciated as being equally important as Single Nucleotide Polymorphism (SNP) variants in the dissection of the genome. We elected to study such structural variants in patients with complex congenital diaphragmatic hernia, i.e. those with multiple anomalies or with syndromic features, with the prediction that this technology will be valuable in the study of patients with congenital anomalies.

Our first analysis of 35 such patients, done using Spectral Array chips with a low resolution of 2 megabases across the genome, detected a microdeletion in chromosome 1q41 42 in a patient with Fryns syndrome, with CDH and additional multiple anomalies (Fig. 1) [9]. The 6.5 megabase deletion was found to be *de novo* (i.e. not present in the parents). It is anticipated that *de novo* mutations will more likely be represented in major congenital anomalies.

Sequencing of genes in microdeleted or microduplicated loci in other patients with CDH is the next step, but expense often precludes extensive or complete sequencing. Prioritization, though risky, is often required. For example, DISP1 (dispatched-1) is a candidate gene found in the 1q41 42 region selected for sequencing because of its importance for release or secretion of sonic hedgehog (SHH), a gene necessary for normal lung development [26,27]. Sequencing of this gene in 24 complex CDH patients revealed a heterozygous substitution which changed alanine to glycine in an evolutionary conserved position (A1471G). Further analysis revealed that this mutation was mosaic and *de novo*. This gene and a number of others in this region are being resequenced in a larger population of both complex and isolated cases. Resequencing of

genes in the 15q426 deletion interval, particularly a prime suspect, COUPTFII, has so far yielded no sequence changes that lead to amino acid changes (nonsynonymous SNPs).

Homozygosity Mapping of Consanguineous Families with Multiple Children Affected with CDH

We searched the world for families in which more than one child was affected with Congenital Diaphragmatic Hernia and found a large family in the Middle East from a consanguineous kindred which had four affected children with CDH as part of a broader syndrome, called Donnai-Barrow syndrome [33]. We studied these children by homozygosity mapping using single nucleotide polymorphism (SNP) arrays which revealed several regions of shared homozygosity, the largest of which was on chromosome 2q23.3–31.1 (Fig. 2) [1]. This region was further narrowed to an 18 megabase region of shared homozygosity. When we sequenced the genes in that region of 51 known genes we found a mutation in the largest gene, Low Density Lipoprotein Receptor related Protein 2 (LRP2) which expresses the protein Megalin (a 600 kDa, 4655 amino acids). Megalin is the receptor for over 50 ligands, including lipoproteins, vitamin-binding proteins, and hormone binding proteins. It interacts with Sonic hedgehog and with a Receptor- Associated Protein (RAP) [1] found in the 4p region associated with the Wolf-Hirschhorn syndrome, which includes CDH. Mice knockouts of Megalin produced phenotypes of agenesis of the corpus callosum, which is a prominent feature of patients with Donnai-Barrow syndrome. These mice also excrete high levels of low molecular weight proteins including Vitamin D and Retinol Binding Proteins which we detected in all patients found to have Donnai-Barrow syndrome. Thus, this particular proteinuria can serve as a surrogate marker for Donnai Barrow syndrome [1,33]. A number of families with Donnai-Barrow syndrome from the UK, Europe, and the Middle East have been found to have mutations in different parts of the Megalin gene. Megalin, along with its interacting proteins, RAP and SHH, are being resequenced in a larger cohort of both syndromic and isolated cases of CDH.

Genotyping CDH Patients Reveals both SNP and CNVs which can be Integrated by Novel Bioinformatic Strategies

We are currently studying our entire cohort of CDH patients, from whom we have extracted DNA, for SNP arrays using the Affymetrix 6.0 Chip with approximately a million SNPs across the whole genome and a similar number of CNVs. Similarly, we recently studied 52 CDH patients who are syndromic or complex, with a higher resolution array CGH platform, Agilent 244k, and uncovered approximately 60 microdeletions or microduplications. All of this data is being analyzed in a meta analysis in which SNP and CNV discovery will be superimposed and integrated with a database that evaluates data [34] pooled from a number of protein-protein interaction databases, including information on a number of model organisms. An updated version contains more than 400,000 unique interactions between 10,000 human proteins. We then seeded this network with a set of 54 input proteins derived from the previously mentioned eight different criteria for CDH associated genes, i.e. knockout models and ENU screens, chromosomal intervals associated with CDH, pathways revealed by genes in homozygosity mapping, and pathways implicated from experimental data such as Vitamin A deficiency and nitrofen-induced CDH models, etc. Compared to multiple sets of 54 randomly chosen proteins, this CDH network scored as highly significant ($P < 1.0 \times 10^{-5}$). These findings indicate that a network approach will identify a number of proteins in one or several shared pathways, thus accounting for the heterogeneity in the genetic causes of CDH. We are also doing a meta analysis which superimposes the genes found in areas of microdeletions or microduplications on this protein-protein interaction network. In so doing, we have identified a prioritized list of genes recapitulated both in the protein-protein interaction of candidate genes and of genes found in regions with Copy Number Variations (CNV). The SNP and CNV discovery can be

expanded by computational analyses that integrate these two powerful detection engines across the genome, which in turn can be integrated with multiple public gene expression libraries and annotated text libraries to reveal logical pathways for gene discovery.

Genes prioritized by this approach will be validated in functional assays to determine if they truly play a role in the pathogenesis of CDH. We anticipate that this approach will increase our understanding of both the genetic basis of Congenital Diaphragmatic Hernia, and of the pathogenesis of other major congenital anomalies. The hope is that these approaches will lead to theoretical and, ultimately, experimentally validated treatment strategies.

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Fig. 1.

Analysis of 35 patients, using Spectral 2600™ Array aCGH chips with a resolution of 2 megabases across the genome, detected a *de novo* microdeletion in chromosome 1q41 42 in a patient with Fryns syndrome, with CDH and additional multiple anomalies.

SNP genotyping

Fig. 2.

Affymetrix GeneChip® Homozygosity mapping over the entire genome using single nucleotide polymorphism (SNP) arrays shows that the largest block of shared homozygosity is on chromosome 2q23.3–31.1. **I, II, III & IV** are surviving patients with Donnai-Barrow Syndrome (DBS) in a kindred from the United Arab Emirates. **C** is a control patient without DBS.