Translational Oncology
www.transonc.com

Volume 9 Number 3 June 2016 pp. 173–178 173

Genomic Alterations in Biliary Tract Cancer Using Targeted Sequencing[1]

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Abstract

Background: Biliary tract cancers (BTCs) are rare and heterogeneous group of tumors classified anatomically into intrahepatic and extrahepatic bile ducts and gallbladder adenocarcinomas. Patient-derived tumor cell (PDC) models with genome analysis can be a valuable platform to develop a method to overcome the clinical barrier on BTCs. *Material and Methods:* Between January 2012 and June 2015, 40 BTC patients' samples were collected. PDCs were isolated and cultured from surgical specimens, biopsy tissues, or malignant effusions including ascites and pleural fluid. Genome analysis using targeted panel sequencing as well as digital multiplexed gene analysis was applied to PDCs as well as primary tumors. *Results:* Extrahepatic cholangiocarcinoma ($N = 15$, 37.5%), intrahepatic cholangiocarcinoma ($N = 10$, 25.0%), gallbladder cancer ($N = 14$, 35.0%), and ampulla of Vater cancer ($N = 1$, 2.5%) were included. We identified 15 mutations with diverse genetic alterations in 19 cases of BTC from primary tumor specimens. The most common molecular alterations were in TP53 (8/19, 42.1%), including missense mutations such as C242Y, E285K, G112S, P19T, R148T, R248Q, and R273L. We also detected two NRAS mutations (G12C and Q61L), two KRAS mutations (G12A and G12S), two ERBB2 mutations (V777L and pM774delinsMA) and amplification, and three PIK3CA mutations (N345K, E545K, and E521K). PDC models were successfully established in 27 of 40 samples (67.5%), including 22/24 from body fluids (91.7%) and 5/16 from tissue specimens (31.3%). *Conclusions:* PDC models are promising tools for uncovering driver mutations and

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[1] This work was supported by a grant from the Korea Health Industry Development Institute funded by the Ministry of Health & Welfare, Republic of Korea (HI14C0072 and HI14C3418) and by the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (NRF-2013R1A1A2013441).
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Received 22 October 2015; Revised 12 January 2016; Accepted 19 January 2016

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1936-5233/16
http://dx.doi.org/10.1016/j.tranon.2016.01.007

identifying rational therapeutic strategies in BTC. Application of this model is expected to inform clinical trials of drugs for molecular-based targeted therapy.

Translational Oncology (2016) 9, 173–178

Introduction

Biliary tract cancers (BTCs) are a heterogeneous group of tumors that affect the intrahepatic and extrahepatic bile ducts and gallbladder [1]. BTCs are rare, but global incidence is rapidly increasing, with greater frequency in Asia than in Western countries [2,3]. BTCs have poor prognosis characterized by early lymph node and distal metastases [1]. Although the clinical features of BTCs vary by primary site, surgical resection is a preferred therapy for all subtypes and offers a potential cure [4,5]. Because BTCs frequently recur after surgery, radiation therapy has been suggested for localized disease [6]. Currently, however, there is no effective adjuvant systemic therapy to our knowledge [7]. In recurrent or metastatic disease, cytotoxic agents including 5-fluorouracil, gemcitabine, and platinum have demonstrated survival benefits over the best standard in supportive care but show only limited efficacies [8,9]. Recent studies revealed molecular aberrations associated with BTC carcinogenesis that may provide molecular targets for treatment [10–12]. However, because BTCs are diverse diseases, with different genetic alterations observed for different subtypes, establishing clinical trial models for targeted therapy is difficult [13]. In addition, tissue sampling from the biliary tract is challenging because of its anatomic location [14,15].

Recently, patient-derived tumor cell (PDC) models have been suggested as preclinical tools for genome-directed targeted therapy. PDCs are *in vitro* cell models generated from freshly resected patient tumors or malignant body fluids that can preserve the histologic and genomic features of primary tumor cells [16]. The time required to establish a PDC model is much shorter than that for a patient-derived xenograft [17]. Furthermore, PDC models can be applied to identify rational therapeutic options through drug sensitivity tests [16]. In this study, to overcome the clinical barrier for genetic profiling of BTCs, we established PDC models from body fluids or tumor tissues from BTC patients and examined genetic alterations using various sequencing methods.

Materials and Methods

Patient Consent and Study Inclusion

Between January 2012 and June 2015, 40 patients with BTC were enrolled in the SMC Oncology Biomarker study as previously described [16,18–20]. All patients were at least 18 years old with pathologically or cytologically confirmed BTC, which includes intrahepatic and extrahepatic cholangiocarcinoma, distal common bile duct cancer, gallbladder adenocarcinoma, and gallbladder neuroendocrine carcinoma. Tissue specimens were obtained by surgical resection or liver biopsy, and effusions were percutaneously drained for therapeutic purposes and analyzed after obtaining informed consent. All procedures were carried out according to guidelines from the Declaration of Helsinki. The Institutional Review Board at the Samsung Medical Center approved the protocol.

Primary Cultures of Tumor Specimens

For malignant effusions, collected effusions (1 to 5 l) were divided into 50-ml tubes, centrifuged at 1500 rpm for 10 minutes, and washed twice with PBS. For surgical specimens, tumors were removed from surgical specimens then homogenized. Cell pellets were resuspended in culture medium and plated into 75-cm^2 culture flasks. Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco BRL, Paisley, UK) and 1% antibiotic-antimycotic solution (Gibco BRL). The medium was changed every 3 days, and cells were maintained at 37°C in a humidified 5% CO_2 incubator. PDCs were passaged using TrypLE Express (Gibco BRL) to detach cells when the cells reached 80% to 90% confluence.

Targeted Sequencing

Genomic DNA was extracted, and a SureSelect customized kit (Agilent Technologies, Santa Clara, CA) was used to capture 381 cancer-related genes. An Illumina HiSeq 2500 was used for sequencing with 100-bp paired-end reads. The sequencing reads were aligned to the human genome reference sequence (hg19) using BWA (v0.7.5) with the "MEM" algorithm. We used SAMTOOLS (v0.1.18) and Picard (v1.93) for sorting SAM/BAM files and duplicate marking, respectively. Local realignment and base recalibration by GATK (v3.1.1) were carried out based on dbSNP137, Mills indels, HapMap, and Omni. Single-nucleotide variations and insertions/deletions were identified using Mutect (v1.1.4) and Pindel (v0.2.4), respectively. ANNOVAR was used to annotate the detected variants. Only variants with >1% allele frequency were included in the results.

Ion AmpliSeq Cancer Panel v2

Adapters 1-96 Kit for the nonbarcoded adapter mix was supplied in the Ion AmpliSeq Library Kit. The ligated DNA underwent nick translation and amplification to complete the linkage between adapters and amplicons and to generate sufficient material for downstream template preparation. Two rounds of Agencourt AMPure XP Reagent binding at 0.6 and 1.2 bead-to-sample volume ratios removed input DNA and unincorporated primers from the amplicons. The final library molecules were 125,300 bp in size. We then transferred the libraries to the Ion OneTouch System for automated template preparation. Sequencing was performed on the Ion PGM sequencer according to the manufacturer's instructions. We used IonTorrent Software for automated data analysis. A new pipeline was designed for highly sensitive identification of single-nucleotide variations for passages 0, 1, and 2. Varscan2 SNP calling was performed with the following options: min-coverage, 50; min-var-freq, 0.01; and *P* value, .1. Variants around the insertions/deletions were filtered out. Variants were annotated using Oncotator. Detailed procedures are described in our previous report [21].

nCounter Copy Number Variation CodeSets

For detection of copy number variations, 300 ng purified genomic DNA extracted from PDCs was analyzed using nCounter Copy Number Variation CodeSets. DNA was fragmented by AluI digestion

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