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Chapter

Genetics of Biliary Atresia: A Work in Progress for a Disease with an Unavoidable Sequela into Liver Cirrhosis following Failure of Hepatic Portoenterostomy

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Abstract

The bile duct development may not be fully completed at birth, and this is quite a common event. Moreover, bile formation is immature, and there is a propensity for the neonate to develop cholestasis in the presence of a wide variety of insults that can damage the liver. A biliary atresia is a correctable form of infantile cholangiopathies. The Kasai hepatic portoenterostomy (HPE) is often performed and is successful if it is done at an early stage. However, HPE can fail, and the liver fate is inevitably a cirrhotic change. Biliary atresia is heterogeneous and may result from a combination of genetic factors, vascular, infective or toxic insults with activation of different genetic and immunological pathways. In this chapter, we will review some genes that may be highly relevant to biliary atresia, including not only PKHD1, JAG1, and CFTR, but also GPC1, ADD3 and others. Four genetic loci are considered as predisposition loci in biliary atresia, despite the absence of an etiologic mutation. The rare occurrence of biliary atresia in well-known genetic syndromes seems to suggest coincidental finding, but epigenetic aspects might play a significant role in contributing to the increase of biliary atresia rate.

Keywords: biliary atresia, cirrhosis, kasai failure, genes, bioinformatics

1. Introduction

Biliary atresia (BA) is a necroinflammatory process of the intrahepatic (inside of the liver) and extrahepatic (outside of the liver) biliary system with a various etiologic background. Since the introduction of the yellow card registries in Taiwan in 2002 as a pilot study and then in other countries, the awareness and the proper management of this disease has increased. In the Western countries, BA occurs in about 1:18,000 live births, but the reported incidences vary from 5 to 32 per 105 live births [1]. The incidence of BA is highest in Asia and in some countries of the Pacific region comparing to the rest of the world with male infants more often affected than females. The clinical triad includes neonatal jaundice (conjugated hyperbilirubinemia that is considered lasting beyond 2 weeks of postnatal life), acholic (pale) stools and dark urine, and hepatomegaly. The ultrasonography and

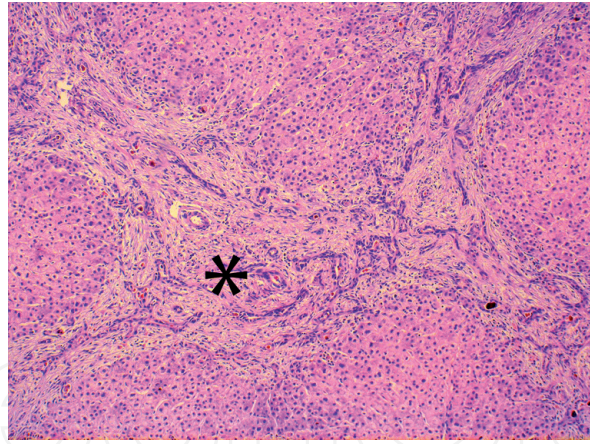


Figure 1. Biliary atresia with extensive fibrosis and bile duct proliferations (asterisk) (Hematoxylin and eosin staining, x100 original magnification).

the endoscopic retrograde cholangiopancreatography (ERCP) are essential to address the diagnosis, which relies on the microscopic examination. Histologically, BA relies on the inflammatory injury of the intra- and extrahepatic biliary tract with sclerosis, luminal narrowing, and final obliteration of the biliary tree. BA lead to liver cirrhosis, if no treatment is initiated. Also, death can occur within the 2 years of life or first infancy. Although BA is, currently, not known to be hereditary, some families with recurrence of this disease have been reported in the literature. BA requires an immediate shunt to promote the discharge of bilirubin from the progressive accumulation in the liver and Professor Morlo Kasai (1922–2008) invented a technique (Kasai hepatic portoenterostomy or HPE), which is currently in use and the first line of surgical therapy for these patients [2–4]. Subsequently, a liver transplantation may be needed, particularly in infants who underwent to surgery not early in life as it should be. The delay of the surgical hepatic portoenterostomy is still a problem in some regions not only in underdeveloped countries, but even in USA and Canada. Thus, the awareness of this condition is crucial to save lives and money. Liver transplantation may be crucial needed to restore the flow of bile or if liver cirrhotic complications occur (**Figure 1**). Currently, nearly 90% of BA infants survive. The majority of these patients have normal quality of life. Prof. Kasai is considered one of the greatest innovators in the field of pediatric surgery and numerous thousands of babies and their families will be always grateful to him.

2. Bile duct development

The development of the biliary tree is crucial to understand and interpret the categories of neonatal and infantile cholangiopathies. This aspect is particularly important if the patient is a preterm baby or a baby considered small for gestational age [5]. At the 3rd week of the post-ovulatory period, endodermal cells sprout from the primitive foregut at the cranial portion. These cells grow towards a loosely arranged mesoderm. The direction is the *plexus vitellinus* of the embryo. Similarly, a bud arises caudally from the foregut and form the primordium (anlage) of the extrahepatic biliary system [5–8]. After a sub-massive and massive liver necrosis, a ductular reaction is observed in the survived liver tissue. This phenomenon is seen in individuals with fulminant hepatitis and endorses the theory recommending the presence of ordinary progenitor cells that may totally differentiate in bile duct epithelial cells, specifically along the portal vein branches [9]. At the hilum of the liver between the 6th and the 9th week of the postovulatory period, progenitor

cells, which are in contact with the mesenchyme that surrounds the primitive portal vein, form first a mono-layered and then later a double-layered cell cords harboring a “slit-like” lumen [5, 10]. This structure is the fundamental (primitive) intrahepatic biliary structure, which has been labeled (bilaminar) “ductal plate.” At the 12th week of intrauterine gestation and on, a continuous remodeling of this prototypic structure takes place. A few parts of the primitive biliary structures dilate and slightly roam toward the center of the portal field. These structures are called “peripheral tubular or ductular structures.” These biliary structures are the immature form of the interlobular bile ducts. Subsequently, one or two immature structures, mostly peripherally located ductular structures transmute into mature interlobular bile ducts. In the meantime, most of these peripherally located structures will gradually disappear, and an essential contribution to this process is due to the apoptosis, which was initially hypothesized by Professor Valeer Desmet (Leuven, Belgium) as early as 1985 [11–16]. Subsequently, Meckel syndrome, a genetic syndrome with autosomal recessive inheritance, occipital encephalocele, postaxial polydactyly, diffuse cystic renal dysplasia, and malformation of the orthologue development of the ductal plate of the liver, was targeted by my research group in the late years of last century [17]. The malformed ductal plates in the fetal livers with Meckel syndrome showed a marked decrease in the apoptotic rate and Fas expression, a pro-apoptotic marker, and an increase in proliferative activity and expression of Bcl-2, which has an antiapoptotic significance [18]. The conversion of the ductal plate into mature and bile draining interlobular bile ducts is conveyed by the appearance of specific intermediate filaments of the cytoskeleton or keratins or cytokeratins (CKs) [19, 20]. The epithelial cells that are forming the interlobular bile ducts start expressing K-7 and K-19 in addition to K-8 and K-18. The keratins K-8 and K-18 are positive in normal hepatocytes of the adult. Quantification of structures of the biliary system and their maturity may be beneficial in the assessment of the maturation of the intrahepatic biliary tree in neonatal and infantile cholangiopathies, and the lack of an interlobular bile duct is the most worrisome prognosticator in a cholangiopathy independently from the specific etiology or

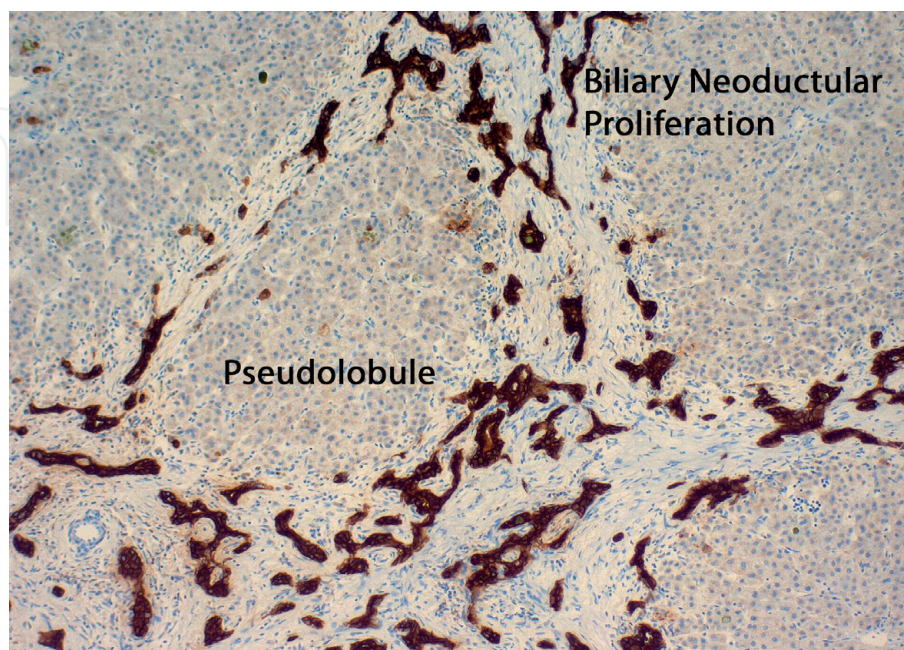


Figure 2.
Keratin 7-immunostaining of the biliary proliferating neoductules (Anti-cytokeratin 7 immunostaining, Avidin-Biotin-Complex, x100 original magnification).

labeling of the disease [21]. **Figure 2** shows the immunohistochemical staining of the biliary proliferation using an antibody against cytokeratin 7.

3. Liver biopsy

Currently, the liver biopsy remains the gold standard for determining the diagnosis of surgical conditions with neonatal cholestasis, such as BA, which is the most critical surgically correctable form of persistent conjugated hyperbilirubinemia. There is an incredible rate of accuracy that comes with the experience of the pathologist and liver biopsy is precise in probably more than 9 cases out of 10, in most tertiary centers of pediatric healthcare, provided that the liver tissue encompasses at least six portal tracts. The experience of the pathologist may be crucial to address the patient to a pediatric surgeon or genetic counseling for neonatal cholestatic liver diseases. The challenge may be quite high that some argued that there might be a continuum with a single underlying cause. According to our previously published data [21], patterns of ductular reactions may muddle the pathologist, and ductal plate remnants, often seen in the liver histology, may indeed recapitulate the embryonic anlage. As a pediatrician and pathologist, the urgency of critical reports is well understood [22]. Medical and surgical causes of neonatal cholestasis need to be ruled out promptly. The central differential diagnoses of BA that need to be kept in mind are: Alagille syndrome, alpha-1-antitrypsin deficiency, cystic fibrosis, sclerosing cholangitis with neonatal onset, and more rarely progressive familial intrahepatic cholestasis (PFIC) types I-III. The differential diagnosis may be complicated and ancillary studies may need mass spectroscopy or other sophisticated techniques.

4. Genetics

As indicated early, BA is usually not hereditary, but some cases and families with recurrence rate have been observed [23]. Four genetic loci are considered as predisposition loci in BA, although no etiologic mutation was identified. The rare occurrence of BA in well-known genetic syndromes seems to suggest coincidental finding, but epigenetic aspects might play a major role in contributing to the increase of BA rate. Chromosomal alterations reported in patients with BA include duplication, deletion, and single copy number variation. Duplications include trisomy 18, trisomy or tetrasomy 22, trisomy 21, trisomy 10q and trisomy 11q23. In trisomy 18, BA has been associated in individuals with congenital heart disease and facial dysmorphism. In trisomy or tetrasomy 22, cat eye syndrome occurs. Facial dysmorphism, congenital heart disease, and cleft palate occurred in trisomy 11q23, while coarctation of the aorta, anal ante-position, and mental retardation have been recorded in patients harboring trisomy 10q. Patients with trisomy 21 syndrome (Down syndrome) showed accompanying atresia of the duodenum and esophagus as well as a heterotaxic setting, which is a condition in which the internal organs are abnormally arranged in the abdomen and chest. Deletion of chromosomal portions has been identified on 18p, 2q73.3, 18q21, 17q12, 1p36, and 20p11.21. Facial dysmorphism, mental retardation, hypothyroidism, and polysplenia were found in deletion of the 2q37.2, while hyperechogenic kidney and pancreatic hypoplasia were found in the deletion of 17q12. Facial dysmorphism and classic Pitt-Hopkins syndrome were found in the deletion of 1p36 and 18q21, respectively. Laterality defects and panhypopituitarism were findings in the setting of deletion of 20p11.21. Pitt-Hopkins syndrome is a condition characterized by developmental delay and intellectual disability with individuals with breathing problems, recurrent seizures, and distinctive facial

features [24–27]. There is a delay in the development of mental and motor skills with numerous affected individuals having features of autistic spectrum disorders. Copy number variations (CNVs) are defined as a type of structural variation: specifically, i.e., a type of duplication or deletion event that affects a considerable number of base pairs. CNVs are a phenomenon in which sections of the genome are repeated, and the number of repeats in the genome varies between individuals in the human population with a specific neurological disorder as seen in Huntington's disease. In this disease, CAG (i.e., cytosine-adenine-guanine) repeats of less than 26 have no effect, but between 27 and 35 there is a risk for the offspring, 36–29 CAG repeats may (or may not) be affected by the disease but harbors 50% of risk to offspring, while 40 or more has a consequence with full penetrance of the disease and a risk of 50% to offspring. CMVs have been identified in a cohort of patients with BA with 29 specific CNVs involving the *JAG1* gene and immunity-related genes. Genome-wide association studies performed in patients affected with BA identified four loci, of which one is in the Han population and three in Caucasians. In the Han ethnics of China, noncoding single nucleotide polymorphisms (SNPs) have been localized to *ADD3* and *XPNPEP1* genes. Noncoding SNPs and a heterozygous deletion have been localized in *ARF6*, *EFEMP1*, and *GPC1* genes, respectively [28–30]. There have been some case reports of BA associated with identified mutation or diagnosed syndrome. The gene *SERPINA1* was considered an aggravating factor for BA, and *JAG1* was questioned in a few patients [31–34]. A compound heterozygous (one copy each of two different alleles) mutation was also questioned in case of *PKHD1* gene [35]. A heterozygous mutation of the gene *MYO5B* was found in a patient suffering from microvillus inclusion disease and progressive familial cholestasis [34]. The Dubin-Johnson syndrome was also found in a patient with BA implying the gene *ABCC2* [34]. Progressive familial cholestasis without microvillus inclusion disease was found in a patient with BA as well. The gene involved *ABCB11* harbored a heterozygous mutation. Heterozygous and hemizygous mutations of the genes *CFC1* and *ZIC3* were found in patients with laterality defects [36, 37]. To be hemizygous for a gene means to have only one copy of one allele of that gene. The Fumarase gene was seen in a patient having a homozygous mutation [38]. The gene *GATA3* was involved as a heterozygous mutation in a patient with HDR syndrome (hypoparathyroidism, sensorineural deafness and renal abnormalities) [39], while the gene *FGFR3* was involved in a patient with achondroplasia [40]. The Mitchell-Riley syndrome was found in an individual with BA and a homozygous mutation of the gene *RFX6* [41]. Fanconi anemia and BA with a biallelic mutation on *ERCC4* were also found [42]. Kartagener syndrome and BA also occurred in the literature [43]. The Zimmermann-Laband syndrome was present in an individual with BA and a heterozygous mutation of *KCNH1* [44]. Kabuki syndrome and BA also occurred in the literature [45, 46]. The Mowat-Wilson syndrome was found in a patient with BA and a heterozygous deletion in the *ZEB2* gene. Two more genes were also seen having a heterozygous mutation in patients with BA including *UGT1A1* and *MLL2* [34]. Finally, three multiple congenital anomaly syndromes have been reported with BA, including Mutchinick syndrome, Goldenhar syndrome, and caudal regression syndrome. Although the presentation of case reports and small case series may indicate a genetic background suggesting that a genetic element plays a significant role in the pathogenesis of BA, we need to argue that the genetics is probably only one of the multiple factors that may occur coincidentally or sequentially. The few reported familial cases of BA have been supportive [46–50] although conflicting sets of monozygotic twins have also been identified [51–53]. Another important aspect is the variations in the incidence of BA among different ethnics and the incidence of HLA B12 and, of course, haplotypes A9-B5 and A28-B35 were also found to be higher in infants with BA compared to a control group [54]. The high incidence rates of BA

in some areas of Southeast Asia, as well as Polynesia, are evocative of an inherited predisposition, although a local eating style or viral factor cannot be ruled out [51].

The original dichotomic description of BA in embryonic and fetal type or syndromic and non-syndromic forms is still valid [47]. The reader may need to keep in mind that most probably the term “atresia” is a misnomer and a necroinflammatory process of obliterative nature is different from an atretic process in origin from the embryologic point of view. In consideration of the period in which “atresia” occurs, it may be classified as embryonic or fetal and perinatal. In approximately 20% of patients with BA, the embryonic form is responsible, while the rest is due to fetal form, which is also called perinatal form. In the early form, the extrahepatic biliary tree might have undergone abnormal morphogenetic processes. BA patients suffering from this form of BA have associated non-hepatic structural anomalies, of which the most common multiple congenital anomalies is the polysplenia syndrome, which is found in 8–12% of patients with BA. This syndrome is characterized by polysplenia/asplenia associated with cardiac defects (dextrocardia, tetralogy of Fallot, anomalies of the pulmonary vasculature, atrio-/ventricular septal defects) a midline liver, preduodenal portal vein, interruption of the inferior vena cava, and *situs viscerum inversus*, and intestinal malrotation. Other congenital malformations can be detected, such as cardiac anomalies, annular pancreas, immotile cilia syndrome (pointing to Kartagener syndrome), duodenal atresia (leading to trisomy 21 syndrome), esophageal atresia, polycystic kidney disease, cleft palate, and jejunal atresia. The fetal or perinatal form is characterized by patency of the extrahepatic and intrahepatic biliary system at birth, but an inflammatory and sclerosing reaction, caused by perinatal injury, results in the obstruction of the biliary tree. The fetal or perinatal form accounts for four out five cases of BA, and it is not typically associated with congenital anomalies [48, 49].

If about 10% of patients with BA have multiple congenital anomalies, it has been argued that the laterality defects have a phenotype like that detected in the ciliopathies, which is a heterogeneous group of disorders caused by structural and functional abnormalities in genes that encode cilia proteins [23, 50–55]. Embryologically, cilia are evolutionarily conserved. The cilia support in founding the left-right axis in vertebrates and are present on numerous cell types. The cilia are also present in the apical surface of biliary cells or cholangiocytes. In these cells, cilium function is integral to bile flow, bile ductular maturation, and neoductular formation [49]. The *PKHD1* gene is responsible for the autosomal recessive polycystic kidney disease (ARPKD). The expression of the *PKHD1* is polycystin, which was found reduced in the livers of infants with BA with and without associated renal cysts by comparing with liver resections of patients without BA [25, 46]. Studies involving rhesus rotavirus murine models of BA have found the extensive loss of primary cilia from extrahepatic cholangiocytes, which was verified by the finding of decreased ciliation in extrahepatic biliary ducts in infants with BA of perinatal type, although cilia appeared normal in neighboring peribiliary glands [50].

There is quite an overwhelming evidence against a Mendelian paradigm of inheritance of BA. The argument against a Mendelian inheritance is supported by the lack of familial penetrance, despite rare cases of familial BA [52, 53], and discordant presentation of BA among twins, including monozygotic twins [54–57]. On the other side, a growing principle of developmental diseases exhibits unusual patterns of inheritance, including Bardet-Biedl syndrome. The Bardet-Biedl syndrome is a ciliopathy, which is clinically heterogeneous and specifically marked by oligogenic inheritance. The mutations in multiple genes cooperate to generate the phenotype of Bardet-Biedl syndrome. It has been suggested that a similar singularity related to gene-gene interactions, or epistasis, may account for the diversity of presentations in BA [23]. Non-Mendelian inheritance is also detected in Alagille syndrome, which is the

result of a haploid insufficiency of *JAG1* (alternatively its receptor NOTCH2). There is a inconstant intrafamilial clinical presentation [58, 59].

Microchimerism is clearly defined as the occurrence of two genetically different cell populations in the same person. There are a few etiologies, in which it can arise and include blood transfusion, organ transplantation and the bidirectional transfer of cells between mother and fetus during pregnancy as well as the twin-to-twin transfer in utero [56]. Microchimerism has been suggested to explain the phenotypic heterogeneity and nonclassical genetic inheritance of BA [60, 61].

Recently, genome-wide association studies (GWASs) have been milestones in identifying some genes and pathways of several diseases. A GWAS is an observational study of a genome-wide set of genetic variants in diverse individuals to understand if any modification is associated with a trait. Generally, GWASs emphasize the associations between single-nucleotide polymorphisms (SNPs) and traits like major human diseases but can equally be useful for any other genetic variants and any other organisms. SNPs are DNA sequence variations occurring probably 1 in every 100, 200–300 bases along the 3-billion-base human genomic sequence and at least 1% of the population. SNPs make up about 90% of DNA sequence variation as indicated by the Human Genome Project (<http://www.ornl.gov>, <http://linkage.rockefeller.edu/soft/>). On the other side, if one of the possible sequences is present in less than 1% of the population (99.9% of people have a C, and 0.1% have a G), then the variation is called a mutation [57, 58].

The Han Chinese population is particularly susceptible to GWAS because of the large size of individuals and homogeneity of this population. A recent study using a GWAS found a potential susceptibility locus for BA between the genes *ADD3* and *XPNPEP1* located with the chromosomal localization of 10q25.1 with replication in independent Chinese and Thai specimens and identification of BA in a Zebrafish model [59–61]. Interestingly, sequencing of a Han Chinese sample identified that a 5-SNP risk haplotype to be associated with BA and the genotype correlated with reduced levels of *ADD3* expression [61]. The study was attempted to be replicated in a Caucasian cohort [60]. The authors found a stronger signal in the first intron of *ADD3*, although the exact genotype at this SNP was not predictive of the degree of *ADD3* expression. The gene under intense investigation is *ADD3* or adducin 3. Adducins are heteromeric proteins constituted of different subunits (alpha, beta, and gamma). The three subunits are involved in the assembly of the spectrin-actin network in red blood cells and at sites of cell-cell contact in epithelial tissues. Adducins alpha and gamma are universally expressed, while adducin beta is found in the brain and hematopoietic tissues. Adducin 3 is a cytoskeleton-associated protein that endorses the assemblage of the spectrin-actin network. Adducin 3 plays a role in actin filament capping and binds to calmodulin [62–63]. In **Figure 3**, the interaction of adducin 3 with other markers is depicted. This representation is the product of the interaction of several molecules, including *ADD1*, *ADD2*, *ANKRD29*, *C1orf85*, *DDX10*, *MSANTD1*, *PSIP1*, *XPNPEP1*, and *XP07* [64]. Apart of adducin 1 and 2 that belong to the same family of adducins (heteromeric cytoskeletal proteins), the other genes/proteins are extremely intriguing to study in BA patients and may give us hints to understand the pathogenesis of this challenging cholangiopathy [65]. *ANKRD29* (Ankyrin Repeat Domain 29) is a protein-coding gene with the gene located on chromosome 18q11.2. *C1orf85* is a glycosylated lysosomal membrane protein with gene ontology annotations including DNA binding transcription factor activity and, of course, ligand-dependent nuclear receptor transcription coactivator activity. *DDX10* is a DEAD box protein, characterized by the conserved motif Asp-Glu-Ala-Asp (DEAD). This class of proteins are putative RNA helicases and are implicated in many cellular processes involving alteration of RNA secondary structure (e.g., translation initiation, nuclear and mitochondrial splicing,

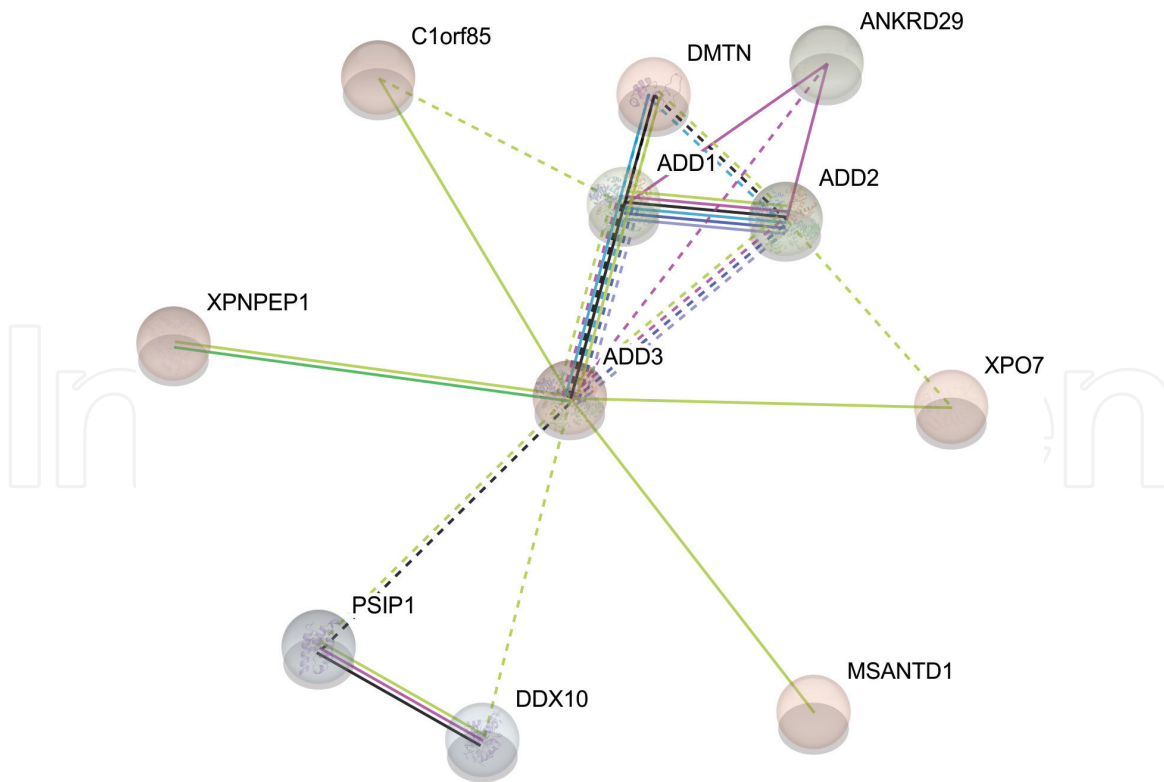


Figure 3.

Interactome of the ADD3 protein with splice isoforms or post-translational modifications are collapsed, while each node represents all the proteins produced by a single, protein-coding gene locus. The edges represent protein-protein interactions with different color according to the interaction type. A red line indicates the presence of fusion evidence, green line a neighborhood evidence, blue line a cooccurrence evidence, purple line an experimental evidence, yellow line a textmining evidence, light blue line a database evidence, and black line a coexpression evidence (see text for details).

and ribosome and spliceosome assembly). In consideration of the distribution patterns, some members of this family are held to be involved in embryogenesis. They may play a role in spermatogenesis, and, overall, cellular growth and division. *MSANTD1* is Myb/SANT DNA Binding Domain Containing 1 and is a protein-coding gene, which is also known as chromosome 4 open reading frame 44. *PSIP1* is PC4 and SFRS1 Interacting Protein 1, which is a protein-coding gene. *PSIP1* is a transcriptional coactivator involved in the differentiation and neurogenesis of neuroepithelial stem cells and specifically in gene regulation and stress responses of lens epithelial cells. The shielding role during stress-induced apoptosis may raise the suspicion that this pattern may be particularly relevant to investigate in the future. Diseases associated with *PSIP1* include atopic dermatitis. *PSIP1* also seems interesting because two related pathways with *PSIP1* are ERK Signaling and Akt Signaling. *XPNPEP1* is a protein coded from a gene *XPNPEP1*. The *XPNPEP1* gene encodes the form in cytosol of a metalloaminopeptidase that catalyzes the cleavage of the N-terminal amino acid adjacent to a proline residue. It seems that this gene product plays a role in the degradation and maturation of tachykinins, neuropeptides, and peptide hormones. There are multiple transcript variants linked to alternative splicing. Finally, *XPO7* or Exportin 7 is coded by the *XPO7* gene. The protein has binding and nuclear export signal receptor activity. It serves for the transport of protein and large RNAs through the nuclear pore complexes in an energy-dependent and regulated process. *XPO7* mediates the nuclear export of proteins (cargos), which have broad substrate specificity. In the nucleus, this protein binds cooperatively to its cargo and the GTPase Ran in its active GTP-bound form. After curbing of this trimeric complex to the nuclear pore complex through binding to nucleoporins, *EXPO7* translocated into the cytoplasm, and the disassembling of the complex and

hydrolysis of Ran-GTP to Ran-GDP determines the release of the cargo from the export receptor. Following this action, XPO7 shows an iterative procedure returning to the nuclear compartment and mediate another round of transport.

The GWAS was also able to identify an additional putative gene, which is called glypican 1 (*GPC1*). *GPC1* is located on chromosome 2q37.3 and has been replicated in an independent cohort of patients demonstrating heterozygous deletion of this gene as the sole gene in this region [62]. Glypicans are heparan sulfate proteoglycans. Glypicans are bound to the external surface of the plasmatic membrane of a cell by a glycosylphosphatidylinositol (GPI) linkage [62, 63, 66]. Homologs of glypican molecules have been identified throughout the Eumetazoa, although clear glypican homologs are not definitely found outside the Metazoa. The family of these proteoglycans includes six members (GPC1–GPC6). Glypican family members are tangled in numerous signaling and developmental pathways in hepatocytes and cholangiocytes, and there is role of GPC-3 for being a marker and a therapeutic target of hepatocellular carcinoma [66], In 2018, Sangkhathat et al. used a whole exome sequencing approach to look for other cholestasis entities in 20 cases diagnosed with BA in Thailand. These authors targeted well 19 genes associated with infantile cholestasis syndromes. Variant selection focused on those with allele frequencies in dbSNP150 database of less than 0.01. A polymerase chain reaction (PCR)-direct sequencing was used to verify all selected variants. Of the 20 cases studied, 13 rare variants were detected in nine genes: four in *JAG1* (Alagille syndrome), two in *MYO5B* (progressive familial intrahepatic cholestasis [PFIC] type 6), and one each in *ABCB11* (PFIC type 2), *ABCC2* (Dubin-Johnson syndrome), *ERCC4* (Fanconi anemia), *KCNH1* (Zimmermann-Laband syndrome), *MLL2* (Kabuki syndrome), *RFX6* (Mitchell-Riley syndrome), and *UG1A1* (Crigler-Najjar syndrome). The authors concluded that severe inflammatory cholangiopathy in BA might be a shared pathology among several infantile cholestatic syndromes [34]. Although these results may be controversially discussed, there is time to verify these conclusions. However, far to be univocal the genetic research on BA showed that other genes involved in biliary tract dysmorphogenesis and cholestasis, the immunologic response, vasculogenesis, and left-right patterning might contribute to this disease with worrisome complications and prognosis. Currently, clinical investigations and nonhuman model systems are focusing on *CFC1*, *CFTR*, *JAG1*, *IFN- γ* , *INV*, *MIF*, *VEGF*, *SOX17*, and *ZIC3* [34–40, 65, 67]. Currently, the finalization of genomic studies has not been reached, but the application of GWAS points incontrovertibly toward two candidate genes that may underlie the development of BA in these patients, including *ADD3* and *GPC1*. It may be important to cross the ethnics and investigate other populations. The use of next-generation sequencing (NGS) has been a throughput in technology in the last decade and will have enormous value in BA research. NGS technologies are miniaturized and parallelized sequencing platforms are targeting from 1 million to 43 billion short reads (50–400 bases each) per instrument run. The massive parallel sequencing is carried out via spatially separated, clonally amplified DNA templates or single DNA molecules localized in a flow cell. NGS is different from the PCR-based Sanger sequencing that is based on polyacrylamide gel electrophoretic separation of chain-termination products available in individual sequencing reactions. NGS strengths include the possibility to detect abnormalities across the entire genome, including substitutions, deletions, insertions, duplications, copy number changes (gene and exon), and chromosome inversions/translocations using less DNA than required for traditional DNA sequencing approaches (e.g., Sanger sequencing). NGS gives data on some molecular aberrations with no currently clinical significance. It requires sophisticated bioinformatics systems, fast data processing, and large data storage capabilities, which can be costly without adequate bioinformatics support. Despite drawbacks, NGS has been used extensively in research and diagnostics [68–72].

5. Epigenetics and genetic modifiers of biliary atresia

Epigenetic modification (e.g., methylation) may be at the basis of some BA patients [73]. Epigenetics is the investigation of inherited variations in the expression of genes that do not involve deviations to the underlying DNA sequence. It means that there is a change in phenotype without evidence of a deviation in genotype. Epigenetics specifically affects how cells translate the genes. There is a natural occurrence of epigenetics, but several factors including age, lifestyle, environment, and disease state influence the epigenetic flow in an organism. Epigenetic changes can affect how cells terminally differentiate or how cancer can occur. There are at least three systems including DNA methylation, modification of histones, and non-coding RNA (ncRNA)-associated gene silencing that are considered to initiate and endure epigenetic change. Epigenetic studies in BA may be particularly challenging because hepatocyte or cholangiocyte DNA of patients may be needed. It has been hypothesized that some genes that are relevant for the biliary function may be involved. These genes may be *A1AT*, *JAG1*, and *CFTR*, but also targeting the bile canaliculus transporters [50, 74–77].

6. Non-genetic contributive factors of biliary atresia

It would be incomplete this chapter, if BA would not be discussed exploring the non-genetic causes of this condition. Although poorly understood, some non-genetic factors may contribute to the development of this surgically correctable neonatal biliary disease. There are several categories, including viruses, toxins, immunological dysregulations, and fetomaternal factors [1, 78–82]. Cytomegalovirus (CMV) is a genus of viruses in the order *Herpesvirales*, in the family *Herpesviridae*, in the subfamily *Betaherpesvirinae*. Both monkeys and humans serve as natural hosts and eight species in this genus are known. Among them, there is the human cytomegalovirus, which is the species that infects humans. CMV has been implicated in BA a few instances in the literature [83–91]. Challenges are the inconsistency of its detection in clinical samples or routine liver biopsy. Other viruses include rotavirus and reovirus, but also other viruses, such as hepatotropic viruses, have been questioned as etiologic factors for this obstructive cholangiopathy of the neonatal age [83, 92–103]. Another etiologic consideration are toxins. Bush tea or other components containing pyrrolizidine alkaloids, deriving mostly from the *Senecio* species, *Crotalaria* species, *Heliotropium lasiocarpum*, or *Symphytum* (comfrey), are a known source to hepatologists and toxicologists [104]. Hepatotoxic pyrrolizidine alkaloids have been found in 150 species of plants. BA may be considered in analogy to the obliterative process seen in pyrrolizidine alkaloids or similar plants [105]. In Australia, toxins present in plants of the *Dysphania* species have been associated with a damage of the biliary system in animals, but the significance for humans is still uncertain [106, 107]. The incidence of viral infection is not epidemic, and BA does not occur outside of the perinatal period. Thus, a virologic etiology may not be widely supported by a strong evidence. An immune-related or an autoimmune disorder is also still plausible due to the absence of recurrence after transplantation [50]. In some infants from Egypt, the cause of BA has been demonstrated to be result of aflatoxin-induced cholangiopathy acquired prenatally in infants who harbor glutathione S-transferase M1 deficiency [108].

7. Final remarks and future perspectives

BA remains a very challenging disease not only from the clinical point of views but also for the pathological one and transcriptome analysis cannot provide a univocal

answer [109]. The importance to differentiate surgically correctable cholangiopathies in the neonatal age is crucial for the prognosis of these patients. The introduction of the Kasai HPE has revolutionized the outcome of patients harboring this condition. The timing when the Kasai HPE is performed has been emphasized as critical. Alternatively, liver transplantation is the only hope for liver cirrhosis developed on the ground of a BA. The underlying cause(s) and outcome contributor(s) to BA remain poorly understood, but new technologies are on the front to target this field. Two genes that we currently consider of significant interest for BA are *ADD3* gene and *GPC1* gene. The feasibility of animal models may also be extremely used in studying the cohorts of animals that may develop this condition in prospective multigeneration investigations. The use of in-depth learning methodologies will be vital in analyzing the massive mole of data that can come out from these experiments, but the use of highly sophisticated computational platforms is opening the horizon for this disease.

Acknowledgements

This work is dedicated to the career of Prof. Dr. J. Hager (**Figure 4**), formerly Director of the Department of Pediatric and Youth Surgery, of the University of Innsbruck, Austria. Prof. Hager was born in 1946 and his career has been fulfilled of enormous and vivid achievements as well as honors, such as the European pediatric surgery diploma, Theodor-Billroth Prize of the Austrian Society for Surgery, and the Spitzzy Prize. He has more than 150 peer-reviewed publications with countless lectures and presentations. His mentoring and teaching activity resulted in numerous fellows, who came out from his school in Innsbruck.



Figure 4.
Professor Dr. med. Josef Hager during a presentation.

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