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Expression of HLA and Autoimmune Pathway Genes in Liver Biopsies of Young Subjects With Autoimmune Hepatitis Type 1

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ABSTRACT

Objectives: To test the hypothesis that autoimmune hepatitis (AIH type I) in young subjects is due to genetic differences in proinflammatory genes responding to viral triggers in patients and controls.

Methods: Intrahepatic gene expression was compared between AIH type I (n = 24, age 9–30 years) patients (hereafter referred to as the AIH group) and controls (n = 21, age 4–25 years). RNA sequencing was performed on complementary DNA (cDNA) libraries made from total RNA extracted from formalin-fixed paraffin-embedded (FFPE) liver biopsy samples. Gene expression levels were quantified, and differentially expressed genes were functionally analyzed. Pathway analysis was performed using the databases Kyoto Encyclopedia of Genes and Genomes (KEGG) and PANTHER. The remaining sequences were mapped to the RefSeq complete set of viral genomes.

Results: Differential gene analysis identified 181 genes that were significantly differentially expressed (136 upregulated in the AIH group). Autoimmune pathway genes such as *CD19* and *CD20* which are important in B cell regulation and maturation as well as, *CD8* and *LY9*, which are T-cell related, were upregulated in our AIH group. Genes implicated in AIH pathogenesis including *CXCL10*, which is thought to be associated with AIH severity and progression, complement genes (*C1QA, C1QB,* and *C1QC*), and human leucocyte antigen (*HLA*) genes (*HLA-DRB1, HLA-DRA, HLA-B,* and *HLA-C*) were upregulated in samples from the AIH group. Specific viral etiologies were not found.

Conclusions: Unbiased next-generation sequencing and differential gene expression analysis of the AIH group has not only added support for the role of B cells in the pathogenesis and treatment of AIH but also has introduced potential new therapeutic targets: *CXCL10* (anti-*CXCL10*) and several complement system–related genes.

Key Words: autoimmune liver disease, liver biopsy, pediatric liver disease, RNA sequencing, transcriptomics

Autoimmune hepatitis (AIH) is a chronic liver disease whose etiology is largely unknown. The predominant hypothesis posits that there may be an environmental trigger resulting in an uncontrolled

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What Is Known

- AIH type I in young subjects have been linked to major histocompatibility complex (MHC) class II genes.
- Both B- and T-cell activations in AIH have been well characterized.
- Most studies of pathogenesis have focused on circulating markers and liver histology.

What Is New

- Both MHC class I genes (*HLA-B,* and *C*) and MHC class II genes were upregulated in the AIH group.
- Our studies identified new potential therapeutic targets: *CXCL10* and some of the complement system genes.

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proinflammatory environment in a genetically susceptible individual (1–5). The 10-year transplant-free survival in untreated patients is only 27% (5). Although the majority of patients can be treated with steroids and antimetabolites, these medications carry with them significant side effect profiles, and most AIH patients require lifelong treatment (3, 6, 7). Long-term steroid therapy can have side effects although long-term outcome can be excellent even in young subjects (8). The aforementioned issues underscore the need to further investigate the complex pathophysiology of this disease to subsequently develop new therapies.

AIH is a polygenic disorder in which the genetic risk factors are largely unknown. However, in the past decade, the use of unbiased next-generation sequencing (NGS) techniques such as genomewide association study (GWAS) and NGS has brought us closer to understanding the pathophysiology of this disease. Genetic predisposition to AIH has been linked mainly to major histocompatibility complex (MHC) class II genes, more specifically

the human leucocyte antigen (*HLA*)–*DR* locus (7, 9), *HLA-DR3* and *DR4*. In children, *HLA-DRB1*1301* is related to susceptibility as well as prognosis and response to treatment (10–12).

In addition to genetic susceptibility, the pathophysiology of AIH may require an environmental trigger, such as a viral infection, with subsequent development of molecular mimicry and cross-reactivity with eventual loss of self-tolerance (13–15). So far, several viruses have been associated with the development of AIH (3, 16, 17). The purpose of our study was to utilize NGS to investigate the genetic susceptibility/infectious trigger hypothesis of AIH utilizing liver biopsies from children and young adults and age-appropriate controls.

METHODS

Ethical Statement

This study was reviewed and approved by the Johns Hopkins Medicine Institutional Review Board. The study was exempt from the informed consent process.

Subjects

The inclusion and exclusion criteria for the AIH group are shown in Table S1 (*http://links.lww.com/MPG/C869*). The diagnosis of AIH was by characteristic findings on liver biopsy (meeting criteria for the diagnosis of AIH using the International Autoimmune Hepatitis Group (IAIHG) scoring system (18) usually in association with F-actin reactive smooth muscle autoantibody, antinuclear autoantibody, and/or autoantibody to soluble liver antigen/liver pancreas). None of the AIH group was on therapy for AIH at the time of biopsy except for one subject who needed an urgent liver transplant for acute liver failure. We included patients of all ethnicities as well as both male and female. The control group was a group of patients of similar male-to-female ratio and biopsy year who received liver biopsies for noninfectious causes such as evaluation of nonalcoholic fatty liver or patients with sickle cell disease and transfusion related iron overload. The inclusion and exclusion criteria for the control group are shown in Table S1 (*http://links.lww.com/MPG/C869*).

Important to note is that our study's AIH group did have 2 patients who most likely had drug-induced AIH (both on minocycline and remain without flares off minocycline for several years). One AIH patient's specimen was not from an initial liver biopsy but that of the explanted liver from when this individual required transplantation due to initial severe presentation of acute liver failure.

Tissue Preparation

Requests for pathology slides and blocks were submitted for retrieval in groups of 5–10 samples at a time. With each submission, we attempted to match the age range and male-to-female ratio of the AIH and control groups. A total of 15 pairs of AIH and control subjects were matched within 1 year of age and were the same sex. For the remainder, there were 9 in the AIH group that were in the 10–30-year-old group (2 males and 7 females); in the control group, there were 6 in the 4–25-year-old group (1 male and 5 females.) Two cored tissue samples were obtained from each subject's tissue block using the services of the Johns Hopkins Tissue Microarray Lab. Tissue coring was only done if it was determined that the block would not be exhausted of all tissue, as per institutional protocol. Coring produced samples that were approximately 1-mm diameter × 1–2-mm height, and these cores were placed into RNase DNase-free microcentrifuge tubes.

Histopathological Examination

All of the pathology slides for both the AIH group and controls were reviewed with a pathologist (L.D.W.) to identify the ideal location for coring into the blocks. The ideal location was an area with the most hepatocytes and least amount of fibrosis, fat, and other cells that could interfere with the analysis. The AIH group's slides were scored using the IAIHG Revised Scoring System's liver histology portion (score −6 to 5) (18) and graded on the intensity of the necroinflammatory activity using the Ishak's Modified Hepatic Activity Index grading system (maximum score of 18) (19) (data not shown but available on request).

RNA Extraction and Messenger RNA Sequencing

In order to perform this study, we first had to develop a method for extracting high-quality RNA from stored formalin-fixed paraffin embedded (FFPE) liver biopsies. We have published the details of this methodology (20) that was developed using the same tissue samples we analyzed in this current report.

The QIAGEN miRNeasy FFPE kit (Hilden, Germany) was used to extract the total RNA, which was then quantified and analyzed for quality using Nanodrop ND-8000 and Agilent 2200 TapeStation High Sensitivity RNA Screen Tape, respectively. Complementary DNA (cDNA) libraries were constructed using the Illumina TruSeq Stranded Total RNA Sample Preparation Kit with Ribo-Zero to remove cytoplasmic and mitochondrial rRNA. Library quality was assessed by TapeStation D1000 Screen Tape. Indexed paired-end sequencing was performed on an Illumina HiSeq2000 with 90 million paired reads per sample.

Analysis of RNA-seq Data

Following initial quality control, adapter sequences and bases with low-quality were trimmed using Fqtrim (DOI: 10.5281/zenodo.593893). Preliminary metagenomic classification using Kraken (21) was performed to determine bacterial contamination. Reads were then mapped to the human genome GRCh38 using the spliced alignment tool Tophat2 v. 2.0.14 (Johns Hopkins Center for Computational Biology, Baltimore, MD) (22) and assembled into transcripts with CLASS2 v2.1.4 (Github, San Francisco, CA) (23), a transcript assembler that is well suited to FFPE data. Of the 24 AIH group and 21 control samples, only 18 AIH group and 15 control samples had sufficient gene information (>20,000 introns from spliced alignments) to allow the reconstruction and quantification of genes and were included in the subsequent analyses. Cuffdiff2 v.2.2.1 (24) was used to quantify gene expression levels and to determine differentially expressed genes, which were then functionally analyzed with the web-based gene functional classification tool Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov). For the differential expression analysis, a q value of 0.05 or lower was considered significant. The q value was calculated based on the false discovery rate as defined by Benjamini and Hochberg (25). Gene ontology (GO) terms were inspected, and pathway analysis was performed using the databases Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp › kegg › pathway) and PANTHER (https://www.pantherdb.org). A Benjamini-Hochberg–corrected P value <0.05 was considered significant.

Additionally, RNA-seq reads for the 24 AIH patients were analyzed by sequential in silico subtraction of human reads and known microbial reads using publicly available software and methods as outlined in (26), employing human genome GRCh38, CLC Genomics Workbench Version 6 (www.clcbio.com) (Qiagen, Hilden, Germany), and the NCBI Reference Sequence database (RefSeq; http://www.ncbi.nlm.nih.gov/refseq/). To determine pathogens potentially implicated in the etiology of the disease, the remaining sequences were mapped to the RefSeq complete set of viral genomes (ftp://ftp.ncbi.nlm.nih.gov/refseq/release/viral/).

RESULTS

A total of 45 FFPE liver biopsy samples were studied. Twenty-four biopsies were from AIH type I patients aged 9–30 years old [median 16.5; interquartile range (IQR) 13–20]. The male to female ratio was 6:18. Biopsies were performed at the time of diagnosis, years 1996–2014. Twenty-one biopsies were from controls aged 4–25 years old (median 15; IQR 12–16.5. The male to female ratio was 5:16. Biopsies were performed during 2001–2014. Biopsies were made into total RNA libraries and analyzed using RNA sequencing. The two groups were similar in terms of gender ratio, age range, and biopsy year (see Table 1).

Sequencing produced between 22–305 million paired-end 100 base pair reads per sample, of which 17.5%–91% mapped to the human genome. These rates were concordant with the numbers of reads metagenomically classified as human with Kraken. The fraction of exonic reads varied between 1% and 36%, consistent with the exonic fraction typically found in total RNA libraries, which include a large amount of unprocessed (intronic) RNA. A total of 33 samples (18 AIH group and 15 controls) had a sufficient number of splice junctions (>20,000) from spliced reads to allow gene and transcript reconstruction, resulting in 4300–31,838 multiexon transcripts per sample (Supplementary Table S2, *http://links.lww.com/MPG/C869*).

Comparative Transcriptome Profiling of AIH Group and Controls

Cuffdiff2 differential gene analysis identified 181 genes at 174 loci that were differentially regulated in AIH group compared to controls with log2 fold change ranging from 1 to 6 (Supplementary Table S3, *http://links.lww.com/MPG/C869*). Of the 181 genes, 136 were upregulated and 45 were downregulated in the AIH group when compared with controls.

Functional Gene Analysis

Signaling in immune system–related processes was enriched among the genes that were upregulated in the AIH group compared with controls (Table 2). The biological processes of the genes that were upregulated in the AIH group compared with the controls revealed by GO analysis included leukocyte- and lymphocyte-mediated immunity, adaptive immunity, Ig-mediated immune response, and B-cell–mediated immunity, with the top 15 GO categories shown in Table 2. Those genes with the highest expression in the AIH group as shown by GO analysis were genes involved in antigen expression (*HLA-DRB1*, *HLA-DRA*, and *HLA-B*), as well as genes involved in immune activation of T and B cells, the chemokine *CXCL10*, and the complement system genes *C1QA*, *C1QB*, and *C1QC*. The GO analysis was able to classify 47% of the total number of genes found to be upregulated in the AIH group. Lipid metabolism and steroid pathway genes were enriched among the genes downregulated in the AIH group compared with controls (Table 3).

The online gene functional analysis tool DAVID was used to study the enrichment of differentially expressed genes in a particular pathway. Analysis of the genes that were upregulated in the AIH group compared with the controls identified the top 11 KEGG pathways with *P* values ≤0.05 (Table 4). Those genes that had the

TABLE 1. Clinical features

AIH group (median age: 16.5; IQR: 13–20)		Controls (median age: 15; IQR: 12–16.5)		
Age at biopsy/sex	Biopsy year	Age at biopsy/sex	Biopsy year	Histology diagnosis
18/M	1996	10/F	2006	Congenital hepatic fibrosis
9/F	2004	9/M	2014	Steatosis
12/F	2000	16/F	2014	Steatosis
17/F	2008	6/F	2002	Iron overload
16/M	2001	14/M	2009	Iron overload
16/F	2011	15/F	2005	Steatosis
11/M	2006	16/M	2005	Steatosis
15/M	2009	4/M	2009	Iron overload
12/M	2012	17/F	2012	Steatosis
14/F	2011	12/F	2013	Steatosis
15/F	2012	16/F	2014	Glycogen accumulation
10/M	2012	18/M	2014	Glycogen accumulation
17/F	2012	17/F	2007	Sickle cell hepatopathy
30/F	2003	25/F	2011	Granulomatous liver
19/F	2011	12/F	2010	Steatosis
21/F	2012	12/F	2007	Steatosis
15/F	2012	15/F	2012	Steatosis
29/F	2004	16/F	2001	Steatosis
17/F	2007	12/F	2001	Iron overload
25/F	2004	17/F	2007	Iron overload
12/F	2002	14/F	2014	Steatosis
29/F	2006			
17/F	2014			

TABLE 2.   Enriched gene ontologies* for biological processes of upregulated genes in patients as analyzed by DAVID

Term	*P* value	Genes†
GO:0002449: lymphocyte-mediated immunity	4.52E-07	C1QA, C1QB, CD8A, LY9, SLAMF7, C1QC, CD74, HLA-DRA
GO:0006955: immune response	7.37E-07	POU2AF1, HLA-DRB1, CD8A, SLAMF7, HLA-B, LY9, C1QC, CD74, CXCL10, C1QA, C1QB, RGS1, LAX1, MS4A1, CD24, FCGR3A, IGLC1, IFI6, HLA-DRA
GO:0002443: leukocyte-mediated immunity	1.85E-06	C1QA, C1QB, CD8A, LY9, SLAMF7, C1QC, CD74, HLA-DRA
GO:0002252: immune effector process	3.48E-06	C1QA, C1QB, CD8A, LAX1, LY9, SLAMF7, C1QC, CD74, HLA-DRA
GO:0002460: adaptive immune response (based on somatic recombination of immune receptors built from immunoglobulin superfamily domains)	1.35E-05	C1QA, C1QB, CD8A, LY9, C1QC, CD74, HLA-DRA
GO:0002250: adaptive immune response	1.35E-05	C1QA, C1QB, CD8A, LY9, C1QC, CD74, HLA-DRA
GO:0016064: immunoglobulin-mediated immune response	3.17E-05	C1QA, C1QB, LY9, C1QC, CD74, HLA-DRA
GO:0002684: positive regulation of immune system process	3.39E-05	C1QA, C1QB, CD19, EREG, LAX1, LCK, CD24, C1QC, CD74, HLA-DRA
GO:0019724: B cell–mediated immunity	3.79E-05	C1QA, C1QB, LY9, C1QC, CD74, HLA-DRA
GO:0050778: positive regulation of immune response	5.76E-05	C1QA, C1QB, CD19, EREG, LAX1, CD24, C1QC, HLA-DRA
GO:0000279: M phase	7.78E-05	MEI1, PRC1, EREG, BUB1, BUB1B, CENPF, TTK, ANLN, PIM2, AURKB, ASPM
GO:0022403: cell cycle phase	1.12E-04	MEI1, PRC1, EREG, BUB1, BUB1B, CENPF, TTK, ANLN, ID4, PIM2, AURKB, ASPM
GO:0007155: cell adhesion	2.56E-04	SIGLEC10, MFGE8, SLAMF7, CLDN11, LY9, DDR1, SIGLEC1, ITGB8, GPR56, DSC3, CD24, GPNMB, THBS2, NTM, SPP1
GO:0022610: biological adhesion	2.60E-04	SIGLEC10, MFGE8, SLAMF7, CLDN11, LY9, DDR1, SIGLEC1, ITGB8, GPR56, DSC3, CD24, GPNMB, THBS2, NTM, SPP1
GO:0046649: lymphocyte activation	4.09E-04	CD8A, LAX1, LCK, IL21R, MS4A1, SLAMF7, CD24, CD74

*Enriched gene ontologies were determined with the tool DAVID †Please note that there are several genes that are in multiple categories

TABLE 3.   Enriched gene ontologies* for biological processes of downregulated genes in patients as assessed by DAVID

Term	*P* value	Genes†
GO:0008203: cholesterol metabolic process	0.010	EBP, DHCR7, CYP7A1
GO:0016125: sterol metabolic process	0.012	EBP, DHCR7, CYP7A1
GO:0008610: lipid biosynthetic process	0.017	EBP, DGAT2, DHCR7, SCD
GO:0055114: oxidation reduction	0.0213	DHCR7, CYP7A1, SCD, DAO, ALDH9A1
GO:0006695: cholesterol biosynthetic process	0.043	EBP, DHCR7
GO:0008202: steroid metabolic process	0.046	EBP, DHCR7, CYP7A1
GO:0006081: cellular aldehyde metabolic process	0.047	DAO, ALDH9A1

DAVID = Database for Annotation, Visualization and Integrated Discovery, GO = gene ontologies. *Enriched gene ontologies were determined with the tool DAVID. †Please note that there are several genes that are in multiple categories.

highest upregulation were involved with B cell maturation and function—*BTK*, *CD19*, *CD8A*, *JAK3*, and *LCK*. *HLA-C* was further identified by KEGG pathway enrichment analysis of the upregulated genes (Table 4).

Analysis of the genes that were downregulated in the AIH group compared with the controls identified KEGG pathways involved in cholesterol/lipid metabolism as well as iron related pathways, as expected for the conditions that our controls had, namely fatty liver disease and transfusion related iron overload due to sickle cell disease (data not shown).

DISCUSSION

As demonstrated by the enriched gene ontologies (GOs) in the AIH group compared with the controls, our study emphasized that AIH is a disorder that is largely due to a genetic predisposition to develop dysregulation of immune-mediated pathways. Although several different groups of investigators (27–35) have investigated genetic determinants of AIH, there are only a few studies (36) utilizing state of the art sequencing techniques to investigate genetic susceptibility to AIH in pediatric patients.

The association of AIH with HLA genes, especially MHC class II, has been well described in the literature (11) and most recently by Ma et al (27) in the largest pediatric series linking HLA genotype to severity of liver disease in pediatric AIH with a 40-year follow-up. Especially, *HLA-DRB1 *0301* (28) has been shown to have a strong association with the pediatric form of AIH, and our study also showed a relationship with this HLA type in our AIH group. However, the MHC class I genes (*HLA-B*, and *C*) were also significantly upregulated in our AIH group. This supports the findings of Podhorzer et al (12), in which pediatric AIH patients were noted to

TABLE 4.   KEGG pathways of upregulated genes in patients and controls

KEGG pathway*, upregulated in pts. vs controls	*P* value	Genes†
Primary immunodeficiency	9.5E-5	BTK, CD19, CD8A, JAK3, LCK
Cell adhesion molecules	2.8E-4	CD8A, CLD11, ITGB8, HLA-DRA, HLA-B, HLA-C, HLA-DRB1, HLA-DRB4, SIGLEC1
Systemic lupus erythematosus	5.9E-4	FCGR3a, C1QA, C1QB, C1QC, HLA-DRA, HLA-DRB1, HLA-DRB4
Antigen processing and presentation	2.6E-3	CD74, CD8A, HLA-B, HLA-DRA, HLA-DRB1
Hematopoietic cell lineage	3.0E-3	CD19, CD8A, HLA-DRA, HLA-DRB1, MS4A1
Complement and coagulation cascades	1.2E-2	C1QA, C1QB, C1QC, THBD
Prion diseases	2.5E-2	C1QA, C1QB, C1QC
Allograft rejection	2.6E-2	HLA-B, HLA-DRA, HLA-DRB1
Graft versus host disease	3.1E-2	HLA-B, HLA-DRA, HLA-DRB1
Type 1 diabetes	3.5E-2	HLA-B, HLA-DRA, HLA-DRB1
Autoimmune thyroid disease	5.0E-2	HLA-B, HLA-DRA, HLA-DRB1

CAM = cell adhesion molecules, KEGG = Kyoto Encyclopedia of Genes and Genomes. *Enriched pathways were determined with the tool DAVID. †Please note that there are several genes that are in multiple categories.

have an increased frequency of the functional form of *KIR2DS4*-Full Length [an natural killer (NK) cell receptor] as well as an increased frequency of this receptor's putative ligand *HLA-C02, 04*, and *06*, which was not seen in the adult AIH patients. In addition, the Podhorzer et al (12) study showed a synergistic effect of *KIR2DS4-FL* with the *HLA-DRB1*1301* with an odds ratio of 36.4.

With our AIH group population being largely of the pediatric age range, it is important to note the finding of *HLA-C* as well as *HLA- B* upregulation. The gene *FCGR3A* on Chr1 (log$_2$FC 1.8, *P* value 1.5×10^{-4}) (Supplemental Table S3, *http://links.lww.com/MPG/C869*), which was significantly up regulated in our AIH group population, is known to be intimately involved in the process of removal of antigen antibody complexes (37). AIH patients may possess HLA alleles that cause them to be susceptible to aberrant clearance function, prolonging exposure to foreign antigens that can lead to molecular mimicry and subsequent upregulation of *FCGR3A* in attempts to clear the Ag-Ab complexes.

The role of T cells in the pathogenesis of AIH has long been recognized, and our data add support to the role of T cells in the pathogenesis of AIH in young subjects. As shown in Table 2, there was upregulation of *CD8A* and *LY*9 (a T lymphocyte surface antigen) as well as the autoimmunity-associated gene *RGS1*, which affects the frequency of T follicular helper cells. In AIH, helper T cells are involved in recognizing autoantigens, which triggers the self-attack process and, in experimental animal depletion of B cells, induces remission of AIH by reduced antigen presentation and help to T cells (38).

Our data also support a role for B cells in AIH in our AIH group. Several of our findings may carry therapeutic implications for AIH including anti-B cell, anti-CXCL10, and complement inhibition. First, many genes that are important in B-cell development and regulation (*POU2AF1* [Table 2], CD *19* and *BTK* [Table 4] and *CD24* [Supplemental Table S3, *http://links.lww.com/MPG/C869*] were upregulated in the AIH group population. Our data are in agreement with Taylor et al (39) who described the involvement of B cells on the pathogenesis in AIH. Interestingly, there are several reports of anti B-cell therapies including rituximab (*anti-CD19/20*) as a successful treatment for refractory AIH in pediatric patients (40, 41) as well as belimumab, an inhibitor of B-cell–activating factor (42).

Our study also noted an increased expression in the AIH group population of *CXCL10*, which is thought to be associated with AIH severity and progression (43, 44). This chemokine has been shown in murine models of nonalcoholic steatohepatitis to be one of the chemokines responsible for hepatic inflammation, and

deletion of CXCL10 is hepatoprotective (45). Chemokine-directed therapies such as peptides that block chemokine receptors (44), and medications that are directed at chemokine suppression (rosiglitazone and methimazole) (46) are being studied in various immune-mediated disorders such as rheumatoid arthritis and Crohn disease. A clinical trial of humanized antibodies to *CXCL10* is being considered for autoimmune liver disease (47).

The complement system genes including *C1QA, C1QB,* and *C1QC* were significantly upregulated in the AIH group. This finding supports the study done by Tu et al (48) who reported that the introduction of polyclonal rabbit anti-ovalbumin (OVA) antibody into Hep-OVA Tg mice (in whom OVA is expressed on the hepatocyte surface), resulting in excessive complement activation and hepatocyte injury. However, when the complement component is depleted by cobra venom factor or by treating the mice with a decay accelerating factor, a native complement inhibitor, the hepatocytes were protected from anti-OVA IgG-induced injury. Thus, the complement system may be targeted in future therapies for AIH.

Limitations of our study would include small sample size and the fact that many of the biopsy samples were very limited in size and amount as well as inclusion of the one patient on immunosuppression. Furthermore, both our AIH group and control group included patients that were older than 18, which could suggest that our findings may not apply to just a pediatric patient pool and warrants further evaluation of the findings between subgroups of ages. The IAIHG scoring system was used for diagnosis of the AIH group at the time. The scoring system for the diagnosis of autoimmune liver disease in children which allows differentiation between AIH and autoimmune sclerosing cholangitis which was described by European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) in 2018 (49) may have been more appropriate. Our deliberate selection of what we considered the ideal location of the AIH liver biopsy—"an area with the most hepatocytes and least amount of fibrosis, fat and other cells that could interfere with the analysis"—may well have exaggerated the difference in lipid metabolism genes between the AIH group and controls and minimized inflammatory genes related to stellate cell activation. An additional limitation is the lack of correlation between the genetic data and clinical outcomes. The recent finding that formalin-fixation of murine livers does induce transcriptomic effects has two implications for using FFPE livers for human clinical research as we did in this study. It is essential to compare findings in FFPE livers from AIH group to control livers processed the same way (50). The other implication is that findings in FFPE human livers should

be validated in fresh frozen liver before embarking on clinical trials based on findings in FFPE livers such as anti-*CXCL10*. However, given the difficulties of obtaining fresh frozen liver and the much greater availability of clinical FFPE livers vs fresh, we believe the FFPE livers provide valuable clinical data as long as the investigators are aware of the limitations.

CONCLUSIONS

In conclusion, differential expression analysis of RNA-seq data comparing AIH group with controls did identify several genes known to be involved in the autoimmune pathway with few of them already studied in AIH. Our data provide strong support for anti-B cell therapies as shown by others as well as more novel therapies such as anti CXCL10 or complement depletion. It is hoped that these finding may lead to novel therapeutic targets for a disorder that subjects most pediatric patients to lifelong treatment with immunosuppressants and their adverse side effects as well as studies in the future of the relationship to intrahepatic gene expression and clinical outcomes.

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