Genomic Approaches to Surgical Diseases

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In the new millennium, surgery is rapidly incorporating novel technological and conceptual paradigms, one of the most important of which is the evolving field of genetics and genomics. In the not-too-distant future, everyone may have a haplotype map, a record of all the single nucleotide polymorphisms (SNPs) spanning each individual’s genome. The patterns of polymorphisms of all chromosomes then can be interrogated for associations with complex or monogenetic diseases, either enhancing or minimizing their severity. Pediatric surgeons became aware of the power of genetics during intense investigations of children with congenital anomalies and realized that with each condition there was a variation in the severity or phenotypic profile of the abnormality. This phenotypic variation also became apparent in transgenic animal models produced by homologous recombinations in which single genes were inactivated (knockouts) or overexpressed. Many of the phenotypes and the timing of their presentation varied with strain background but, in general, recapitulated the diseases that surgeons care for routinely, leading us to conclude that most congenital anomalies encountered in the surgical repertoire of pediatric surgeons have a genetic cause. It is likely that adult surgical diseases such as cancer, arthritis, heart disease, diabetes mellitus, and pancreatitis also have genetic causes, with multiple genetic risk factors contributing to the heterogeneity of disease phenotypes. Therefore, it behooves adult and pediatric surgeons to comprehend the tools of genetics that will contribute to understanding the molecular etiology of patients’ diseases. We must understand how to incorporate strategies to detect genetic causes into the clinical care of our patients and into the training of our future academic and clinical surgeons. In an era of “the genomic revolution,” we can remain the specimen collectors or we can play a strong role in determining the direction of research for our specialty in the future.

THE IMPORTANCE OF GENETICS AND GENOMICS

The face of research and its effect on surgical care are changing rapidly. In the 1950s, studies of metabolism became possible because of the post–World War II availability of radioisotopes. This, in turn, led in the 1960s to an era of intense study of renal, vascular, pulmonary, cardiovascular, gastrointestinal, and neurological physiology. In the 1970s, biochemistry and cell biology contributed to our understanding of disease processes at the cellular and molecular levels. The introduction of immunology in the 1970s and 1980s led to our understanding of tolerance and rejection, eventually making routine clinical transplantation a reality. The 1980s and 1990s highlighted molecular biology, which brought understanding of disease etiologies to the gene and protein levels. As we entered the 2000s, the power of genetics and genomics made possible the cloning of the entire genome of many species, including humans. This effort was capped by the recent completion of the HapMap Project, a

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comparison of the patterns of SNPs across several different populations from Asia, Africa, Europe, North America, and South America. These discoveries, coupled with tissue engineering, stem cell biology, outcomes analyses, and complex bioinformatics, allow us to examine our patients using new tools, with different expectations from patients and clinicians about diseases in the context of individual genetic footprints.

We must incorporate these techniques into our already lengthy training programs so that surgeons can participate in the discovery and the changes of the future and even lead in this scientific revolution. We must devise an infrastructure that offers our residents research exposures and then protects our highly schooled junior faculty from the ravages of managed care, oppressive compliance, and increasing stress on surgical and support staff, to carry out in-depth creative contributions important to our field. We must prevent economic pressures from adversely affecting the care of our patients by consuming the analytical time of physicians and other medical professionals.

**PEDIATRIC CONGENITAL ANOMALIES**

The congenital anomalies cared for by pediatric surgeons fall into major embryological categories, including foregut anomalies (such as esophageal atresia, cystic adenomatoid malformations, lobar abnormalities, and congenital diaphragmatic hernia [CDH] with lung hypoplasia); midgut abnormalities of the liver, pancreas, and small bowel; hindgut anomalies (such as anal atresia, cloacal deformities, and urogenital sinus abnormalities); dysmaturity disorders (such as Hirschsprung disease and neuronal intestinal dysplasia); abdominal wall defects; urological and reproductive abnormalities; cardiac anomalies; muscular and skeletal hypoplasia; and neurological anomalies. Our aim is to discover the pathogenesis of these anomalies at the molecular level and to convert molecular etiologies to new therapies.

**CONGENITAL DIAPHRAGMATIC HERNIA**

A 1996 study by Langham et al indicated that CDH, a common congenital anomaly, had an incidence of 1 case per 2500 live births. Using fetal ultrasonography, the detection rate is estimated to be higher, 1 case per 1000 live births, indicating that because of severity or complexity many pregnancies are resulting in spontaneous abortion or that patients are being advised to terminate pregnancies by physicians who may be unaware of the improved outcomes of newer treatment strategies, particularly for patients with isolated CDH. Fifty percent of the cases involve isolated CDH defects, indicating that improved outcomes could result in an excellent quality of life.

Considerable progress has been made in the treatment of children with CDH that trades early surgical intervention for delayed surgical intervention after the child is stabilized and uses novel gentle ventilatory strategies or extracorporeal membrane oxygenation to avoid the barotrauma that has been the cause of morbid broncho-pulmonary dysplasia. As a result of these changes, survival has improved from 50% to as high as 80% to 90% in tertiary care centers in the developed world. However, the percentages of pulmonary, cardiac, gastrointestinal, neuromuscular, and neurological complications remain formidable, and the morbidity due to pulmonary hypoplasia and pulmonary hypertension remains considerable. Congenital diaphragmatic hernia results from a defect predominantly in the muscular portions of the diaphragm, with the most common, Bochdalek hernia, occurring posterolaterally, usually on the left side. Anterior Morgagni hernias make up 10%, and a small number can occur as deficiencies in the central tendon, causing an eventration of the diaphragm. The embryological origins of the diaphragm are complex: the central tendon arises from the septum transversum over the liver, the dorsolateral muscles arise from the pleuropertoneal folds, the dorsal crural form around the esophageal mesentry, and the lateral and anterior muscular rims develop from the intercostal muscles. The embryological relationship between diaphragm development and lung development remains obscure. The genes important for lung development are conserved throughout evolution, making it conceivable that results of studies of CDH in animal models will provide new directions to the care of CDH in humans. An association of CDH with chromosomal abnormalities has been recognized for some time, with known hot spots in 15q, 8p, and 12p.

To incorporate the tools of genetics with the objective of understanding CDH at the molecular level, investigators in Boston initiated a large program funded by the National Institute of Child Health and Human Development, Bethesda, Md, using multiple animal models, based on the evolutionary conservation of the genes involved in tracheal and lung development. The aim of this project was to devise therapeutics based on molecular and genetic findings to improve the future care of patients with CDH and use the knowledge gained and the infrastructure devised to study other congenital anomalies. The animal models included chick, *Drosophila* (fly), and rodents in which toxins induced the phenotypes of CDH and lung hypoplasia.

Animal models were examined in parallel as human patients with CDH were studied at the MassGeneral Hospital for Children, Massachusetts General Hospital (MGH), and Children's Hospital Boston, where Jay Wilson, MD, and his colleague Russell Jennings, MD, operate one of the first interdisciplinary clinics in the nation to follow up children who have survived CDH. Other patients, particularly those from families with multiple affected members, were identified through an active organization of parents with children with CDH called CHERUBS. A network of national and international clinical geneticists was approached by the lead geneticists of this project, Lewis Holmes, MD, and Barbara Pober, MD. Wilson and I were joined in this effort by all of the pediatric surgeons at the MassGeneral Hospital for Children (David Lawlor, MD; Allan Goldstein, MD; Peter Masiakos, MD; Daniel Doody, MD; Daniel Ryan, MD; Jay Vacanti, MD; Jay Schnitzer, MD; and Rafael Pieretti, MD) and Children's Hospital Boston (Russell Jennings, MD; Craig Lillihei, MD; Robert Shamberger, MD; Steve Fishman, MD; Mark Puder, MD; Terry Buchmiller, MD; Tom Jakicic, MD; Heung Bae Kim, MD; Katherine Chen, MD; and David Mooney, MD). Human studies protocols and consent forms for patients, parents, and sib-
lings were written for each center by geneticists and by the study coordinator, Meaghan Russell, MPH. Mothers with prenatally diagnosed CDH were also contacted at the MGH and Brigham and Women’s Hospital.

After consent forms had been signed, skin biopsy specimens were obtained at the time of surgery; blood samples were drawn from patients, parents, and siblings; and cell lines were established. Neonates who were being treated with extracorporeal membrane oxygenation or whose clinical course was precarious had only fibroblast cell lines established from skin biopsy specimens. Lymphocytes harvested from blood were immortalized with Epstein-Barr virus, and cultures were expanded and frozen in multiple aliquots for iterative DNA extractions. Whole-genome amplification was used on DNA extracted from paraffin blocks, buccal smears, or small stored samples of DNA. DNA was quantitated in preparation for sequence analysis, array-based comparative genomic hybridization (aCGH), genomewide SNP analysis, or multiplex ligation-dependent probe amplification (MLPA).

ASSUMPTIONS

The first assumption was that congenital anomalies, such as CDH, result from mutations of any one of multiple single genes in a pathway (ie, the same phenotype can occur whether ligand, receptors, downstream signaling factors, transcription factors, or regulated genes are mutated). The second assumption was that genes and molecules that are found to rescue or complement the mutation can serve as potential therapeutics; once causative genes are known, treatment strategies can be designed to correct the CDH phenotype. Because CDH can be diagnosed with high accuracy by ultrasonography in utero and postnatal treatment of infants with CDH has dramatically improved, the third assumption was that only a small margin of improvement in the lung hypoplasia would have a large effect on survival and reduce morbidity. Furthermore, replacement downstream of a molecular defect by gene therapy or by treatment with an appropriate pharmacological agent could be delivered by minimally invasive techniques in utero or after birth.

ANIMAL MODELS

Chick, rodent, and Drosophila animal models were used to identify or to analyze candidate genes. The chick model is particularly advantageous because the shell can be partially removed to provide a window for access to the embryo for overexpression or inactivation of genes of interest that are introduced as the lung buds begin to develop from the foregut. Differentiation of the distal airway has been studied extensively in the chick, and interactions have been discovered between sonic hedgehog (Shh), bone morphogenesis protein (Bmp4), wingless (Wnt5a), and fibroblast growth factor (Fgf10) pathway genes, which have been found to be evolutionarily conserved in chick, mammalian, and Drosophila species during lung development. Using these pathway candidate genes, the strategy was first to establish normal expression patterns in the chick and then to alter expression by retroviral overexpression or inactivation to identify function. Once a gene leading to lung hypoplasia was identified, its expression pattern in human fetal lungs was then studied by Drucilla Roberts, MD, chief of prenatal pathology at the MGH, in lungs of human fetuses with CDH and lungs of age-matched healthy control subjects for comparative expression studies of candidate genes. Maria Liscertales, PhD (unpublished data, 2006) found that early overexpression of Wnt5a in the chick caused lung hypoplasia with down-regulation of sonic hedgehog, Fgf10, and Bmp4 expression. These results suggest that an activating mutation in an upstream molecule or in Wnt5a could be a cause of the hypoplasia phenotype. Similar hypoplasia phenotypes resulted from overexpression of Bmp4 and of Fit68, a novel protein being studied in the Roberts laboratory by Akemi Kawaguchi, MD. Knockout of these candidate genes using RNA interference (RNAi) technology in the chick, now under way in the laboratory, will help to delineate further the function of these genes, their possible contributions to pulmonary hypoplasia, or how they might be manipulated to decrease the severity of the hypoplastic lung.

A study of tracheal morphogenesis in Drosophila is being led by Lizabeth Perkins, PhD. As with the chick, molecules found to be important in branching morphogenesis in the fly are preserved in the mammalian lungs. Perkins performed a genetic screen of the entire fly genome to find regions that can correct or worsen a mild phenotype caused by mutation of a phosphatase called corkscrew, which is essential for regulation of growth factor activation. Mutants of thrombospondin, a cell adhesion molecule with calcium binding and epidermal growth factorlike domains, and a thrombospondin type III repeat domain were found to worsen the hypomorphic phosphatase phenotype. Thrombospondin was then found to interact in fly cells with the insulinlike growth factor receptor and integrins, molecules known to be important in migration events required in branching morphogenesis. Perkins found other genes of interest, in addition to thrombospondin, which are being evaluated by RNAi in cell-based assays. The RNAi screens will be used to identify new candidates for subsequent testing. Tracheal branching assays established in whole embryos have shown that vitamins C and E and retinoic acid, when used alone or together, can enhance tracheal branching. Therefore, Drosophila systems can be used to identify mutations and, conversely, to test the efficacy of various agents as potential therapies for clinical lung hypoplasia.

The rodent embryo has been studied extensively by Schnitzer and colleagues since it was discovered that a toxicant such as nitrofen, given to pregnant dams before fetal lung development commences, crosses the placenta and causes lung hypoplasia and diaphragmatic hernia in about half of the litters. Lungs removed after budding from the foregut and placed in organ culture can be followed ex vivo to observe patterns of branching morphogenesis. Hypoplastic lungs were found to be rescued with antioxidants such as vitamin C, N-acetyl cysteine, vitamin E, and glutathione. Growth stimulatory pathways are important in branching morphogenesis, as was proven by blocking the mitogen-activated protein kinase pathway with U0126, which resulted in lung hypoplasia in organ culture. Therefore, this model can be used to test therapeu-
tistics for lung hypoplasia that have potential for application in humans. Anselmo et al, led by T. Bernard Kinane, MD, showed that migration molecules such as Slt and Robo are essential for formation of alveolar septa. Because a phenotype of diaphragm and lung hypoplasia occurs when Slit3 is inactivated, it is believed that these molecules are also important in migration of muscle cell progenitors in the diaphragm.

N-ethyl-N-nitrosourea, a DNA intercalating agent causing random DNA breaks, has been used to induce a broad range of congenital abnormalities resulting from mutations in mice. Those that cause diaphragm defects were studied by Ackerman et al, who identified a mutation in FOG2, which was confirmed when this mutation was introduced as a transgene into mice. Testing for this gene in severely affected human patients who died with evagination of the diaphragm disclosed a de novo mutation of FOG2 in one of these patients, the first associated with human CDH. This finding confirmed the feasibility of a genetic approach to study congenital anomalies such as CDH. FOG2 (friend of GATA) interacts with the transcription factors GATA4 and GATA6, compound knockouts of which produce the same diaphragm and lung defects. The GATAs, in turn, interact with COUP-TFII, which is an important transcription factor in the retinoic acid pathway. Knockout of COUP-TFII causes diaphragm defects in transgenic mice. Therefore, mouse models have been important in elucidating genes required for lung development and in providing clues regarding potential genetic causes of CDH.

**CANDIDATE Genes FROM ANIMAL STUDIES INSTRUCT HUMAN STUDIES**

During the past 5 years, we devised an infrastructure and recruited 200 patients for genetic analyses. All patients with CDH admitted to the MGH or Children's Hospital Boston are phenotyped. A family history is obtained, and all clinical information is recorded in a database. Lymphoblasts or fibroblast cell lines are established and stored for multiple studies, and DNA is extracted and accurate linear genomewide amplification performed when appropriate.

Guided by the animal models and classic studies of lung development in the literature, we focused on 22 candidate genes that are transcription factors (such as GATA4 and its binding partner FOG2); genes in the shh, fgfio, or Bmp4 pathways; retinoic acid pathway genes; migration molecules; insulilike growth factor receptor 1; and a chromodomain molecule that affects migration. After DNA amplification, the exonic regions, the exon and intron boundaries with 50 base pairs on either side of each exon, and 5’ and 3’ untranslated regions were sequenced in 66 patients or, for some genes, in 101 patients. Single nucleotide polymorphisms were chosen for further study as potential mutations after meeting the following criteria: the polymorphisms of interest must be (1) absent from existing databases, (2) absent in a healthy population such as the populations used to establish the HapMap, and (3) absent in unaffected controls that are simultaneously sequenced. Furthermore, those SNPs for which molecular modeling predicts a potentially damaging molecular change are considered candidates for testing in functional assays appropriate for the molecular species to determine whether they could be causative in CDH.

**Comparative Genomic Hybridization**

Complete sequencing of a large number of genes is costly. However, newer sequencing technologies are reducing the cost, which may make this approach more economically feasible in the future. Therefore, as the first cohort of patients was undergoing mutational analysis, novel cytogenetic techniques were used based on the hypothesis that microdeletions or microduplications, too small to be seen on routine karyotype analysis, can be the cause of CDH. Array-based comparative genome hybridization (aCGH), which uses differentially fluorescent-labeled bacterial artificial chromosomes that span all the chromosomes for detection, was performed by Sibel Kantarci, PhD, and by David Casavant, MD, under the direction of Charles Lee, PhD, in the cytogenetics laboratory at Brigham and Women’s Hospital.

Eleven of the first 29 cases with normal karyotypes examined by aCGH were classified as isolated and had no abnormalities detected. Two abnormalities were found in 18 cases classified as complex CDH. One microdeletion was found in the 1q41–42 region, which is known to be associated with Fryns syndrome, in a sample from a patient with CDH. Fluorescence in situ hybridization was performed to confirm the deletion, and a 6.5-megabase region was further delineated with MLPA. Multiplex ligation-dependent probe amplification is a powerful inexpensive technology that will be used to screen this region in the entire 182-patient cohort to help identify CDH-associated genes. Array-based comparative genomic hybridization also identified a 22q11 duplication in a region in which deletion has been associated with DiGeorge syndrome. The duplication was again confirmed by fluorescence in situ hybridization, and MLPA was used to identify accurate break points involved in the duplication. Therefore, after examination of every chromosome using aCGH, each microdeletion or microduplication found is confirmed using fluorescence in situ hybridization and then is further delineated using MLPA, which can then be used to genotype the entire CDH cohort. When used in tandem, these powerful techniques can be informative. Further evaluation of genes in the identified regions may yield causative genes. For example, in a study in Rotterdam, the Netherlands, aCGH was used to delineate a small region in 15q26 that has been known for years to be a hot spot for CDH. Mutational analysis of candidate genes in this region and MLPA are being performed.

**Genomewide SNP Analysis**

Traditional linkage analysis has been successful in delineating monogenic, or single gene, defects in large families with multiple affected members. Because of high mortality rates, such families are usually unavailable for the study of gene defects causing congenital anomalies such as CDH. Recent sequencing of the entire human genome has permitted genomewide SNP analysis, which can identify areas of loss of heterozygosity (or shared homozygosity) present in affected family members com-
pared with nonaffected family members. Correlations of these variations with the presence of disease is being assessed among patients with multifactorial diseases such as asthma, hypertension, diabetes mellitus, coronary artery disease, and psychiatric diseases. Results of a study of normal variations in different human ethnicity groups have recently been published as the HapMap, which will be important for comparison of haplotypes of a diseased population with the pattern of SNPs or large-scale variations in healthy populations. Patients can be analyzed using this array-based technology with ever-increasing numbers of SNPs, from 10,000 to 300,000 per chip.

Ten thousand SNP chips were used to analyze a United Arab Emirates family, identified by Pober and Al Ghazali, in which multiple members had CDH associated with Donnai-Barrow syndrome. This family had a high degree of consanguinity, which, in effect, changes a recessive disorder to a dominant disorder. Single nucleotide polymorphism analysis allowed detection of an area of loss of heterozygosity or shared homozygosity in the centromeric region of chromosome. Microsatellite markers are being used to further delineate the region and to establish a logarithm of odds (LOD) score by Kantarci, working in the laboratory of Chris Walsh, MD, who previously (along with their colleagues) had success with this approach to identify genes responsible for congenital brain malformations. It is anticipated that this approach will be successful in delineating the 2p and region of shared homozygosity in this family. Genes in this region will be further examined by mutational analysis. Application of this methodology to the study of several monogenic twins who are discordant for CDH is also expected.

**SUMMARY**

Powerful tools are available with which to analyze patients with CDH. Mutational or sequence analyses were performed in patients and families using candidate genes generated from screens in chicks, rodents, and flies. Other candidate regions are being identified using aCGH, fluorescence in situ hybridization, MLPA, and genomewide SNP hybridization analyses. Further delineation of the regions found in these studies will provide other candidate genes for study. Rather than fully sequencing the entire patient population, these patients can be evaluated with the less expensive alternative of MLPA as a novel method of genotyping.

The etiology of CDH is not expected to be a single gene defect; rather, the definition of disrupted genes along a pathway that is important for lung development is sought. Understanding these pathways will help in developing therapeutics based on molecules or proteins that are already known to create perturbations in that particular pathway. For example, the origin of chronic myeloid leukemia was found to be a translocation causing activation of the tyrosine kinase gene, ABL. Tyrosine kinase inhibitors such as imatinib mesylate (Gleevec; Novartis Pharmaceuticals Corporation, East Hanover, NJ) that were subsequently synthesized have been efficacious in treating patients with this disease. It is expected that important pathways will be identified that will allow creation of novel treatment strategies for CDH. The infrastructure used to study CDH could then be applied to the analysis of other congenital anomalies. The future of medicine and surgery holds exciting promise; surgeons can partake of this glorious adventure if they make a commitment to use genetics and genomics in the future care of their patients.

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**REFERENCES**