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REVIEW ARTICLE
Multiple Hits during Early Embryonic Development: Digenic Diseases and Holoprosencephaly

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Introduction

Tremendous advances have been made over the past two decades in determining the molecular genetic basis for human inherited diseases. Mutations in an impressive number of genes have been linked with specific clinical conditions. Much of this work has focused on elucidating the genes associated with disorders that demonstrate Mendelian inheritance. Disorders with Mendelian inheritance are, by definition, "single-gene" disorders; that is, individuals with the mutated gene are at risk of having the disease, whereas individuals in the same kindred who do not carry the mutation are not at risk.

One hope of molecular genetic diagnosis has been that it may offer the ability to predict clinical status from the genotype. However, for many of these Mendelian conditions, genotype-phenotype correlations have proved elusive (Beaudet et al. 2001; Scriver 2002). In other words, the factors that govern translation of risk for a disease, which is conferred by genotype, into phenotypic clinical disease have often remained unclear. The imprecise prediction of phenotype on the basis of the presence of a mutation is clinically apparent in the frequent occurrence of broad variability in phenotypic expression within a single family. A mutation in a gene that can either cause clinical signs or have no recognized phenotypic effect is not uncommon, and this fact is reflected in the concept of reduced penetrance. Thus, not only is the severity of disease often difficult to predict on the basis of the specific mutation but, in some cases, whether or not someone will even be affected can also be unclear. It is well recognized that for several monogenic conditions in which mutations in a single gene are believed to account for all cases of the disease (e.g., cystic fibrosis and phenylketonuria), there may be important genetic modifiers of expressivity (Haldane 1949; Scriver and Waters 1999; Dipple and McCabe 2000*b*).

For most conditions, the molecular bases of variable expressivity and reduced penetrance are not well delineated. Variation in environmental exposures may contribute to the phenotype (Scriver 2002). In addition, as we highlight in this review, it is likely that at least part of the clinical variability is due to abnormalities in other genes that act in the same or interacting developmental pathways (Vockley et al. 2000; Nadeau 2001; McCabe 2002). Factors that influence phenotypic variability include functional activity thresholds of proteins, modifier genes, and systems dynamics (Dipple and McCabe 2000*a,* 2000*b;* Dipple et al. 2001). Two thresholds of mutant protein function were proposed: a level below which the phenotype was always severe and a level above which the phenotype was always mild (Dipple and McCabe 2000*a,* 2000*b*). The dynamics of biological networked systems predicts that disease is most likely to occur if the activity of a protein that is functionally linked to a large number of other proteins is altered (Dipple et al. 2001). This is supported by the recent descriptions of digenic inheritance in several human diseases (table 1). In digenic inheritance, mutations in each of two unlinked genes are present in a single individual, and the combination of the two genetic hits causes a disease phenotype that is not apparent when an individual carries only one of these gene alterations.

In the present report, we focus on molecular findings demonstrating that, for several human diseases, digenic inheritance can either cause disease or affect the severity of the phenotype. We hypothesize that for some conditions, specifically those that originate during early embryogenesis, alterations in modifier genes or interactions with environmental factors are required for full expression of the disease (multiple-hit hypothesis). We will focus, in particular, on holoprosencephaly (HPE), a severe and common developmental defect of the forebrain. This condition is etiologically heterogeneous, because a mutation in any one of several genes has been identified in affected patients. In addition, a number of

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Table 1

Digenic Inheritance in Human Disease

Effect and Phenotype	Gene 1		Gene 2		Reference
	Mutation	Phenotype	Mutation	Phenotype	
Synergistic:					
RP	*ROM1*$^{+/G80insG}$	Normal	*RDS*$^{+/L185P}$	Normal	Kajiwara et al. 1994
RP	*ROM1*$^{+/L114insG}$	Normal	*RDS*$^{+/L185P}$	Normal	Kajiwara et al. 1994
Bardet-Biedl	*BBS2*$^{Y24X/Q59X}$	Normal	*BBS6*$^{+/Q147X}$	Normal	Katsanis et al. 2001
Deafness	*GJB2*$^{+/35delG}$	Normal	*GJB6*$^{+/-}$	Normal	Lerer et al. 2001
Deafness	*GJB2*$^{+/167delT}$	Normal	*GJB6*$^{+/-}$	Normal	del Castillo et al. 2002
Hirschsprung	*RET*$^{+/I64711}$	Normal	*EDNRB*$^{+/S305N}$	Normal	Auricchio et al. 1999
Severe insulin resistance	*PPARG*$^{+/A553delAAAiT}$	Normal	*PPP1R3A*$^{+/C1984delAG}$	Normal	Savage et al. 2002
Modifier:					
Juvenile-onset glaucoma	*MYOC*$^{+/G399V}$	Adult-onset glaucoma	*CYP1B1*$^{+/R368H}$	Normal	Vincent et al. 2002
Usher 1	*USH3*$^{mut/mut}$	Usher 3	*MYO7A*$^{+/delG\ (exon\ 25)}$	Normal	Adato et al. 1999
Congenital nonlethal JEB	*COL17A1*$^{R1226X/L855X}$	Juvenile JEB	*LAMB3*$^{+/R635X}$	Normal	Floeth et al. 1999
More severe ADPKD	*PKD1*$^{+/mut}$	Less severe ADPKD	*PKD2*$^{+/2152delA}$	Less severe ADPKD	Pei et al. 2001
More severe hearing loss	*DFNA1*	Mild hearing loss	*DFNA2*	Mild hearing loss	Balciuniene et al. 1998
WS2/OA	*MITF*$^{+/944delA}$	?WS2	*TYR*$^{+/R402Q}$	Normal	Morell et al. 1997
More severe WS2/OA	*MITF*$^{+/944delA}$	?WS2	*TYR*$^{R402Q/R402Q}$	Normal	

Note.—The phenotypic description applies to the family reported in the reference only. mut = haplotype consistent with mutation in the gene; + = wild type; − = partial deletion of gene.

environmental exposures can cause HPE. Thus, we suggest that HPE is an example of a condition in which multiple genetic and environmental influences can affect the severity of the phenotype and that multiple hits are required for severe manifestations of the condition.

Digenic Inheritance in Human Diseases

Retinitis Pigmentosa

Retinitis pigmentosa (RP) is characterized by progressive visual loss, night blindness, and pigmentary deposition in the retina. RP is etiologically heterogeneous, in that at least 26 different genes/loci have been described (Wang et al. 2001). Several different genes have been identified in autosomal dominant RP, including *RDS* on 6p21 (MIM #179605). It is presently unclear whether heterozygous mutations in *ROM1* alone can cause RP (Wang et al. 2001). Three pedigrees have been described in which carriers of either a heterozygous *ROM1* or *RDS* mutation were unaffected. However, offspring who were heterozygous for both a *ROM1* and an *RDS* mutation had RP (Kajiwara et al. 1994). Both gene products are involved in formation of a part of the retina. ROM1 protein has been shown to interact with RDS in the formation of an oligomeric transmembrane protein complex in the rod outer-segment disks (Loewen et al. 2001).

Bardet-Biedl Syndrome

Bardet-Biedl syndrome (MIM #209900) is an autosomal recessive disorder characterized by mental retardation, retinal degeneration, polydactyly, and obesity. It has been mapped to at least six loci: *BBS1* (11q13), *BBS2* (16q21), *BBS3* (3p13-p12), *BBS4* (15q22.3), *BBS5* (2q31), and *BBS6* (20p12) (Sheffield et al. 2001). Four genes have been identified to date: *BBS1, BBS2, BBS4,* and *BBS6* (Katsanis et al. 2000; Slavotinek et al. 2000; Mykytyn et al. 2001, 2002; Nishimura et al. 2001). Mutations in *BBS6,* also termed "*MKKS,*" had previously been found to cause McKusick-Kaufman syndrome (Stone et al. 2000). Interestingly, when both the *BBS2* and *BBS6* genes were screened, four pedigrees had two mutated alleles for one of the genes and had one normal and one mutated allele in the second gene (Katsanis et al. 2001). In one of the pedigrees, an individual who had two mutant alleles of *BBS2* and two normal *BBS6* alleles was clinically unaffected, whereas the affected individual had one mutated allele for *BBS6,* as well as the two mutant alleles of *BBS2*. The potential requirement for a third mutation in a second BBS gene in addition to two mutations in the first BBS gene was termed "triallelic" inheritance.

Deafness

Mutations in *GJB2,* which encodes connexin 26, are the most frequent cause of nonsyndromic autosomal recessive hearing loss in certain populations. Although hearing loss associated with homozygous mutations in *GJB2* is prelingual, there is variability in its severity, and it may be only mild (Cohn et al. 1999). Mutant alleles of *GJB6* (connexin 30) have also been associated with hearing loss (Grifa et al. 1999). In an assessment of Spanish families in which the inheritance pattern was consistent with autosomal recessive inheritance and whose members had only one detected mutation in *GJB2*

(the other allele was apparently normal), some had a chromosomal deletion that truncated the *GJB6* gene but did not affect the *GJB2* gene on that chromosome (Lerer et al. 2001; del Castillo et al. 2002). Both connexins are expressed in the inner ear and cochlea. In addition, they can bind to each other to form gap-junction channels. It was hypothesized that loss of any two of the four alleles from *GJB2* and *GJB6* could result in hearing impairment. Thus, either monogenic or digenic inheritance can occur with these genes.

Evidence of digenic inheritance has also been reported for nonsyndromic hearing loss involving the *DFNA12* (chromosome 11q22-q24) and *DFNA2* (chromosome 1p32) loci (Balciuniene et al. 1998), although the genes had not yet been identified at the time of the report. Increased severity of deafness was present in family members who were carriers of both defective alleles.

Hirschsprung Disease

Hirschsprung disease is characterized by chronic constipation due to absence of the ganglion cells in a region of the intestine. It has been classified, by the extent of involvement, into long-segment and short-segment forms. It is an etiologically heterogeneous condition that can be transmitted in an autosomal dominant fashion (with incomplete penetrance) or as a multifactorial trait. At least six different genes have been implicated in human Hirschsprung disease (Parisi and Kapur 2000). In many pedigrees, it shows incomplete penetrance and variable expressivity. Mutations in the *RET* gene have been noted in both familial and sporadic cases (Parisi and Kapur 2000). The penetrance of *RET* mutations is estimated to be 50%–70% (Bolk et al. 2000). *RET* is the major gene involved in Hirschsprung disease, especially the long-segment form. An individual with one abnormal allele of *RET* (resulting in defective splicing) also had a missense mutation in *EDNRB* (encoding the endothelin-B receptor) (Auricchio et al. 1999).

In a few instances, mutations have been detected both in the *RET* gene and in a gene encoding a RET ligand: *GDNF* (which encodes glial cell line–derived neurotrophic factor) or *NTN* (which encodes Neurturin) (Angrist et al. 1996; Salomon et al. 1996; Doray et al. 1998). For *GDNF* and *NTN,* a mutation in either gene alone does not seem to be sufficient for development of Hirschsprung disease in humans, and disease arises only when a mutation in one of these genes occurs in the presence of a *RET* mutation. The exact contribution of *NTN* and *GDNF* to the development of Hirschsprung disease is still unclear. An analysis of the short-segment form demonstrated susceptibility loci at 3p21, 10q11, and 19q12 (Bolk-Gabriel et al. 2002). The gene at 10q11 is most likely *RET,* and the 3p21 and 19q12 loci may be *RET*-dependent modifiers (Bolk-Gabriel et al. 2002). The data were believed to be most consistent with the involvement of all three and with *RET* being the major gene. In a study of Mennonite kindreds, RET, EDNRB, and a locus at 16q23 were identified as susceptibility loci (Carrasquillo et al. 2002).

An example of two genetic hits being found in the same gene is provided by studies of *RET* (Fitze et al. 2002). The c135G/A polymorphism seemed to modify the effect of *RET* germline mutations. The c135A allele was found more frequently with short-segment disease when it was on the same chromosome as the germline mutation, whereas a *RET* germline mutation on the c135G allele was associated with the long-segment phenotype. Fitze et al. speculated that the c135A allele had what might be called a "protective" effect and was modifying the Hirschsprung phenotype. This finding supports the model of Hirschsprung disease in which phenotypes are influenced by differences both between and within genes (McCabe 2002).

Insulin Resistance

Impaired response to insulin, or insulin resistance, is present in individuals with type 2 diabetes. Familial cases of severe insulin resistance, which may or may not be associated with true diabetes, have been described. All five affected members of a kindred with severe insulin resistance had heterozygous frameshift/premature stop codon mutations in two unlinked genes, *PPARG* and *PPP1R3A* (Savage et al. 2002). No other family members were compound heterozygotes for these two mutations. Individuals carrying only one of the mutations had normal fasting insulin levels. *PPARG* encodes peroxisome proliferator–activated receptor γ (PPARγ), a protein that forms a heterodimer with the retinoid X receptor that is activated by antidiabetic agonists. The protein phosphatase 1, regulatory subunit 3 (PPP1R3A) protein plays an important role in regulating glycogen metabolism. The mutant PPP1R3A proteins had abnormal cellular localization and were primarily located in the cytosol, whereas normal protein is normally present in intracellular membranes. It is of interest that PPARγ is primarily expressed in fat (although the protein is also observed at low levels in muscle), and PPP1R3A is expressed only in muscle. Thus, although both proteins are involved in the cellular response to insulin, it seems unlikely that they directly interact.

Early-Onset Glaucoma

Glaucoma is a leading cause of blindness and is characterized by irreversible loss of retinal ganglion cells, visual field deficits, and an abnormal appearance of the optic nerve head. Glaucoma can be characterized by anatomy (open angle vs. closed angle) and time of onset (infantile, juvenile, or adult onset). Onset of glaucoma

at <40 years of age generally causes more severe visual impairment than does glaucoma occurring at a later age (MIM #137750). Juvenile open-angle glaucoma and primary adult-onset open-angle glaucoma can be caused by mutations in the *MYOC* gene, which encodes the myocilin protein, on chromosome 1q25, at the *GLC1A* locus (Alward et al. 1998; Fingert et al. 1999). Mutations in this gene account for ~5% of sporadic primary adult-onset open-angle glaucoma and for as much as 33% of familial cases of juvenile open-angle glaucoma (Shimizu et al. 2000). There is significant inter- and intrafamilial variability in age at onset, rapidity of progression, and severity. Mutations in *CYP1B1,* at the *GLC3A* locus, are present in a substantial proportion of patients with congenital glaucoma (MIM #231300), a recessive disease (Stoilov et al. 1997, 1998). The gene encodes a member of the cytochrome p450 superfamily and is located on chromosome 2p21. There is some degree of variability in age at onset, and incomplete penetrance has been noted (Bejjani et al. 2000). Both genes are expressed in the iris, trabecular meshwork, and ciliary body of the eye. A Canadian family with autosomal dominant glaucoma with both primary adult-onset and juvenile forms of open-angle glaucoma showed segregation of both *MYOC* and *CYP1B1* mutations with disease. All affected family members carried the *MYOC* mutation, and those with both mutations had juvenile glaucoma, whereas those with only the *MYOC* mutation had the adult-onset form. The mean age at onset of disease among carriers of the MYOC mutation alone was 51 years, whereas carriers of both the *MYOC* and *CYP1B1* mutations had an average age at onset of only 27 years (Vincent et al. 2002). Individuals carrying only the *CYP1B1* mutation were not clinically affected. Thus, in this family, *CYP1B1* appears to be acting as a modifier of *MYOC*.

Usher Syndrome

The Usher syndromes are autosomal recessive disorders characterized by sensorineural hearing loss and RP. Three different clinical types have been described: USH1, USH2, and USH3, with USH1 being the most severe. USH1B (MIM #276903) is due to mutations in the *MYO7A* gene, on chromosome 11q13, which encodes myosin VIIA (Weil et al. 1995; Weston et al. 1996). *USH3* maps to chromosome 3q (MIM #276902), and the associated gene has recently been identified (Joensuu et al. 2001). Other researchers have noted apparent digenic inheritance of deafness, involving the *USH1B* and *USH3* loci (Adato et al. 1999). Two affected brothers were described in a Yemenite kindred, in which one brother showed the USH1 phenotype, and the other brother showed the USH3 phenotype. In both individuals, linkage was consistent with the USH3 locus, not the *USH1* or *USH2* loci, and both showed homozygosity for four markers in the region of *USH3*. Mutations in *MYO7A* were screened for, and an allele of *MYO7A* with two nucleotide changes was present in the brother with the USH1 phenotype but not in the brother with the USH3 phenotype. Their mother and two unaffected siblings were double heterozygotes for the *MYO7A* that had the nucleotide changes and the "USH3 haplotype," but they did not have any hearing loss or other signs of Usher syndrome. This suggested that the *MYO7A* mutations may increase the severity of the disease as part of the clinical symptoms associated with homozygous mutations in USH3.

Epidermolysis Bullosa

Epidermolysis bullosa (EB) includes several disorders featuring skin blisters, often provoked by friction or trauma. In junctional EB (JEB), the blisters form between the basal keratinocyte plasma membrane and the basal lamina, and the hemidesmosomes are abnormal. The form termed "generalized atrophic benign epidermolysis bullosa" (GABEB) (MIM #226650) is an autosomal recessive nonlethal subtype that is due, in most cases, to mutations in the *COL17A1* gene encoding collagen XVII (McGrath et al. 1995). In the autosomal recessive Herlitz type of JEB (MIM #226700), blisters develop at birth, and the disease is lethal. Some cases are due to mutations in *LAMB3,* which encodes the β3 polypeptide subunit of laminin 5 (Kivirikko et al. 1996). Thus, mutations affecting both alleles of either *LAMB3* or *COL17A1* can be associated with JEB, although the disease is generally more severe in individuals with the *LAMB3* mutations. One patient was a compound heterozygote for *COL17A1* mutations and also had a heterozygous mutation in *LAMB3* (Floeth and Bruckner-Tuderman 1999). Although there was congenital blistering, the subsequent course was more mild than is typically seen in JEB-Herlitz. Individuals who had one normal allele and one abnormal allele for both *COL17A1* and *LAMB3* were clinically normal. Thus, the *LAMB3* mutation appears to make the severity of EB greater than that seen in most individuals with *COL17A1* mutations. Both laminin 5 and collagen XVII are components of the hemidesmosome-anchoring filament complex at the dermal-epidermal junction.

Autosomal Dominant Polycystic Kidney Disease

Individuals with autosomal dominant polycystic kidney disease (ADPKD) (MIM #173900) are at risk of renal failure. The disease generally presents in the third to fifth decade of life, although presentations in childhood and infancy have been noted. ADPKD is most frequently due to mutations in *PKD1*. Mutations in *PKD2* have also been noted in a minority of patients, and mu-

tations in a third gene have been detected in a small number of patients. Most affected individuals have a single mutation in either *PKD1* or *PKD2*. Although most cysts demonstrate loss of heterozygosity for the remaining allele of *PKD1* or *PKD2,* other cysts have *trans* heterozygous mutations in both *PKD1* and *PKD2* (Koptides and Deltas 2000; Watnick et al. 2000). Thus, at-risk individuals have a germline mutation in either *PKD1* or *PKD2,* and cyst formation occurs after there is a second mutation. Interestingly, in a pedigree with independently segregating *PKD1* and *PKD2* mutations, individuals heterozygous for mutations in both genes were more severely affected than those with only a single gene mutation (Pei et al. 2001). The gene products of *PKD1* and *PKD2,* polycystin 1 and 2, interact to produce cation currents, which neither polycystin is able to produce alone (Hanaoka et al. 2000).

Waardenburg Syndrome and Ocular Albinism

Waardenburg syndrome type II (WS2 [MIM #193510]) is an autosomal dominant condition characterized by hypopigmentation and hearing loss. It is etiologically heterogeneous, and some cases are due to mutations in the *MITF* gene (Tassabehji et al. 1994), which encodes a transcription factor. Ocular albinism (MIM #203100) is also etiologically heterogeneous and can be due to mutations in the *TYR* gene, which encodes the tyrosinase enzyme (Spritz et al. 1990). WS2 in combination with ocular albinism was noted in one kindred (Morell et al. 1997). A TYR^{R402Q} mutation was present in either heterozygous or homozygous form in 13 members of the family. All of the individuals with either hearing loss or ocular albinism had a mutation in *MITF,* in addition to the *TYR* mutation. This suggests that the WS2–ocular albinism phenotype may result from the interaction between MITF and tyrosinase. MITF may act to regulate *TYR* transcription (Bentley et al. 1994).

HPE: At the Crossroads between Mendelian and Multifactorial Diseases

HPE is the most common developmental abnormality of the forebrain (Muenke and Beachy 2001). It occurs as frequently as 1 in 250 conceptuses and ~1 in 16,000 live births (Roach et al. 1975; Matsunaga and Shiota 1977). The condition is characterized by incomplete separation of the forebrain into distinct right and left halves. Affected regions include forebrain derivatives, such as the cerebral cortex and thalamus. Some degree of mental retardation occurs in most affected individuals. There is a continuous gradation of severity of the brain malformation, from complete continuity of the two cerebral hemispheres, to partial separation, to only slight continuity of the most ventral aspect of the hemispheres. Associated craniofacial features are extremely variable and occur in 80%–90% of patients with HPE (Cohen 1989). Facial anomalies can be striking and may include cyclopia (single midline eye), nasal anomalies, and cleft lip and palate. More mild manifestations include ocular hypotelorism (closely spaced eyes), microcephaly, and a single central maxillary incisor (Ming and Muenke 1998). When these milder craniofacial features occur in the absence of HPE, they are termed "microforms" and are generally associated with normal cognitive development. Although HPE usually occurs sporadically, multiple kindreds demonstrating autosomal dominant inheritance have been described. In familial HPE, there can be extremely wide variation in type and severity of brain and/or facial anomaly within a single kindred (Muenke et al. 1994). Patients with HPE, individuals with only microforms who have normal intelligence, and clinically unaffected obligate carriers may be present in a single pedigree. On the basis of analysis of autosomal dominant pedigrees, it is estimated that, among carriers of the abnormal gene, 37% will have HPE, and 27% will have a mild sign (microform). Interestingly, 36% of obligate carriers have no clinical abnormality and have normal intelligence (Cohen 1989).

Genetics of Human HPE

HPE is etiologically heterogeneous, and both environmental and genetic causes have been identified (Ming et al. 1998). Substantial progress has been made towards elucidating the genetic basis of HPE. Our initial studies focused on chromosomal regions in which deletions were associated with HPE (Roessler and Muenke 1998). Analysis of recurrent rearrangements involving 7q36 led to the identification of Sonic hedgehog (*SHH*) as the first gene known to cause human HPE (Belloni et al. 1996; Roessler et al. 1996). Mutations of this gene appear to be the most common genetic cause of human HPE identified to date (Nanni et al. 1999). Mutations have been detected in familial and sporadic cases (Roessler et al. 1996, 1997; Nanni et al. 1999; Odent et al. 1999). A similar strategy was employed to identify mutations in *ZIC2* (Brown et al. 1998, 2001), *SIX3* (Wallis et al. 1999), and *TGIF* (Gripp et al. 2000). Recently, mutations associated with HPE have been found in other genes selected as candidates because of their participation in a signaling pathway important for brain development: *PATCHED1* (PTCH) (Ming et al. 2002) and *GLI2* (M.M., unpublished data) in SHH signaling, and *TDGF1* (de la Cruz et al. 2002) and *FAST1* (M.M., unpublished data) in the Nodal/transforming growth factor β (TGF-β) pathway (fig. 1). The identification of these eight genes associated with HPE demonstrates the complexity of the genetic etiology of this condition. However, mutations in these genes have been identified

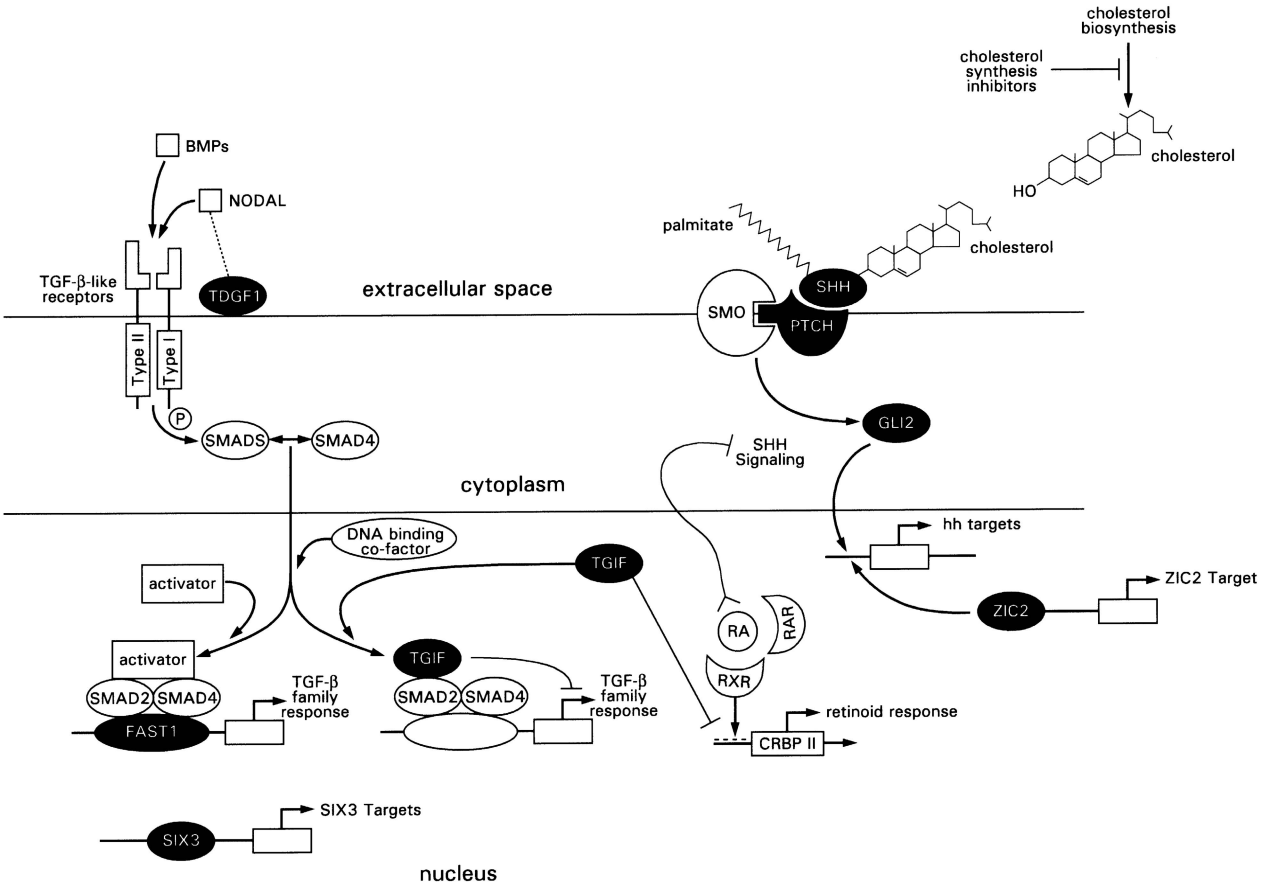

Figure 1 Association of HPE with abnormal signaling pathways and cholesterol biosynthesis and with ZIC2 and SIX3 transcription factors. Some aspects of interactions are speculative, since not all links have been explicitly demonstrated experimentally and many of the links are only suggestive. In addition, results have been synthesized from several species although they are depicted here for humans only. To date, the following genes (blackened symbols) have been implicated in HPE in humans: *SHH, PTCH, GLI2* in Sonic hedgehog signaling, *TDGF1, FAST1, TGIF* in Nodal/TGF-β signaling, *SIX3,* and *ZIC2* (updated and modified from Muenke and Beachy 2001).

in only 15%–20% of the HPE cases in our cohort of patients with HPE in our cohort of affected individuals with normal karyotypes. Thus, a number of other genes are likely to be involved in HPE. Additional candidate genes would include those that map to a chromosomal region in which recurrent rearrangements are associated with HPE, genes in which an animal model shows HPE, genes involved in forebrain development, or genes that encode a component of a signaling pathway associated with HPE.

Is the Intrafamilial Variability in Human HPE Due to Multiple Genetic Hits (Multigenic Inheritance)?

A perplexing question in familial HPE has been the extreme variability, ranging from clinically normal individuals to patients with severe HPE. Of interest, some, but not all, patients with a chromosomal deletion associated with HPE will actually have HPE. Although all known patients with a 2p21 deletion (including *SIX3*) have HPE, only ~50% of patients with del(7)(q36) (including *SHH*) and 10% of those with del(18p) (including *TGIF*) have HPE (M.M., unpublished data). This implies that haploinsufficiency for the respective gene is not generally sufficient to cause HPE and that other genetic or environmental factors are likely to be involved. This concept is supported by molecular genetic analysis of HPE patients. An *SHH* mutation has been detected in multiple pedigrees, demonstrating autosomal dominant inheritance of the HPE spectrum. Within one family, the same *SHH* mutation may be present in individuals with HPE, individuals with microforms, and asymptomatic family members (Roessler et al. 1996). In fact, a clinically normal mother with an *SHH* mutation predicted to result in premature termination of translation had a child with HPE and the same mutation (Roessler et al. 1996). Thus, the phenotype associated with a given *SHH* mutation can be extremely variable, even in a parent and child.

This variability in phenotype has also been noted in

association with mutations in the other HPE genes. We hypothesize that despite its apparent transmission as a "single-gene" autosomal dominant disease, HPE is actually a multifactorial condition, and the phenotype arising in any single individual is the cumulative result of multiple genetic and environmental influences. We hypothesize that, for the severe phenotype, multiple genetic alterations and/or prenatal exposures to teratogens are required. Inheritance of only one abnormal gene may give rise to either a mild manifestation or no clinical abnormality. We will first discuss evidence from animal models that multiple genetic abnormalities may be required for HPE.

Mutations in Two HPE Genes in Individuals with HPE

The phenotypic spectrum of autosomal dominant HPE is quite variable, and we postulate that any given individual's phenotype depends on the number and type of HPE gene mutations present in that person. To date, we have identified three patients who have a mutation in both *SHH* and a second HPE gene (Nanni et al. 1999) (fig. 2). The first patient with HPE has a deletion of 18p11, including the *TGIF* gene, and a mutation in *SHH* (P424A). Interestingly, her phenotypically normal mother has the same *SHH* mutation but carries a balanced translocation involving 18p, t(1;18)(q43;p11.3). Thus, the mother has two copies of *TGIF,* but the daughter has only a single allele (fig. 2*A*).

The second patient with HPE has a mutation in both *TGIF* (T151A) and *SHH* (378-380del). Her clinically normal mother has the mutation in *SHH* but has two normal *TGIF* alleles (fig. 2*B*). The father was not available for study. The third patient with HPE has both a *ZIC2* (polyalanine expansion) and an *SHH* (G290N) mutation (fig. 2*C*). Her parents were not available for study. The fact that these patients have mutations in two HPE genes raises the possibility that the two mutations are combining to give rise to a more severe phenotype than would the single mutation alone. This is supported by the finding that the clinically normal parents that were available for testing carried only one of the mutations. Because a mutation in any of the HPE genes is detected in only ~15%–20% of patients with HPE, it is unlikely that the two mutations occurred by chance in each of three unrelated patients with HPE. Elsewhere, we reported the finding of 23 different *SHH* mutations in 344 patients with HPE (Nanni et al. 1999), 15 *ZIC2* mutations in 509 patients with HPE (Brown et al. 2001), and 4 *TGIF* mutations in 300 patients with HPE (Gripp et al. 2000). Assuming Hardy-Weinberg equilibrium, the expected probability of a double heterozygote for *SHH/TGIF* is 0.0008, and the probability of *SHH/ZIC2* is 0.0018. The difference between the expected and observed (*SHH/TGIF* = 0.0067; *SHH/ZIC2* = 0.0033) values of double heterozygotes is statistically significant ($\chi^2 = 10.343$, $P = .0012$ and $\chi^2 = 4.423$, $P = .034$, respectively). These data further suggest that it is the cumulative effect of multiple genetic defects that may determine the phenotype. Given the large number of HPE-associated genes, the complexity suggested by the variety of mutant alleles, and the fact that mutations have been identified in only 20% of patients with HPE to date, it is intriguing to speculate why, in one family with *SHH* mutation, HPE microsigns (e.g., single central incisor [SCI]) segregate with the mutation (fig. 2*D*); whereas, in an unrelated family with a different *SHH* mutation segregating with SCIs, a child is born with severe HPE (fig. 2*E*).

The finding that two mutations are detected in some patients with HPE may provide insight into why dose sensitivity for *SHH* appears to vary between humans and mice. That is, the *Shh* mutant homozygous mouse has features of HPE and cyclopia and often dies during embryonic development, whereas the heterozygous mouse appears normal (Chiang et al. 1996). This is in contrast to humans, in which haploinsufficiency for *SHH* is associated with HPE. This may reflect the fact that humans with two mutated alleles of *SHH* are not likely to survive past early gestation, and almost all patients studied are live-born infants. We postulate that patients heterozygous for an *SHH* mutation and no other genetic abnormalities in HPE genes will not have HPE and that the patients with HPE also have a second mutation in a distinct gene. The requirement of multiple genetic lesions may be more evident in humans, since it is unlikely that any of these important developmental genes is truly absent in live-born infants.

Animal Models of Digenic Inheritance of HPE

Several mouse mutants demonstrate that HPE can arise from the co-occurrence of mutations in two different genes (table 2). Mice that are mutant for both of the genes may show HPE, whereas a mouse with only one of the genes affected does not have HPE and often appears normal. For example, both *nodal*$^{+/-}$; *HNF3b*$^{+/-}$ (Varlet et al. 1997) and *Otx2*$^{+/-}$; *HNF3b*$^{+/-}$ (Jin et al. 2001) double heterozygotes show cyclopia and HPE. Cyclopia and reduction of anterior head structures were present in a *Smad2*$^{+/-}$; *nodal*$^{+/-}$ double heterozygote (Nomura and Li 1998) and in an *ActRIIA* (activin receptor II) $^{-/-}$; *nodal*$^{+/-}$ double mutant (Song et al. 1999). In addition, both *Noggin*$^{-/-}$; *Chordin*$^{-/-}$ and *Noggin*$^{+/-}$; *Chordin*$^{-/-}$ double mutants show defects consistent with HPE (Bachiller et al. 2000; Klingensmith et al. 2001). Overall, these data demonstrate that HPE can be due to digenic inheritance in mouse models.

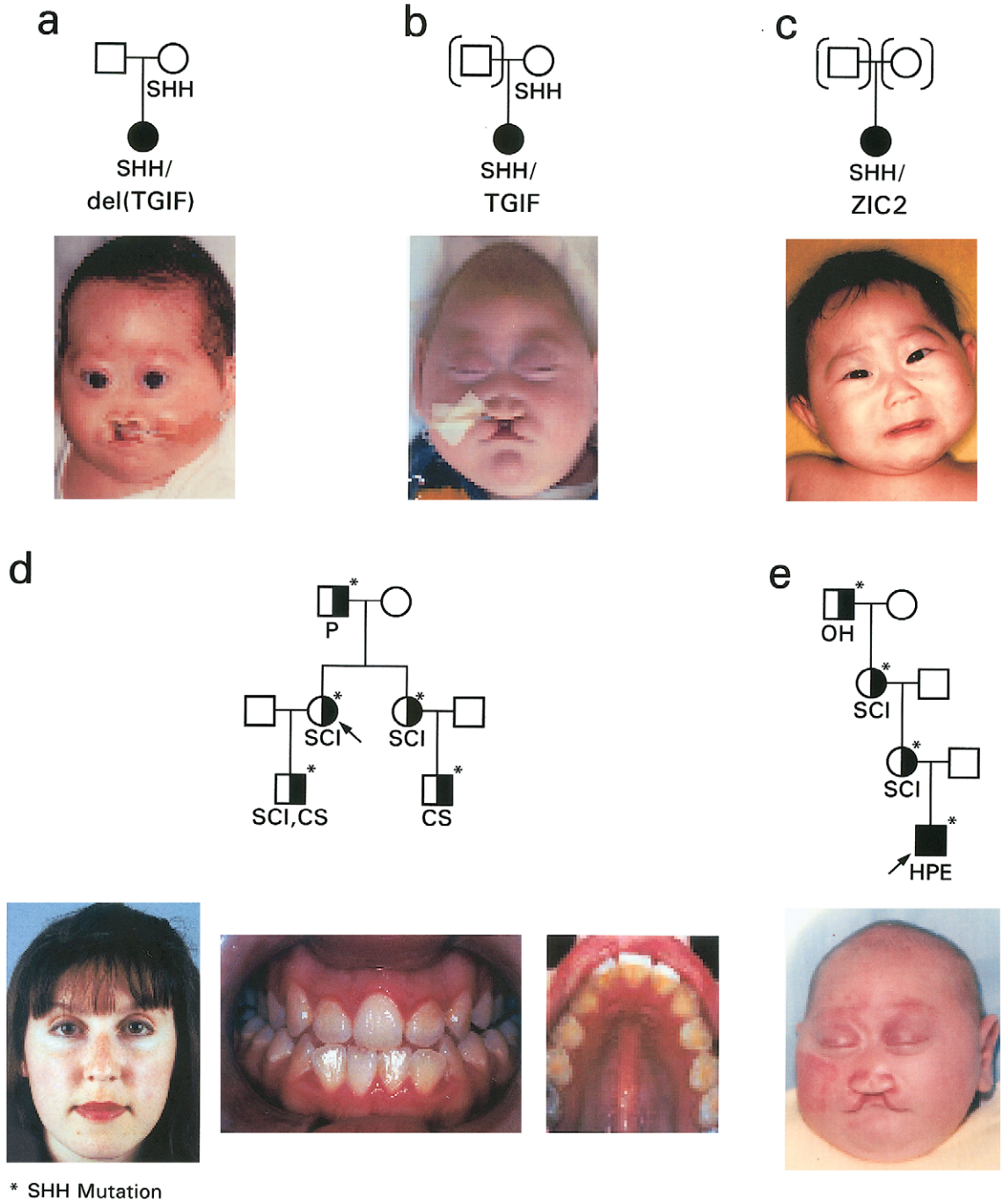

Figure 2 Pedigrees segregating different missense mutations in *SHH*. *A–C,* Patients with HPE and mutations in a second gene. *D* and *E,* Individuals with HPE microsign (CS = choanal stenosis; P = ptosis of eyelids; OH = ocular hypotelorism) are depicted by half-blackened symbols. One pedigree segregates an *SHH* (I111F) mutation (modified from Nanni et al. 2001) (*D*). One pedigree segregates an *SHH* (S236R) mutation (modified from Nanni et al. 1999) (*E*). Patient photographs have been published elsewhere (Nanni et al. 1999 [patients in panels *A*, *C,* and *E*], Nanni et al. 2001 [patient in panel *D*]; Gripp et al. 2000 [patient in panel *B*]) and are used with permission of the publishers.

Table 2

Mouse Models of Digenic Inheritance of HPE

Mutant Mice	Reference
nodal$^{+/-}$; *HNF3b*$^{+/-}$	Varlet et al. 1997
Otx2$^{+/-}$; *HNF3b*$^{+/-}$	Jin et al. 2001
Smad2$^{+/-}$; *nodal*$^{+/-}$	Nomura and Li 1998
Activin receptor II (*ActRIIA*)$^{-/-}$; *nodal*$^{+/-}$	Song et al. 1999
Noggin$^{-/-}$; *Chordin*$^{-/-}$	Bachiller et al. 2000
Noggin$^{+/-}$; *Chordin*$^{-/-}$	Klingensmith et al. 2001

Relationships between the HPE Genes

The human and animal data detailed above indicate that abnormalities in two unlinked genes can have a synergistic effect in causing HPE. Animal models indicate that disruption of a number of distinct developmental processes can bring about the common phenotype of HPE. First, early developmental effects during gastrulation that result in loss of the prechordal plate, an important source of the ventralizing signal Shh, can lead to loss of ventral cell types, cyclopia, and HPE (Rubenstein and Beachy 1998). Chemical or physical ablation of the prechordal plate at gastrulation can induce cyclopia and ventral forebrain defects (Rubenstein and Beachy 1998). Several zebrafish mutants with an abnormal prechordal plate show cyclopia (e.g., *cyclops, squint,* and *one-eyed pinhead*) (Schier et al. 1996; Schier 2001). Second, HPE can arise from a relatively late disruption after neural tube closure. For example, loss of normal dorsal-ventral patterning after neural tube closure can result in HPE (Golden et al. 1999). Also, forebrain truncation defects with cyclopia (Schneider et al. 2001), similar to those seen in mouse models, have also been induced at these later time points.

The relationships between some of the human HPE genes are beginning to be understood (fig. 1). Shh is critical for ventral forebrain induction; Patched is its receptor; and Gli2 is a mediator of Shh target gene transcription. Six3 is also expressed in the ventral forebrain, and it may modulate the effect of Shh on the ventral forebrain (Kobayashi et al. 2002). Any relationship between Zic2 and Shh is likely to be less direct, because Zic2 is primarily expressed in the dorsal neural tube (Nagai et al. 1997) and Shh expression is restricted to the ventral neural tube. Zic proteins can bind to the same target sequences as the Gli proteins, transcription factors that regulate expression of Shh-responsive genes (Aruga et al. 1994; Mizugishi et al. 2001). In addition, Zic2 can alter Gli activity in neural patterning (Brewster et al. 1998).

The functional relationship between Tgif and Shh is unknown. Tgif acts as a transcriptional co-repressor of expression in TGF-β signaling (Wotton et al. 1999) (fig. 1). Disruption of Nodal, a member of the TGF-β superfamily, leads to abnormal prechordal plate formation and results in ventral forebrain deficits and cyclopia (Schier et al. 1997; Zhang et al. 1998). It is of possible relevance that another TGF-β family member, bone morphogenetic protein 7, also acts in ventral forebrain development in conjunction with Shh (Dale et al. 1997, 1999). Tgif has also been shown to act in retinoic acid–receptor signaling (Bertolino et al. 1995), and retinoic acid exposure can decrease Shh activity in the craniofacial region (Helms et al. 1997). Overall, the understanding of the complex interplay among the different HPE gene products is just beginning to emerge. Further experimental information will determine whether these different proteins act directly or indirectly in processes that mediate forebrain development.

Teratogens Cause HPE and May Interact with HPE Genes

Although it appears likely that multiple genes contribute to the phenotype of human HPE, evidence from both human studies and animal models implicate environmental factors in the pathogenesis of HPE as well. Maternal diabetes, including gestational diabetes, increases the risk of HPE to ~1%–2% of pregnancy outcomes (Barr et al. 1983; Cohen 1989; Martinez-Frias et al. 1998). Prenatal human exposure to ethanol (Ronen and Andrews 1991; Croen et al. 2000; Cohen and Shiota 2002) has been associated with abnormalities of the HPE spectrum. Animal models demonstrate that HPE can be induced with ethanol exposure during gastrulation (Sulik and Johnston 1982) and may be due to interference with prechordal plate function (Blader and Strahle 1998). HPE in humans has also been noted in association with prenatal exposure to retinoic acid (Lammer et al. 1985; Cohen and Shiota 2002), although the degree of increased risk is not known. Administration of retinoic acid to pregnant mice induced craniofacial malformations consistent with HPE (Sulik et al. 1995). Retinoic acid may affect Shh signaling (Helms et al. 1997; Schneider et al. 2001).

There are several lines of evidence linking defective cholesterol biosynthesis and HPE. First, ~5% of children with Smith-Lemli-Opitz syndrome (SLOS) show HPE or malformations consistent with the HPE spectrum (Kelley et al. 1996). SLOS is caused by a defect in the final step of cholesterol synthesis, 7-dehydrocholesterol reductase (7-DHCR) (Fitzky et al. 1998; Wassif et al. 1998). Interestingly, this enzyme is also inhibited by the chemical AY9944, and animals exposed to this agent have HPE (Roux et al. 1979; Muenke and Beachy 2001). In addition, the compounds cyclopamine and jervine are plant alkaloids that induced outbreaks of cyclopia in flocks of sheep that ate plants containing these compounds (Kee-

ler and Binns 1968). These compounds inhibit cholesterol biosynthesis (Mann and Beachy 2000). The mature Shh molecule undergoes cholesterol modification (fig. 1; Porter et al. 1996), although it is not clear how defective cholesterol biosynthesis affects Shh signaling, and the effect may actually be on other components of the pathway (Taipale et al. 2000). The observation that only 5% of children with SLOS have HPE (Kelley et al. 1996) suggests that the mutation in 7-DHCR may require a second genetic or environmental hit to produce the HPE phenotype. When families of children with isolated HPE were studied, significantly lower cholesterol values were found in the mothers than in the fathers (M.M. and Kelley, unpublished data). It is possible that low maternal cholesterol levels during pregnancy may influence the risk for HPE in the fetus. Overall, it appears that decreased cholesterol levels during fetal development may have adverse effects on brain development, whether through environmental and/or genetic mechanisms. A retrospective and a prospective study to address this possibility are in progress (M.M. and colleagues).

Taken together, data from both human studies and animal models indicate that prenatal environmental exposures can play a key role in the generation of HPE. This raises the possibility that maternal levels of various compounds could play a role in the pathogenesis of human HPE. Thus, endogenous maternal levels of cholesterol or retinoic acid, exposure to ethanol, or subclinical levels of maternal hyperglycemia may contribute to HPE. We hypothesize that even relatively low doses of these teratogens, which by themselves may not be sufficient to cause HPE or any other clinical abnormality, may act in concert with other environmental or genetic variables to generate the HPE phenotype.

Conclusions

Overlap between Monogenic and Multigenic Diseases

The distinction between monogenic and multigenic inheritance of disease is becoming increasingly blurred. Traditionally, conditions were considered to follow either Mendelian (single gene) or multifactorial (multiple genes) inheritance patterns. However, the findings of incomplete penetrance and variable expressivity for Mendelian disorders clearly indicate that contributions apart from the "single gene" play an important role in the clinical outcomes of these patients (Beaudet et al. 2001). For many Mendelian disorders, the cause of phenotypic variability is likely to be the interplay between multiple genetic and environmental factors. The demonstration of digenic inheritance in human conditions indicates that what are considered single-gene diseases may, in some cases, be due to two or more genetic hits. Of note, many of the conditions discussed (including HPE, Hirschsprung disease, deafness, RP, and Bardet-Biedl syndrome) have significant genetic heterogeneity. In some instances, homozygous mutations in a single gene can also cause the disease in humans or animal models. Since disruption of the function of any of several different proteins can lead to the same phenotype, these proteins are likely to functionally interact with each other. Thus, we would anticipate that alterations of two proteins that act in the same or interacting developmental pathways could have compounding effects. Additional genetic interactions will likely be identified for these diseases.

Classes of Digenic Inheritance

The relationships between the genes in digenic inheritance vary and may be classified into different categories. In the first group, the mutations in the two genes seem to have a synergistic, or multiplicative, effect on the phenotype (table 1). In these cases, mutations in each of two genes are both required for manifestation of the disease, and either of the hits alone is not sufficient to cause symptoms (e.g., *ROM1/RDS*). In this situation, it is not clear whether either gene has a greater influence on the patient's manifestations, and both genes could contribute equally to the phenotypic expression. We have termed these combinations "pathogenic" digenic inheritance, since no phenotypic abnormality is clinically apparent when only one gene mutation is present. The phrase "synergistic heterozygosity" has been employed to describe a similar situation in metabolic diseases (Vockley et al. 2000). In synergistic heterozygosity, concurrent partial defects in more than one pathway, or at multiple steps in a pathway, give rise to a clinically relevant defect in energy metabolism, although the defect in any one of the enzymes is not sufficient to cause a significant deficit.

The question of whether a mutation in a major gene is required for disease or whether different combinations of genetic mutations also give rise to the same phenotype awaits further study. If both mutations appear together in individuals in successive generations, the trait may appear to be transmitted in an autosomal dominant manner. Identification of unaffected individuals who have only one of the mutations will be needed for the digenic inheritance to become apparent.

In the second group, mutations in the two genes may appear to confer additive effects. We have termed these combinations "modifier" digenic inheritance, because a mutation in the first gene is sufficient to cause disease, but the second mutation in a different gene increases its severity (*PKD1/PKD2, COL7A1/LAMB3, DFNA2/DFNA12,* and *MYOC/CYP1B1*). In this case, the second gene may act as a modifier of the first gene.

Cumulative Effects of Genetic Abnormalities

Two genes that affect one another's phenotypic effects presumably must function in some common biologic process (Vockley et al. 2000; McCabe 2002). The different types of interactions could also be classified into different groups. First, the proteins may directly interact with each other to form a structural component (e.g., polycystins 1 and 2, ROM1, and PRP1). Second, both proteins could interact in the same signaling pathway (e.g., RET and its ligand GDNF).

In another class of interactions, the two proteins do not have a direct interaction. This could occur for two proteins that are in the same signaling pathway but act at distinct steps. For example, a ligand could induce, through intracellular signaling, the activity of a transcription factor. The proteins would be in the same pathway but not have any direct physical interaction. The modification, or buffering, of the phenotypic effect of a mutation by an alteration of a second gene in the same pathway or process has been termed "intrinsic" buffering (Hartman et al. 2001).

Alternatively, the proteins may not participate in the same signaling pathway, but both are involved in the same developmental process. There are several different scenarios for this situation as well. First, one protein may affect the cellular response to the second protein. For example, Six3 and Shh are not known to directly regulate one another's expression or to act in the same signaling pathway. However, Six3 seems to allow cells to respond to Shh by expressing markers specific for the anterior forebrain (Kobayashi et al. 2002). Second, the two proteins may not modify each other's cellular responses but, rather, may interact only in a broader sense, in that both proteins may be independently involved in the same developmental process. For example, two proteins may both be involved in ventral patterning of the forebrain but may not directly affect each other's activity. Third, the two proteins may both impact development of the same organ but at different developmental times. For example, abnormalities in either prechordal mesoderm formation or dorsal-ventral specification of the forebrain can lead to HPE in animal models. In some cases, the defect in dorsal-ventral patterning arises as a result of abnormal prechordal mesoderm formation. However, it is possible that mildly abnormal notochord formation could still result in normal brain development. Slightly decreased ventralizing activity in the neural tube with normal prechordal mesoderm formation could, by itself, be sufficient to result in normal forebrain development. However, the two mild abnormalities could synergize by both affecting the same process (ventral patterning of the neural tube) and thus cause HPE. Modification of the effect of one gene by the action of another that is located in a biochemically distinct circuit has been termed "extrinsic" buffering (Hartman et al. 2001). This would apply to scenarios in which processes are redundant or complementary, such as a mutation in an enzyme in DNA repair that exacerbates a defect in DNA replication (Hartman et al. 2001).

For all of the scenarios indicated in which the two proteins do not directly interact, molecular techniques (such as two-hybrid screening) may not reveal the interaction, and developmental biology models may be required to demonstrate the functional interaction.

Environmental Influences and Other Factors

Environmental effects also play an important role in determining phenotypic outcome. As with the interactions between two proteins, environmental agents may influence protein function at several different levels. An agent may reduce the cellular response to a protein by impairing the activity of a signaling pathway (e.g., decreased cholesterol leading to abnormal Shh signaling). Alternatively, the environmental substance may regulate the expression of a gene (e.g., retinoic acid and Shh), or the environmental agent may directly alter the protein's function (e.g., retinoic acid receptor may compete for the same DNA binding site as TGIF in HPE). Lastly, the environmental agent could have a toxic effect on either the proliferation or the survival of critical cell types. Prenatal environmental influences may not always be easy to determine, because different pregnancies presumably have differences in intrauterine exposures. Even MZ twins may have different degrees of blood flow and thus, potentially, different levels of exposure to teratogenic agents.

In addition to environmental and genetic effects, the potential effects of stochastic contributions to development could also play a role (Kirschner et al. 2000; Hartman et al. 2001). Stochastic events in developmental biology are believed to govern immunoglobulin rearrangement on the DNA level, and it appears that the determination of left-right situs is a random process in certain mutant mice (Bisgrove and Yost 2001). Developmental events may not always be very precisely regulated; rather, there may be a range that results in normal development. In the presence of a mutant gene, a low normal level of development that has arisen because of stochastic events may become apparent. For example, if there is imprecision in the development of the prechordal mesoderm such that a range of outcomes may be developmentally normal, it is possible that a low normal amount of prechordal mesoderm activity in combination with a genetic mutation that acts in the same pathway could cause abnormal development.

Summary

Multiple genetic and environmental factors likely play a role in the phenotypic expression of many Mendelian disorders. Understanding the biologic role of the genes associated with human disease may guide future explorations of additional modifier genes. For example, with HPE, there are several possible categories of additional candidate genes and potential sites of interaction. First, there are genes encoding proteins that are in the signaling pathways for identified disease genes (e.g., Shh and nodal signaling). Second, genes involved in the relevant developmental processes are potential candidate genes for human diseases (dorsal-ventral patterning of the forebrain, other processes in midline forebrain development, prechordal mesoderm induction). Third, genes involved in synthesis or metabolism of teratogenic agents (cholesterol biosynthesis, retinoic acid metabolism) could also be potential genetic contributors.

Multiple genetic hits or environmental exposures may be required for clinical expression of many Mendelian disorders. Conceptions of a disease being the result of mutations in a single gene should take into account the overlap between Mendelian and multifactorial disorders. Indeed, as more detail emerges demonstrating the impact of genomic and environmental variability on phenotype, we may reconceptualize classic Mendelian and complex multifactorial disorders as two ends of a continuum of disease causation, with the gradient reflecting the relative independence of a given mutation in producing the disease phenotype. It may be useful to remember that "genomes speak biochemistry, not phenotype" (Plasterk 1999). Future studies will focus both on the identification of additional genes involved with these human diseases and on the understanding of their biologic interactions. Such information will lend greater insight into the complex genetic and environmental influences that lead to phenotypic expression of a trait.

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Electronic-Database Information

Accession numbers and the URL for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM) http://www.ncbi.nlm.nih.gov/Omim/ (for ADPKD [MIM #173900], Bardet-Biedl Syndrome [MIM #209900], congenital glaucoma [MIM #231300], early onset glaucoma [MIM #137750], GABEB [MIM #226650], JEB [MIM #226700], ocular albinism [MIM #203100], *RDS* [MIM #179605], USH1B [MIM #276903], *USH3* [MIM #276902], and WS2 [MIM #193510])

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