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Molecular profiling of intrahepatic and extrahepatic cholangiocarcinoma using next generation sequencing

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ABSTRACT

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.

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).

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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 rehydrated using Xylene and graded ethanol washes, followed by water. Genomic DNA (gDNA) was obtained using the Gentra Pure Gene Kit (Qiagen), and quantified using the Quant-iT ™ PicoGreen® dsDNA Assay Kit (Invitrogen) according to the manufacturer's recommendations.

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 to the data analysis workflow. These metrics included chip loading efficiency, total usable sequences, percent lowquality 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 1
Characteristics and mutation in intrahepatic cholangiocarcinoma patients.

No	Sex	Age (years)	Tumor grade	Mutation	Current status
1	Male	85	Poorly differentiated	*NRAS* (c.35G>T, p.G12V)	Deceased
2	Male	80	Poorly differentiated	wt*	Deceased
3	Female	56	Poorly differentiated	*IDH-1* (c.395G>T, p.R132L)	Deceased

wt = wild type.