Novel resequencing chip customized to diagnose mutations in patients with inherited syndromes of intrahepatic cholestasis

Cong Liu[*], **Bruce J. Aronow**, **Anil G. Jegga**, **Ning Wang**, **Alex Miethke**, **Reena Mourya**, and **Jorge A. Bezerra**
Cincinnati Children's Hospital Medical Center and Department of Pediatrics, University of Cincinnati, Cincinnati, OH, USA

Abstract

Background and Aims—Inherited syndromes of intrahepatic cholestasis commonly result from mutations in the genes *SERPINA1* (α1-antitrypsin deficiency), *JAG1* (Alagille syndrome), *ATP8B1* (Progressive Familial Intrahepatic Cholestasis type 1/PFIC1), *ABCB11* (PFIC2), and *ABCB4* (PFIC3). However, the large gene sizes and lack of mutational hotspots make it difficult to survey for disease-causing mutations in clinical practice. Here, we aimed to develop a technological tool that reads out the nucleotide sequence of these genes rapidly and accurately.

Methods—25-mer nucleotide probes were designed to identify each base for all exons, 10 bases of intronic sequence bordering exons, 280–500 bases upstream from the first exon for each gene, and 350 bases of the second intron of the *JAG1* gene, and tiled using the Affymetrix resequencing platform. We then developed high-fidelity PCRs to produce amplicons using 1 ml of blood from each subject; amplicons were hybridized to the chip, and nucleotide calls were validated by standard capillary sequencing methods.

Results—Hybridization of amplicons with the chip produced a high nucleotide sequence readout for all five genes in a single assay, with an automated call rate of 93.5% (range: 90.3–95.7%). The accuracy of nucleotide calls was 99.99% when compared with capillary sequencing. Testing the chip on subjects with cholestatic syndromes identified disease-causing mutations in *SERPINA1, JAG1, ATP8B1, ABCB11* or *ABCB4*.

Conclusion—The resequencing chip efficiently reads *SERPINA1, JAG1, ATP8B1, ABCB11* and *ABCB4* with a high call rate and accuracy in one assay, and identifies disease-causing mutations.

INTRODUCTION

The discovery of the genetic basis of syndromes of intrahepatic cholestasis allows for the potential for diagnostic and treatment plans tailored to the patient's genetic makeup. Inherited syndromes of intrahepatic cholestasis represent a heterogeneous group of disorders that begin during childhood, most commonly manifesting as neonatal cholestasis, and lead to ongoing liver dysfunction in children and adults.[1] These disorders share many clinical features, yet discrete clinical, biochemical and histological patterns suggest specific entities. Identification of specific diseases as causes of intrahepatic cholestasis began with the report that α1-antitrypsin (α1AT) deficiency can present as neonatal cholestasis.[2, 3] Subsequent studies led

Corresponding author: Jorge A. Bezerra, Cincinnati Children's Hospital Medical Center; 3333 Burnet Avenue, Cincinnati, OH, 45229-3039, USA. Phone: 513-636-3008; Fax 513-636-5581, Email: Jorge.Bezerra@cchmc.org.
[*]Current address: P2D, Inc., Cincinnati, OH, USA.

to the recognition of the inherited nature of syndromic and non-syndromic forms of cholestasis. [4] For example, patients with the Alagille syndrome have chronic cholestasis and variable syndromic features that may be shared by family members. [5, 6] Application of genetic mapping approaches to study these patients discovered disease-causing mutations in the *JAG1* gene. [7, 8] A significant portion of the remaining subjects with non-syndromic forms of intrahepatic cholestasis comprise a group commonly referred to as Progressive Familial Intrahepatic Cholestasis (PFIC). [9–14] Patients with the most common types of PFIC are now known to harbor mutations in genes encoding proteins involved in bile acid transport: 1) *ATP8B1* gene, encoding FIC1 (patients with PFIC1); [15, 16] *ABCB11* gene, encoding the bile salt export pump (BSEP, patients with PFIC2); [17] and *ABCB4* gene, encoding the multi-drug resistance protein-3 (MDR3, patients with PFIC3). [18, 19] Despite the remarkable progress in identifying the genes that cause the most common inherited syndromes of intrahepatic cholestasis, current diagnostic algorithms do not incorporate mutation analysis in the clinical evaluation of affected patients. In addition, the large gene size and the lack of highly predominant mutational hotspots form obstacles to studies exploring gene-gene interactions and genotype-phenotype relationships. To fulfill this technological gap, we developed a customized resequencing gene chip that reads *SERPINA1* (gene encoding α1AT)*, JAG1, ATP8B1, ABCB11,* and *ABCB4* genes simultaneously in a single assay, with high call rate and accuracy.

METHODS

Length of genes and selection of target sequences

A sequence analysis of all exon-intron elements for the *SERPINA1, JAG1, ATP8B1, ABCB11,* and *ABCB4* genes showed that sequencing by standard methods would require the identification of 313,288 bases (data from GenBank at www.ncbi.nlm.nih.gov/entrez). An alternative high-throughput method is resequencing technology using the Affymetrix GeneChip® CustomSeq™ Resequencing Array platform that can identify 30,000 nucleotides of double-stranded contiguous or non-contiguous sequence (60,000 total) (www.affymetrix.com/products/arrays/specific/custom_seq.affx). This resequencing platform identifies individual nucleotides by a comparative, high-fidelity hybridization using four 25-mer probes designed per strand of each individual nucleotide (base), where the central position of the probes varies to incorporate each of the four possible bases (A, T, C, G). In this system, the probe that matches 100% of the target sequence (which occurs in 1 of 4 probes according to the precise match of the central position base) generates a specific signal and identifies the individual base. To select the target sequences of interest, we developed a database for all exons, 10 bases of the intronic sequence bordering exons (potential splice sites), and 280–500 bases upstream from the first exon for each gene. We also selected 350 bases of the second intron of the *JAG1* gene based on its identification as a putative regulatory region as defined by Trafac, an application that surveys for potential regulatory regions by the direct comparison of conserved non-coding DNA sequences between human and mouse orthologous genes [20]; no similar region was identified for any of the other four genes. We included the potential regulatory sequences to facilitate studies addressing whether they may be sites of novel mutations that segregate with disease phenotypes or that modify the level of gene expression. Combined, these fragments formed the target sequences of interest and totaled 26,725 bases. The target sequences were converted to FASTA format and analyzed for repetitive elements, internal duplications and homologous sequences. The entire sequence was then submitted to Affymetrix, Inc (Santa Clara, CA) for primer design and chip assembly.

Amplification of target sequences

DNA was isolated from 1 ml of peripheral blood obtained from subjects with intrahepatic cholestasis evaluated at the Cincinnati Children's Hospital Pediatric Liver Care Center. Informed consent was obtained from the patient's legal guardians as approved by the

Institutional Review Board of Cincinnati Children's Hospital Medical Center. DNA was isolated using the Puregene® Purification Kit (Gentra Systems, Minneapolis, MN), according to the manufacturer's protocol. To produce DNA samples of sufficient amounts for proper labeling and hybridization against the chip, we designed PCR primers to amplify the target sequences from each patient's DNA (herein referred to as "amplicons"). In order to develop a simpler amplification assay with a small number of reactions that can be run simultaneously, we designed short- and long-range, high fidelity PCR protocols. They included an initial incubation of DNA with RNase A solution, followed by a total of 4 μg of purified DNA divided into small aliquots to serve as template in three amplification programs containing 35 individual reactions (Supplementary Figure 1). A 7.5 kb control fragment was amplified using primers and template included in the CustomSeq™ Control kit provided by Affymetrix.

Chip hybridization and analysis

Individual amplicons were identified in 1% agarose electrophoreses and quantified by PicoGreen dsDNA Quantitation Kit (Invitrogen, Carlsbad, CA); equimolar amounts of amplicons were pooled and subjected to fragmentation and labeling according to protocols provided by Affymetrix. In brief, fragmentation was performed with the GeneChip Fragmentation Reaction Kit, which contains an enzyme to fragment amplicons at 37 °C × 15 minutes, followed by labeling with TdT buffer, Labeling Reaction Mix and TdT enzyme at 37 °C × 2 hours, and a denaturing step at 95 °C × 15 min. Prior to hybridization, the chip was incubated with 200 μl of pre-hybridization buffer (Tris, pH7.8 and 1% Tween-20) at 45 °C in a hybridization oven for 15 min. Then, the buffer was removed and a 200 μl of hybridization solution (Tris, pH7.8, Tween-20, acetylated BSA, herring sperm DNA, labeled oligonucleotide control reagent, and labeled fragments of amplicons) was added to the resequencing chip. Following incubation at 45 °C for 16 hours, the chip was washed with decreasing concentrations of SSPE and Tween-20, stained with anti-biotin antibody, and scanned with the Affymetrix GeneChip® 3000 Scanner. Specific signals were captured by the Affymetrix GeneChip® Operating Software (GCOS) and analyzed with the Affymetrix GeneChip® DNA Analysis Software (GDAS). To examine the accuracy of the chip readout, unused fractions of the original amplicons were subjected to automated capillary sequencing using ABI Prism® 3730 DNA Analyzer at the Gene Sequencing Core at Cincinnati Children's Research Foundation.

Statistical analysis

Base call accuracy was expressed as a percentage of the number of base calls correctly classified when compared to automated capillary sequencing. We estimated the need to sequence at least 32,431 bases to enable the detection of an error rate of 0.0005 and a no more than 0.0002 error (95% confidence interval) with 80% power and 5% significance level.[21] The agreement between resequencing and capillary sequencing was measured using intraclass kappa statistics implemented in SAS, Version 9.1 (SAS Institute, Cary, NC).

RESULTS

Assembly of the *Jaundice Chip*

A key feature of CustomSeq™ Arrays is that the target gene sequences do not have to be contiguous, allowing for an efficient method to sequence gene domains that are separated by long intervening DNA sequences that are not the focus of interrogation. This feature allowed us to select 26,725 bases encompassing nucleotide elements that represent the best candidates for mutations that alter the structure/function of the proteins and are likely to cause a clinical phenotype. Analysis of the entire target sequences did not find repetitive elements, internal duplications or highly homologous sequences, which could decrease reading accuracy due to potential cross-hybridization of amplicons with similar gene segments. Therefore, the dataset

was submitted to Affymetrix, where 25-mer probes were designed to identify individual bases in the forward and reverse strands. The primers were tiled onto 25×20 μm features in a standard array format, and the array was assembled into a hybridization cartridge; the protocols for production of 25-mer probes, tiling, and assembling constitute proprietary information of Affymetrix.

Efficient amplification of target sequences

In order to develop a convenient assay that efficiently produces amplicons for all target sequences in a short period of time using a small volume of blood from patients, we designed 35 PCR primer pairs that produced single amplicons in reactions containing optimal amplification buffer and three cycling programs (Supplementary Table 1). The relationship between target sequences, amplicons, fragmentation, labeling, and hybridization with the resequencing chip are summarized in Supplementary Figure 2. Sufficient amounts of DNA for all amplicons were produced in the first run of PCR reactions in ~55% of the samples from subjects included in this report; the remainder required 1-3 additional PCR reactions to produce enough quantities of individual amplicons with low copy number in the first run. The low yield was not related to the same amplicons, but varied among the reactions. Altogether, amplicons were successfully produced at desired quantities within 48 hours.

Highly accurate readout by the resequencing chip

To determine whether hybridization of the chips with fragmented and labeled amplicons identified the target gene sequences, the reference nucleotide sequences for *SERPINA1, JAG1, ATP8B1, ABCB11,* and *ABCB4* were entered in the GDAS software. Then, using the software to make automated base calling according to hybridization intensities, we found high sequence readout from the first chip, with a base call rate of 91.4%. Base call rate was defined as the fraction of individual bases that can be clearly detected and identified (or "called") by the method.[22] Because the GDAS call algorithm computes a background correction that requires multiple samples for an effective estimate, we hybridized amplicons from 15 additional subjects. The average call rate for all 18 samples was 93.5% (range: 90.3–95.7%); the percent of "no calls" was not influenced by the lengths of the amplicons (Supplementary Table 2). The "no call" positions were not sequence dependent (average GC percentage of 49.63%), did not reside near the ends of amplicons, and were dispersed throughout the target sequences of all five genes (Supplementary Figure 3). We were able to manually analyze "no call" regions and identify individual nucleotides; however, because the manual calls could not be uniformly validated by automated capillary sequencing, we decided not to use manual analysis and to accept the standard filter conditions, algorithm parameters, and reliability rules of GDAS.

To determine the accuracy of the automated nucleotide calls, we compared the chip readout with the readout produced by standard automated capillary sequencing (Figure 1). Capillary sequencing was performed for the first and last exons of all five genes and 1–2 other exons randomly chosen for each gene using the amplicons from 10 of the 18 subjects. In the comparison, we included all bases that were identified/called by both methods. Direct comparison of 40,216 bases identified by the chip and capillary sequencing revealed 4 discordant calls in the chip readout, representing an automated call accuracy of 99.99%. Notably, all 4 discordant calls were heterozygous calls, with one of the alleles containing a base that did not match the automated sequence call (Table 1). Both methods were in overall agreement, as supported by a kappa value of 0.7498 (95% CI: 0.644–0.856, P<0.0005). Lastly, the reproducibility by capillary sequencing of 52 single nucleotide mutations (minus the 4 discordant calls) present among the 40,216 bases translates into a positive predictive value of 92.31%.

Detection of disease-causing mutations by the *Jaundice Chip*

To investigate if the chip identifies disease-causing mutations, we analyzed the sequence readout of subjects known to have intrahepatic cholestasis secondary to α1AT deficiency, the Alagille syndrome, or PFIC1-3 based on clinical, histological, and biochemical criteria (reviewed in reference #[1]). We also analyzed the readout from five subjects with biliary atresia, a non-inherited cholestatic disease due to a fibroinflammatory obstruction of extrahepatic bile ducts (as "disease controls"). Each subject with inherited forms of intrahepatic cholestasis was found to harbor at least one disease-causing mutation previously reported or newly identified mutations containing a new stop codon (Table 2 and Figure 2). Interestingly, one of the subjects with PFIC1 (subject #10) presented with neonatal cholestasis and has had progressive liver disease, with severe pruritus that was unresponsive to external biliary diversion. Analysis of the gene readout revealed a homozygous deletion of the 10 intronic bases continuing with the first 17 bases of exon 23 of *ATP8B1*. This finding was validated by capillary sequencing, which further showed that the extent of deletion included 569 bases 5′ of exon 23 (552 bases of intron 22 + 17 bases of exon 23). In subjects with cholestasis due to biliary atresia, non-synonymous polymorphisms were heterozygous and infrequent (Table 2).

Finally, to examine the ability of the chip to detect a broader spectrum of genetic mutations reported for the clinical phenotypes, we developed a dataset of mutations for all 5 genes based on data from the published literature (www.ncbi.nlm.nih.gov/entrez/, December 2005) and from the Human Gene Mutation Database (www.hgmd.cf.ac.uk/ac/index.php). Collectively, the most common types of mutations were base changes and comprised a dataset of 360 positions reported as missense/nonsense mutations (N=200), single nucleotide polymorphisms (N=119), and mutations in splice sites (N=41). Among these positions, only 11 (or 3.06%) resided in regions of the chip that consistently generated failed signals (or "no calls;" Table 3). Thus, the nucleotide sequences identified by the *Jaundice Chip* efficiently screened for >95% of the disease-causing mutations due to single base changes reported to date. A second group of disease-causing mutations consisted of insertions, deletions or duplications, which may be as high as 55% in the *JAG1* gene in patients with the Alagille disease,[23] or cause a more severe phenotype as reported for *ATP8B1* (as illustrated by patient #10 of Table 2).[24] Although most of these insertions, deletions or duplications fall within the reading ability of the chip (Supplementary Table 3), our current dataset does not allow a precise analysis of how reliable the chip is in detecting these types of mutation.

DISCUSSION

We used the resequencing technology to develop a chip that reads the nucleotide signature for five genes known to cause the most common inherited syndromes of intrahepatic cholestasis: *SERPINA1, JAG1, ATP8B1, ABCB11,* and *ABCB4*. The chip readout is highly accurate and has an error rate estimated at 0.001 due primarily to heterozygous base calls when compared to automated capillary sequencing. The readout encompasses all coding sequences, intron-exon boundaries, and >200 bases upstream of the first exon, thus enabling the detection of nucleotide changes that may alter structure/function and/or level of expression of encoded proteins. Detection of individual bases for all five genes occurs simultaneously during a single hybridization assay and without manual editing; automated identification has an average call rate of 93.5%. In addition to the accurate sequence readout with minimal need for data curation, the amplification of all target sequences from as little as 4 μg of whole blood DNA in PCR reactions run in three cycling programs simultaneously form a robust system that yields sequence for mutational analysis in 3–4 days. These features will greatly facilitate the use of the chip as a research tool to study the role of individual or combined genes in determination of clinical phenotypes, and as a potential diagnostic tool for patients with inherited syndromes of intrahepatic cholestasis.

The high-throughput features of resequencing microarrays offer the potential to meet investigative and clinical needs to identify nucleotide variations along different gene segments rapidly and efficiently. This approach is particularly relevant to genomic regions that are not contiguous and/or have high rates of nucleotide polymorphisms. The *Jaundice Chip* reported herein can screen for >95% of disease-causing mutations due to single nucleotide changes and appears to also detect short homozygous deletions. While we expect that the chip will identify other homozygous deletions of short and long DNA segments, it is possible that heterozygous deletions (regardless of the length) or insertions may not be detected by the chip because the strand with normal sequences will produce a normal readout. If this occurs, complementary technologies such as single strand conformational polymorphism, conformation sensitive gel electrophoresis and capillary sequencing of selected regions may be required for the most comprehensive detection of all types of mutations. More accurate evaluation of these scenarios will require formal bench-testing of the chip against DNA from subjects known to carry insertions, deletions or duplications. This notwithstanding, the *Jaundice Chip* allows for the accurate screening for the vast majority of reported mutations due to single nucleotide changes accurately and in a predictable fashion.

The ability to rapidly survey for genetic mutations in children and adults with inherited syndromes of intrahepatic cholestasis generates unique opportunities to investigate the molecular basis of the clinical heterogeneity observed in these patients. For example, although mutations in *ATP8B1* and *ABCB11* have been reported for subjects with severe forms of PFIC with low serum levels of gamma-glutamyltranspeptide (γGTP), specific mutations within each gene have also been associated with milder clinical phenotypes.[15, 17, 24–30] Equally notable is the phenotypic pleomorphism of mutations in *ABCB4*, which ranges from high γGTP-PFIC (or PFIC3), to intrahepatic cholestasis of pregnancy, and gallstone formation,[19, 25, 31–36] and an array of mutations in *JAG1* in subjects with liver and/or non-hepatic malformations (e.g.: cardiovascular and renal defects).[4, 7, 8, 23, 37–39] In view of such variability in clinical presentation, the ability to analyze the nucleotide sequence facilitates studies of phenotype-genotype relationship for individual genes. In addition, we chose to add *SERPINA1* to the chip as an investigative tool for studies examining how α1AT polymorphisms may serve as disease modifiers in subjects harboring mutations in one of the other cholestatic genes, or in subjects with other forms of liver disease in a fashion described for patients with chronic pulmonary diseases.[40–42]

In summary, we customized a state-of-the-art resequencing technology to read a comprehensive nucleotide sequence of the five genes responsible for the most common forms of inherited cholestasis. The combined large size of these genes and the lack of hot spots for disease-causing mutations make it time-consuming and costly to use genetic testing by standard sequencing methodologies in the evaluation of patients with cholestasis. These limitations may be overcome by the resequencing tool here reported, and named *Jaundice Chip*. The immediate patient population that may benefit from the chip consists of children with chronic cholestasis, in whom current diagnostic algorithms are aimed at excluding known diseases and frequently include: 1) extensive biochemical evaluation of blood and urine, 2) radiological studies of the liver and extra-hepatic systems, and 3) liver biopsy for histological examination and electron microscopy. An additional application of the chip is as an investigative tool to study the role of synonymous and non-synonymous polymorphisms of individual genes, or of heterozygous polymorphisms in functionally related genes, in phenotype determination of children and adults with chronic cholestasis, intrahepatic cholestasis of pregnancy, and gallstone formation associated with mutations in *ABCB4*.[43, 44] One specific disease that may benefit from such investigative approach is biliary atresia, in which the systematic analysis of all five genes may provide insight into the potential role of genetic variations in long-term survival with the native liver. For all of these settings, the current version of the *Jaundice Chip* offers a real opportunity to translate laboratory discoveries into a tool that may simplify the diagnostic algorithm at the

bedside, and facilitate the design of treatment protocols that take into account the genetic makeup of the patient.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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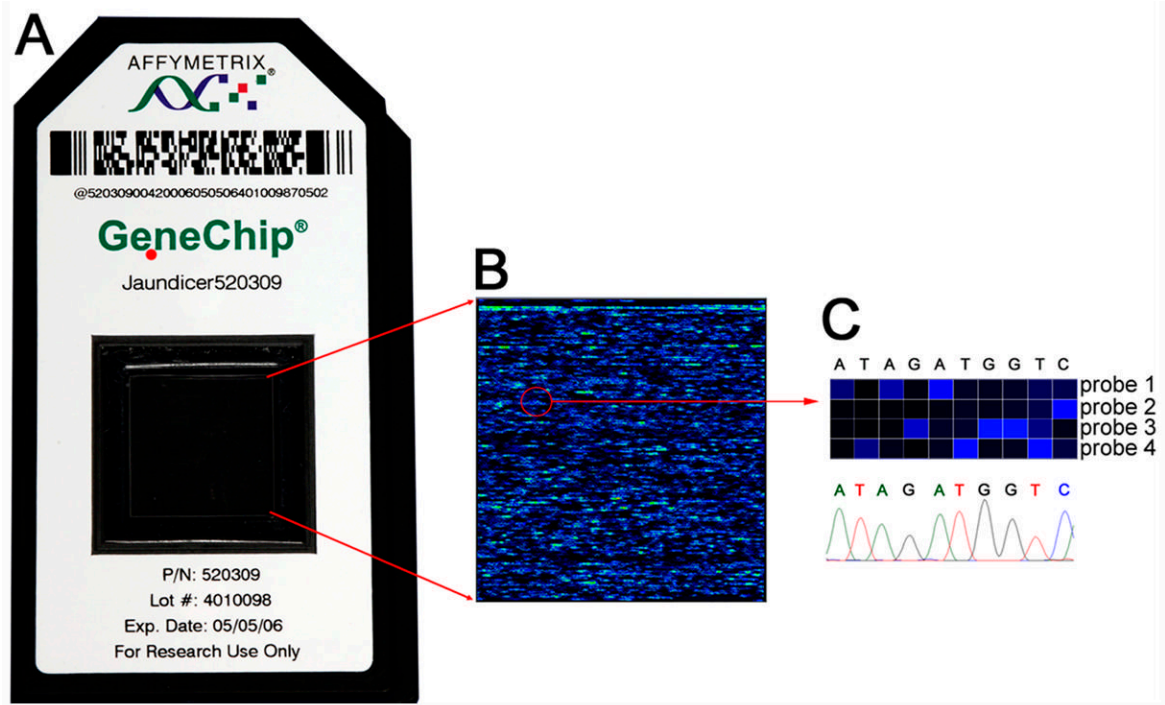
Figure 1. Sequence readout by the resequencing "Jaundice Chip"
Panel **A** displays the hybridization cartridge assembled by Affymetrix, Inc., which contains a solid support featuring 25-mer probes designed to sequence 26,725 nucleotides (bases) of *SERPINA1, JAG1, ATP8B1, ABCB11,* and *ABCB4*. Panel **B** shows signals obtained from a high-resolution scanning after hybridization of a chip with amplicons from a subject. In the top portion of panel **C**, magnification of a region of the chip shows that the brightest signal for each set of four 25-mer probes for each column identifies a base. In the lower panel, identical base calls were made by standard capillary sequencing methodology.
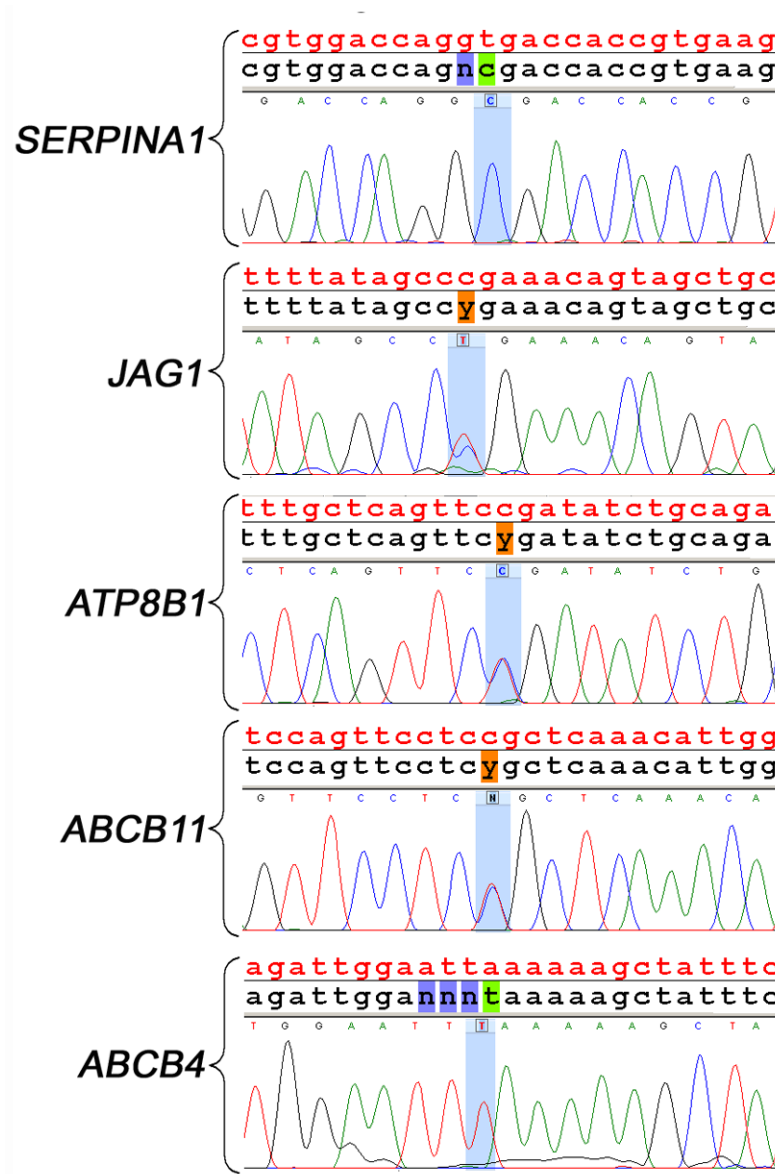
Figure 2. Comparison of nucleotide calls made by the resequencing chip and by standard capillary sequencing

The top panel depicts the reference nucleotide sequence for *SERPINA1* in red, the chip readout in black, and nucleotides identified by capillary sequencing. The T>C homozygous mutation identified by the chip (shaded in green) is reproduced by capillary sequencing. The same sequencing organization is presented for the other genes in the four lower panels. In *JAG1, ATP8B1,* and *ABCB11*, the chip produced heterozygous C/T calls (shown as a typical "y" symbol shaded in orange) that could be identified by red and blue peaks produced by capillary sequencing. In the lower panel, a homozygous A>T mutation in *ABCB4* was identified by both methods. The mutations presented in this figure reflect the mutations described in patients #1,4,8,11 and 13 of Table 2.

Table 1

Performance data for the *Jaundice Chip* with accuracy examined by direct comparison with automated capillary sequencing.

Automated call rate	Mean: 93.5%
	Range: 90.3–95.7%
Number of bases called by both methods	40,216
Number of errors by the resequencing chip	4 [*]
Call accuracy	99.99%

[*] All four errors were heterozygous calls

Table 2

Non-synonymous changes identified by the *Jaundice Chip* in subjects with inherited forms of intrahepatic cholestasis (subjects #1–13). Disease-causing mutations for subjects #1, 4, 8,11 and 13 were reproduced by standard capillary sequencing as shown in Figure 2. Also included are the changes found after the sequence analysis for all five genes in patients with biliary atresia (#14–18).

Patient #	Diagnosis[*]	Mutation
1	α1AT deficiency	*SERPINA1* T638C (Val213Ala, homozygous) and G1024A (Glu342Lys, homozygous)[45, 46],
2	α1AT deficiency	*SERPINA1*T638C (Val213Ala, homozygous) and G1024A (Glu342Lys, homozygous)[45, 46],
3	α1AT deficiency	*SERPINA1* T638C (Val213Ala, homozygous) and G1024A (Glu342Lys, homozygous)[45, 46],
4	Alagille syndrome	*JAG1* C2230T (Arg744stop, heterozygous)[47]
5	Alagille syndrome	*JAG1* IVS19 +1 G to A, heterozygous [48]
6	Alagille syndrome	*JAG1* C2650T (Glu884Stop, heterozygous) [**]
7	Alagille syndrome	*JAG1* C2650T (Glu884Stop, heterozygous) [**]
8	PFIC1	*ATP8B1* C2788T (Arg930stop, heterozygous)[28]
9	PFIC1	*ATP8B1* T1982C (Ile661Thr, heterozygous) [15]
10	PFIC1	*ATP8B1* 569 bp deletion (including first 17 bp in exon 23, homozygous)[**]
11	PFIC2	*ABCB11* C3457T (Arg1153Cys, heterozygous)[17]
12	PFIC2	*ABCB11* C2782T (Arg928Stop, heterozygous)[**]
13	PFIC3	*ABCB4* A1954G (Arg652Gly, homozygous)[49]
14	Biliary atresia	*ATP8B1* IVS 26 +8 G to T, heterozygous [**]
15	Biliary atresia	No non-synonymous polymorphism
16	Biliary atresia	No non-synonymous polymorphism
17	Biliary atresia	*JAG1* C2612G (Pro871Arg, heterozygous) [50]
18	Biliary atresia	*SERPINA1* G302A (Arg101His, heterozygous) [51]

[*] Clinical criteria according to references [1] and [52];

[**] Not previously reported

Table 3

Relationship between the number and type of mutations reported for each gene and the number of regions that consistently failed to generate signals ("no calls").

Type of mutation	Number of mutations	Number of mutations in "no call" regions
Single nucleotide changes		
Missense/nonsense	200	6
Single nucleotide polymorphism	119	4
Splicing	41	1
Total number of mutations	*360*	*11*[*]
Insertion and deletion[**]		
Insertion	50	1 (?)
Insertion/deletion	5	0
Small deletion	86	4 (?)
Large deletion (>20 nucleotides)	6	0
Duplication	2	0

[*] 6 mutations in *SERPINA1*, 4 mutations in *JAG1*, and 1 mutation in *ABCB11*

[**] The resequencing technology may not reliably identify insertion, deletion, or duplication. Although most of these mutations reside in regions of the gene fully sequenced by the chip, the ability to identify these mutations requires further validation.