Molecular profiling of intrahepatic and extrahepatic cholangiocarcinoma using next generation sequencing

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A B S T R A C T

Cholangiocarcinoma is a heterogeneous malignant process, which is further classified into intrahepatic cholangiocarcinoma (ICC) and extrahepatic cholangiocarcinoma (ECC). The poor prognosis of the disease is partly due to the lack of understanding of the disease mechanism. Multiple gene alterations identified by various molecular techniques have been described recently. As a result, multiple targeted therapies for ICC and ECC are being developed. In this study, we identified and compared somatic mutations in ICC and ECC patients using next generation sequencing (NGS) (Ampliseq Cancer Hotspot Panel v2 and Ion Torrent 318v2 chips). Eleven of 16 samples passed internal quality control established for NGS testing. ICC cases (n = 3) showed IDH1 (33.3%) and NRAS (33.3%) mutations. Meanwhile, TP53 (75%), KRAS (50%), and BRAF (12.5%) mutations were identified in ECC cases (n = 8). Our study confirmed the molecular heterogeneity of ICC and ECC using NGS. This information will be important for individual patients as targeted therapies for ICC and ECC become available in the future.

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1. Background

Cholangiocarcinoma is a malignant process which arises from the bile duct epithelial cells. It may originate within the liver as an intrahepatic cholangiocarcinoma (ICC) or involve large hilar bile ducts and extrahepatic biliary tree as an extrahepatic cholangiocarcinoma (ECC) or bile duct carcinoma. Both types of cholangiocarcinoma are biologically distinctive as they have different risk factors, genetic mutations, expression profiling, and clinical outcomes (Sempoux et al., 2011). The overall incidence and mortality rates of cholangiocarcinoma have been increasing over the past few decades. It has been reported that the occurrence of ICC has increased in the United States, while ECC has declined or remained stable. A recent study revealed that the increasing rate of ICC was partly due to the misclassification of Klatskin/perihilar tumors as intrahepatic instead of extrahepatic tumors (Khan et al., 2012).

The established risk factors for cholangiocarcinoma include primary sclerosing cholangitis, parasitic biliary infection (Opisthorchis viverrini in Thailand and Laos; Clonorchis sinensis in Southwest China), choledochal cysts, Caroli’s disease, and toxins. Furthermore, patients with chronic hepatitis C infection and hepatolithiasis are at risk for ICC, while pancreaticobiliary maljunction with bile duct dilatation, cholelithiasis, and cholecystectomy are mainly risk factors for ECC (Cardinale et al., 2010). The majority of cholangiocarcinoma cases occur sporadically despite the well-established risk factors.

Surgery is the only curative option for early-stage ICC and ECC patients. ICC patients undergo either segmental or lobe resection, while pancreaticoduodenectomy is the mainstay treatment for resectable ECC cases. Unfortunately, most cholangiocarcinoma patients present with advanced and unresectable disease. Moreover, local recurrence and distant metastasis are frequently seen after the surgical resection.

The disease prognosis varies; hilar cholangiocarcinoma is associated with slightly better prognosis even in the locally advanced disease setting with liver transplantation, while suboptimal outcome is seen in ICC patients with similar treatment (Churi et al., 2014). Interestingly, distal ECC shows a similar clinical course with pancreatic adenocarcinoma. The 5-year survival rates for localized ICC and ECC are 12% and 30%, respectively. Meanwhile, ICC and ECC patients who develop metastatic disease have a 5-year survival rate of 2% (http://www.cancer.org/acs/groups/cid/documents/webcontent/003084-pdf.pdf).
Limited understanding of the pathogenesis is partly responsible for the overall low survival rates in both ICC and ECC. Available palliative chemotherapy has not improved the outcome of these patients and no molecular targeted agents have been approved for cholangiocarcinoma treatment. Recent studies have described the utility of different molecular techniques in the identification of gene alteration in this entity (Churi et al., 2014; Jiao et al., 2013; Miller et al., 2009; Ross et al., 2014; Sia et al., 2013, 2015; Turaga et al., 2013). The long-term objective of these studies is to find actionable genes which may improve the management and outcome of ICC and ECC patients.

Next generation sequencing (NGS) is an affordable technology which consolidates a broad range of molecular oncology testing into a single platform and single assay (Tsongalis et al., 2014). In the era of personalized medicine, NGS plays an important role in identifying mutations which may predict the prognosis or alter the management for cancer patients. In this study, we identified and compared somatic mutations in ICC and ECC patients using NGS. Recent advances and molecular insights on cholangiocarcinoma will also be discussed.

2. Materials and methods

2.1. Case selection

Sixteen patients with ICC (n = 8) and ECC (n = 8) who underwent biopsy and/or resection at Dartmouth-Hitchcock Medical Center (DHMC) from 2005–2013 were selected for our study. The histologic slides (hematoxylin and eosin-stained slides) were retrieved and the diagnosis for each case was confirmed by 2 pathologists (J.P. and A.A.S.). Histologic characteristics, demographic and clinical information for each patient were recorded. This study was approved by the Committee for the Protection of Human Subjects at Dartmouth College.

2.2. Sample collection and DNA extraction

The appropriate formalin-fixed paraffin-embedded (FFPE) tissue block was selected for each case. Sixteen unstained FFPE tissue sections of 5 μm each were obtained. One case was excluded because of exhausted tissue in the paraffin-block. The lesional area and the percent tumor cell content for each case were identified and assessed by a pathologist (J.P.).

Before DNA extraction, all samples were evaluated to ensure each case contained a minimum of 10% tumor content, previously established during the validation process (Reitman and Yan, 2010). Two samples were excluded from the study because of their low tumor content. Unstained slides from 13 cases were deparaffinized and assessed by a pathologist (J.P.).

2.3. Next generation sequencing and data analysis

Next generation sequencing was performed using the Ion AmpliSeq™ Cancer Hotspot Panel v2 which consists of 50 oncogenes and tumor suppressor genes (Table 1), covering approximately 2800 Catalogue of Somatic Mutations in Cancer (COSMIC) mutations. In 2013, the molecular pathology laboratory at DHMC validated (Tsongalis et al., 2014) and incorporated sequencing as a routine clinical test for somatic mutational screening in patients with metastatic carcinoma. Since 2013, the laboratory has received over 1,100 FFPE clinical samples, including non-small cell lung carcinomas (NSCLC), colon adenocarcinomas, gliomas/glioblastomas, melanomas, breast carcinomas, and samples consisting of other tumor types (sarcomas, uterus, kidney, pancreas), as well as almost 500 FFPE samples for research projects.

Barcoded libraries were prepared using at least 10 ng of gDNA. They were quantified using the Ion Library Quantitation qPCR Kit (Life Technologies) and combined to a final concentration of 100 pM each. Two samples failed the qPCR minimum threshold due to a lack of amplification (< 10 pM each). Eleven samples were sequenced on the Ion Torrent Personal Genome Machine (PGM™) using Ion 318™ chips.

Sequencing reads were aligned to hg19, and variant calling was performed using Torrent Suite (v4.0.2) and the Variant Caller Plugin (v4.0). Variant annotation and functional predictions were performed using Golden Helix’s SNP and Variation Suite Software SVS (v.8.2.1). Some variants were also manually interrogated using the Broad Institute’s Integrative Genomics Viewer (IGV).

In order to ensure the overall quality of the test, post-sequencing QC metrics were incorporated into the data analysis workflow. These metrics included chip loading efficiency, total usable sequences, percent low-quality reads, percent of aligned reads, percent of aligned bases, on target reads, and coverage uniformity. Sequencing runs and/or samples that did not pass one of the QC metrics above were not included in this study. Also, only variants detected at more than 5.0% allelic frequency, and covered at more than 500× were reported (cutoffs determined during validation process) (Tsongalis et al., 2014).

3. Results

Eleven patients were analyzed after passing the internal quality control established in-house for NGS testing. These patients included 8 patients with ECC and 3 patients with ICC. Nine of the samples used in the sequencing were derived from biopsy/resection specimens (3 ICC cases and 6 ECC cases) and the remaining 2 samples were from fine-needle aspirates (2 ECC cases).

3.1. Histological morphology of ICC and ECC

Histological examination showed poorly-differentiated lesions in all ICC (100%) and ECC cases (100%). Fig. 1 showed a hematoxylin and eosin (H&E) stained image of ICC. The lesion was comprised of dense poorly-formed glands with desmoplastic stroma in the background. The nuclei were pleomorphic and mitotic activities were noted. An example of ECC was shown in Fig. 2. Multiple angulated, mucin-producing glands were seen with desmoplastic stroma and striking irregular and hyperchromatic nuclei. Immunohistochemical studies were performed in challenging cases to distinguish these lesions from the common differential diagnosis, such as hepatocellular carcinoma and metastatic adenocarcinomas.

<table>
<thead>
<tr>
<th>No</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Tumor grade</th>
<th>Mutation</th>
<th>Current status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>85</td>
<td>Poorly differentiated</td>
<td>NRAS (c.35G&gt;T, p.G12V)</td>
<td>Deceased</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>80</td>
<td>Poorly differentiated</td>
<td>wt*</td>
<td>Deceased</td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>56</td>
<td>Poorly differentiated</td>
<td>IDH1 (c.395G&gt;T, p.R132L)</td>
<td>Deceased</td>
</tr>
</tbody>
</table>

wt = wild type.
3.3. Somatic mutations in ECC patients

The predominant ECC patients were male patients (M:F = 3:1) with an average age of 72.6 ± 8.5 years. Tumor protein p53 (TP53) was the most frequent mutated gene in these patients (75%). Other mutated genes identified in ECC included Kirsten rat sarcoma viral oncogene homolog (KRAS) (50%) and v-raf murine sarcoma viral oncogene homolog B (BRAF) (12.5%). Two of the eight patients (25%) were wild-type (wt) for the gene sequences present in the AmpliSeq Hotspot Cancer Panel v2. In addition, potential benign polymorphisms were identified in these patients. The demographic information, histologic grade, clinical follow-up, and the mutated genes in patients with ECC are shown in Table 3.

3.4. Clinical follow-up

Most of the patients in the study (83.3%) were deceased at the time of writing. All 3 ICC patients (100%) were deceased. Only 1 of the ECC patients survived (12.5%), while 6 patients were deceased (75%) and 1 patient was lost to follow-up (12.5%). The causes of death and time interval between the diagnosis and death were not analyzed since most of this information was not available.

4. Discussion

Recent technical advances in molecular analysis with reduced costs of sequencing have resulted in a paradigm shift of cancer management (Churi et al., 2014). A series of studies using traditional and advanced technologies have described a variety of gene mutations and genomic alterations in cholangiocarcinoma, particularly ICC, to further explore the disease mechanism and to develop potential targeted therapies (Ross et al., 2014).

Comparative genomic hybridization and gene expression data has shown an overlap in pathogenic mechanism for all subsets of cholangiocarcinoma; however, significant diversity of mutational findings between individual patients is also apparent (Miller et al., 2009). Here we demonstrate the utility of NGS in detecting somatic mutations for both ICC and ECC patients. Using a 50-gene panel, we identified IDH1 and NRAS gene alterations in patients with ICC and mutated KRAS, TP53, and BRAF genes in ECC cases.

IDH1 is one of the most commonly altered genes in ICC patients (Ross et al., 2014). It is identified in 10–20% of ICC and the mutation is not usually seen in ECC patients (Borger et al., 2012; Wang et al., 2013; Ward et al., 2010). Using DNA sequencing of hybridization-captured libraries in 28 ICC cases, Ross et al. reported that altered IDH1/2 alteration was identified in 36% of the cases, similar prevalence to AT rich interactive domain 1 A (ARID1A) (36%), TP53 (36%), and myeloid cell leukemia 1 (MCL1) (21%) alterations (Ross et al., 2014).

Similar to one of our ICC patients, the majority (99%) of somatic mutations in IDH1 is found at codon R132, which is functionally conserved and aligns with R172 of IDH2 (Rohle et al., 2013). A heterozygous mutation at IDH1 codon R132 will cause defective IDH1 enzyme activity which results in a decrease of cellular antioxidant activities (Reitman and Yan, 2010). The mutant enzyme induces the reduction of a-ketoglutarate to 2-hydroxyglutarate, with the conversion of NADPH to NADP+ (Rohle et al., 2013). Accumulation of this potential oncometabolite, 2-hydroxyglutarate, has been demonstrated in cancers with IDH mutation. In addition, increased levels of p53 and DNA hypermethylation are seen in IDH1-mutant tumors (Wang et al., 2013).

IDH1 mutations are associated with poorly differentiated tumors with clear cell changes histologically (Kipp et al., 2012). However, IDH1 genetic alteration has not shown any prognostic significance (Churi et al., 2014). Preclinical evidence suggests that targeted therapy is potentially applicable for IDH1/2-mutant cancers. In gliomas, AG1–5198, a selective IDH1 inhibitor, blocks the 2-hydroxyglutarate production in a dose-dependent manner and stunted the growth of IDH1-
Table 3
Characteristics and mutation in extrahepatic cholangiocarcinoma patients.

<table>
<thead>
<tr>
<th>No</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Tumor grade</th>
<th>Mutation</th>
<th>Current status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Female</td>
<td>74</td>
<td>Poorly differentiated</td>
<td>KRAS (c.35G&gt;T, p.G12V), TPS3 (c.428G&gt;A, p.E143V and c.224C&gt;T, p.P75L)</td>
<td>Deceased</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>57</td>
<td>Poorly differentiated</td>
<td>KRAS (c.35G&gt;A, p.G12D)</td>
<td>Deceased</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>64</td>
<td>Poorly differentiated</td>
<td>TP53 (c.818G&gt;A, p.R273H)</td>
<td>Deceased</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>78</td>
<td>Poorly differentiated</td>
<td>TP53 (c.844C&gt;T, p.R282V)</td>
<td>Deceased</td>
</tr>
<tr>
<td>5</td>
<td>Female</td>
<td>73</td>
<td>Poorly differentiated</td>
<td>TP53 (c.200G&gt;T, p.G66V)</td>
<td>Unknown</td>
</tr>
<tr>
<td>6</td>
<td>Male</td>
<td>73</td>
<td>Poorly differentiated</td>
<td>Benign polymorphism</td>
<td>Deceased</td>
</tr>
<tr>
<td>7</td>
<td>Male</td>
<td>74</td>
<td>Poorly differentiated</td>
<td>wt*</td>
<td>Deceased</td>
</tr>
<tr>
<td>8</td>
<td>Male</td>
<td>88</td>
<td>Poorly differentiated</td>
<td>BRAF (c.1742A&gt;C, p.N581T)</td>
<td>Deceased</td>
</tr>
<tr>
<td>9</td>
<td>Male</td>
<td>88</td>
<td>Poorly differentiated</td>
<td>TP53 (c.657C&gt;T, p.R211)</td>
<td>Deceased</td>
</tr>
<tr>
<td>10</td>
<td>Male</td>
<td>88</td>
<td>Poorly differentiated</td>
<td>KRAS (c.35G&gt;A, p.G12D)</td>
<td>Living</td>
</tr>
<tr>
<td>11</td>
<td>Female</td>
<td>78</td>
<td>Poorly differentiated</td>
<td>TP53 (c.658T&gt;A, p.Y220N)</td>
<td>Living</td>
</tr>
</tbody>
</table>

wt = wild type.

mutant tumor cells sparing the IDHI wild-type cells (Rohle et al., 2013). Currently, IDHI inhibitors are being investigated in clinical trials. AG-120, a specific IDHI inhibitor, was reported by Rizvi et al. to show a clinical benefit in 70% of phase-1 trial patients with IDHI-mutant associated solid tumors including cholangiocarcinoma (Rizvi et al., 2014).

NRAS mutation is less frequently identified in ICC cases, with a prevalence of 3% in recent studies (Jiao et al., 2013; Zhu et al., 2014). Due to the low frequency, no information on clinicopathological correlation is available for tumors with this particular gene mutation and thus it would be considered a variant of unknown clinical significance in this tumor type.

Mutation of KRAS and loss-of-function mutations of TPS3 are frequently seen in both ICC and ECC (Churi et al., 2014). TPS3 mutations were reported in 21% of cholangiocarcinoma cases in a review of studies with 229 patients (Khan et al., 2005). A direct DNA sequencing analysis of 69 cholangiocarcinomas identified KRAS and BRAF mutations in 22% and 45% of the tumors (Tannapfel et al., 2003). Both KRAS and BRAF are members of mitogen-activated protein kinase/extracellular-signal-regulated kinases (MAPK/ERK) pathway which mediate cellular response to growth signals. Neither the status of KRAS, BRAF, or both alterations influenced the survival of patients with cholangiocarcinoma (Tannapfel et al., 2003). The strategy for KRAS-mutated tumor targeted therapy has focused on the downstream effector pathways of KRAS. Phase II studies of a MAPK/ERK kinase (MEK) inhibitor in biliary tract cancer cases that included cholangiocarcinomas have been promising. Furthermore, a direct KRAS inhibitor may be useful when it becomes available in the future (Rizvi et al., 2014).

Sia et al. recently proposed a molecular classification of ICC into proliferative and inflammatory-type ICC based on high-throughput genomic data (Sia et al., 2013). Proliferation-type ICC, the more common type which usually shows aggressive clinical behavior and similar genomic profile to poor-prognosis hepatocellular carcinoma, is characterized by induction of cellular signal in cell cycle progression and proliferation. Meanwhile, an enriched inflammation-related pathway, mainly interleukin (IL)-10/-6 and signal transducer and activator of transcription 3 (STAT3), is the characteristic of inflammatory-type ICC (Sia et al., 2013).

Inactivating mutations of multiple chromatin-remodeling genes have also been reported in ICC. Through exome sequencing of 32 ICCs, Jiao et al. reported 1,259 somatic mutations in 1,128 genes; Frequent inactivating mutations in multiple chromatin-remodeling genes (BRCA-associated protein 1 (BAP1), ARID1A, and polybromo-1 (PBRM1)) and mutation in one of these genes occurred in 47% of the carcinomas sequenced (Jiao et al., 2013). Subjects with a mutation in any one of these genes trended toward worse survival, although no significant differences were seen in the survival time. Histone deacetylase inhibitors, which target chromatin remodeling genes, have been proposed as potential targeted therapies (Jiao et al., 2013).

A novel recurrent oncogenic fusion, fibroblast growth factor receptor 2 (FGFR2) — periphilin1 (PPHLN1), gene and damaging mutations in the V-raf murine sarcoma 3611 viral oncogene homolog (ARAF) gene were recently described in 16% and 11% of 122 ICC cases (Sia et al., 2015). The transforming and oncogetic activity of the FGFR2-PPHLN1 fusion can potentially be inhibited by a selective FGFR2 inhibitor (Sia et al., 2015).

A recent study which utilized a high throughput mass spectrometry-based platform showed different mutational patterns between ICC cases that arise in normal liver and those which are associated with preexisting chronic liver disease (Jang et al., 2014). In the study, phosphatidylinositol-4,5-bisphosphonate 3-kinase, catalytic subunit alpha (PIK3CA), phosphatase and tensin homolog (PTEN), cyclin-dependent kinase inhibitor 2A (CDKN2A), and TP53 mutations were harbored exclusively in ICCs with normal background liver. Meanwhile, epidermal growth factor receptor (EGFR) mutation was seen more frequently in ICCs with chronic liver disease. Most of these EGFR mutations were located at exon 19, identical to deletions identified in non-small cell lung carcinomas.

V-erb-B2 avian erythroleukemia viral oncogene homolog 2 (ERBB2) was reported to be one of the common genes altered in ECC patients (Churi et al., 2014). A study from Churi et al. (2014) showed that ERBB2 mutations were seen in the kinase domain (V777L) and extracellular domain (S310F). However, immunohistochemistry for ERBB2 overexpression was negative in all of these cases.

Another study which utilized immunohistochemistry showed higher ERBB2 expression in 300 ECC cases (8.5%) compared to 106 ICC cases (0.9%). They also analyzed the expression of EGRF and vascular endothelial growth factor (VEGF) between these two groups. They reported that EGRF expression was associated with tumor progression and VEGF expression was involved in hematogenic metastasis (Yoshikawa et al., 2008). Some of these molecular studies have led to the development of sorafenib (multikinase inhibitor) as a treatment option for cholangiocarcinoma patients (El-khoueiry et al., 2012).

Our study confirms the molecular heterogeneity in ICC and ECC. Currently, no effective therapy is available for patients with unresectable tumors. The majority of patients do not survive in order to assess follow-up of the disease, independent of the somatic mutations identified. This finding is consistent with the reported 5-year survival rates of 12 and 30% in localized ICC and ECC and extremely low survival rates (2%) in advanced diseases. Therefore, there is an urgent need to characterize the molecular profile and to develop targeted therapies for these patients.

5. Conclusions

Many targeted therapies are being developed for ICC and ECC based on the findings of advanced molecular technologies. Our study supports the premise that NGS is an effective diagnostic tool to identify multiple somatic mutations in different genes simultaneously. NGS testing will be an important part of patient management strategies when targeted therapies for cholangiocarcinoma become available in the near future.
References


