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Mutations of candidate tumor suppressor genes at chromosome 3p in intrahepatic cholangiocarcinoma

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ABSTRACT

The genetic status of candidate tumor suppressor genes (TSGs) at chromosome 3p has not yet been elucidated in intrahepatic cholangiocarcinoma (iCCA). Herein, we retrospectively investigated 32 fresh iCCA case samples from a single medical institution to clarify mutations of 11 TSGs by next-generation sequencing. Validation of the mutations was performed on the MassARRAY platform or by high-resolution melting curve analysis. We then integrated the gene mutations into copy number alterations at chromosome 3p that had been generated in a previous study using the same fresh iCCA samples, and correlated the integration results with the clinicopathologic features. Nine of the 32 (28.1%) iCCA patients had gene mutations at chromosome 3p, totaling 11 mutations across five genes. Those included five (15.6%) *BAP1* mutations, two each (6.3%) of *CACNA2D3* and *RASSF1* mutations, and one each (3.1%) of *ATG7* and *PLCD1* mutations. Six (18.8%) cases had concurrent loss of chromosome 3p and gene mutations. Patients with TSG mutations had shorter disease-free and survival times than those without the mutations. Our data showed that iCCA patients with TSG mutations at chromosome 3p faced an adverse prognosis. *BAP1* was the common target of mutational inactivation and may be a principal driver of 3p21 losses.

1. Introduction

Loss of chromosome 3p is a known feature in many human cancers, and is associated with tumor progression and poor clinical outcomes (Zabarovsky et al., 2002). Cytogenetic and molecular studies have revealed the loss or altered expression of several candidate tumor suppressor genes (TSGs) at 3p in colon cancer (Tsai et al., 2011), lung cancer (Tai et al., 2006), esophageal squamous cell carcinoma (Qin et al., 2008), and renal cell carcinoma (Kroeger et al., 2013).

Inactivation of TSGs through loss of heterozygosity (LOH) is a common mechanism leading to the development of cancer. In intrahepatic cholangiocarcinoma (iCCA), mutation of the *P53* gene has been found in 28–77% of cases and is associated with low overall survival (Khan et al., 2006; Tannapfel et al., 2000b). The candidate TSGs at 9p21 ($p16^{INK4a}$/$p14^{ARF}$ and $p15^{INK4b}$) are involved in 37–83% of iCCA by LOH or promoter hypermethylation (Momoi et al., 2001; Tannapfel et al., 2000a; Yang et al., 2005).

Chromosome 3p loss, with a focus on the Ras association domain family member 1 (*RASSF1A*) and fragile histidine triad (*FHIT*) genes, has been studied in a few iCCA cases. Wong et al. (2002) reported 40% of 13 iCCA cases with 3p loss and 69% with a methylated *RASSF1A* promoter region. *FHIT* was shown to have losses of exons 5 and 6, and hypermethylation of the promoter in 19 iCCA cases (Foja et al., 2005). The BRCA1-associated protein 1 (*BAP1*) gene is another candidate TSG reported in iCCA, with a wide variation of mutation frequency (9.1–25.3%) (Churi et al., 2014; Jiao et al., 2013; Simbolo et al., 2014).

Previously, we had conducted a genome-wide single nucleotide polymorphism (SNP) array study using 32 fresh iCCA samples and found a high frequency of recurrent 3p losses associated with larger tumor size, high histologic grade, and vascular invasion (Huang et al., 2014). However, the genetic status of candidate TSGs at 3p was not explored. Unlike oncogenes, TSGs require two mutational event "hits"

Abbreviations: VHL, von Hippel-Lindau tumor suppressor; PPARG, peroxisome proliferator-activated receptor gamma; PLCD1, phospholipase C delta 1; RASSF1, Ras association domain family member 1; BAP1, BRCA1-associated protein 1; HYAC1, hyaluronoglucosaminidase 1; ACY1, aminoacylase 1; FHIT, fragile histidine triad; FOXP1, forkhead box P1; ATG7, autophagy-related 7; CACNA2D3, calcium voltage-gated channel auxiliary subunit alpha2delta 3

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for carcinogenesis. Further mapping of genetic alterations on chromosome 3p may help delineate the molecular features of candidate TSGs and their clinical significance in iCCA. In the present study, we aimed to examine mutations of TSGs at 3p and whether such aberrations correlate with LOH. To this end, we first used the same cohort of 32 fresh iCCA samples to detect mutations of 11 TSGs by next-generation sequencing (NGS). Validation of the mutations was performed on the MassARRAY platform or by high-resolution melting (HRM) curve analysis. Furthermore, the mutations of target genes and LOH were integrated to correlate with clinicopathologic features.

2. Materials and methods

2.1. Intrahepatic cholangiocarcinoma cases

The study was approved by the Ethics Committee of Chang Gung Memorial Hospital, Kaohsiung, Taiwan (Approval No. 103-6997B). We enrolled the same 32 fresh cases of iCCA as used in our previous study (Huang et al., 2014) for this current NGS analysis. The American Joint Committee on Cancer (7th edition) staging system was adopted for the staging of iCCA.

2.2. DNA preparation

The fresh liver samples were assayed using an Ion Torrent Next-Generation Sequencing kit (Thermo Fisher Scientific Inc). For fresh frozen tissue, DNA was extracted using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quantity and integrity of the genomic DNA were determined using the Qubit (Invitrogen, Carlsbad, CA, USA) and Fragment Analyzer (Advanced Analytical Technologies, Inc., Ankeny, IA, USA) systems, respectively.

2.3. Customized target panel sequencing

An Ion Torrent adapter–ligation library was made, following the manufacturer's protocol (Life Technologies, Camarillo, CA, USA). In brief, 20 ng of genomic DNA was amplified using customized panels of two pools of 193 primer pairs (designed by AmpliSeq.com; Thermo Fisher Scientific Inc., Waltham, MA, USA) to target all coding exons of 11 genes; namely, von Hippel-Lindau tumor suppressor (*VHL*), peroxisome proliferator-activated receptor gamma (*PPARG*), phospholipase C delta 1 (*PLCD1*), *RASSF1*, *BAP1*, hyaluronoglucosaminidase 1 (*HYAL1*), aminoacylase 1 (*ACY1*), *FHIT*, forkhead box P1 (*FOXP1*), autophagy-related 7 (*ATG7*), and calcium voltage-gated channel auxiliary subunit alpha2delta 3 (*CACNA2D3*). The amplicons were ligated with barcoded adapters using the Ion Amplicon Library Kit (Thermo Fisher Scientific Inc.). The barcoded libraries were subsequently conjugated with sequencing beads by emulsion PCR and enriched using Ion Chef (Thermo Fisher Scientific Inc.) according to the Ion Torrent protocol (Thermo Fisher Scientific Inc). The quality and quantity of the amplified library were determined using the Fragment Analyzer and Qubit systems, respectively. Sequencing was performed on an Ion Proton sequencer using an Ion PI chip (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol.

2.4. Bioinformatic analysis

Ion Torrent platform-specific pipeline software (Torrent Suite version 4.2; Life Technologies Corporation, Carlsbad, CA, USA) was used to generate the sequence alignment with the hg19 human genome reference. The coverage depth was calculated using the Torrent Coverage Analysis plug-in. Single nucleotide variants and short insertion/deletions were identified using the Torrent Variant Caller plug-in (version 4.4). The Variant Effect Predictor toolset was used to annotate every variant in the COSMIC (version 70), dbSNP 138, and 1000 Genome Project (phase 1) databases. A variant coverage of < 50 or variant frequency of $< 5\%$ was filtered out. The subset of candidate mutations was validated using Ion Reporter software (version 4.0; Life Technologies Corporation) based on published biological evidence. Variants were reported according to Human Genome Variation Society nomenclature and classified into three categories: pathogenic, variants of uncertain significance, and benign. All potentially causative mutations were further investigated with MassARRAY or by HRM curve analysis, using standard protocols.

2.5. Target mutation site validation using MassARRAY

The mutation analysis was performed using the MassARRAY platform with iPLEX gold chemistry (Agena, San Diego, CA, USA). The assay was designed using MassARRAY Assay Design 4.0 software. The PCR primer sequences and extension primer sequences used are listed in Supplementary Tables S1 and S2, respectively. The multiplex PCR was performed in a 5-μL volume containing 10 ng/μL of genomic DNA, 0.2 units of Taq DNA polymerase, 2.5 pmol of each PCR primer, and 25 mM of dNTPs (PCR Accessory and Enzyme Kit; Agena). The thermal cycling conditions for the PCR were as follows: 4 min at 94 °C; followed by 45 cycles of 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 1 min. The program was terminated after a final incubation at 72 °C for 3 min. After completion of the PCR, unincorporated dNTPs were deactivated using 0.3 U of shrimp alkaline phosphatase. The single-base primer extension reaction was then performed using the iPLEX mass terminator mixture, thermo sequenase, and extension primer mix. The thermal cycling program for the reaction included an initial denaturation for 30 s at 94 °C, followed by 40 cycles at 94 °C for 5 s, with five nested cycles of 52 °C for 5 s and 80 °C for 5 s. The single-base extension was completed with a final incubation at 72 °C for 3 min (iPLEX Pro Kit; Agena). The samples were then cooled to 4 °C, following which the reaction products were desalted by cation-exchange resin chromatography. Seven microliters of the purified primer extension reaction product was loaded onto a matrix pad of a SpectroCHIP (Agena), which was then processed and analyzed using MassARRAY Analyzer 4. The allele calling and frequency were acquired with MassARRAY Typer 4.0 software (Agena).

2.6. Target mutation site validation by high-resolution melting curve analysis

Real-time PCR and HRM curve analysis were performed on a StepOnePlus™ Real Time PCR system (Applied Biosystems, Foster City, CA, USA) to amplify the target region (Supplementary Table S3). The target was amplified in a 10-μL reaction volume containing 20 ng of template DNA, 200 nM of specific primer pair, and 2 × HRM qPCR Fast Master Mix Reagent (Topgen Biotechnology Co., Ltd., Taiwan). The HRM curve was analyzed with ThermoFisher Cloud HRM software (Thermo Fisher Scientific).

3. Results

3.1. Ion AmpliSeq sequencing of human iCCA

Genomic DNA from 32 iCCA patients was sequenced using the NGS approach to identify mutations in 737 loci of 11 TSGs. Sequencing via the Ion Proton sequencer generated a total of 38,798,026 mapped reads (Supplementary Table S4). Over 7 Gb of bases were detected, with an average read length of 200 bp (designed amplicon size: 125–275 bp), and 96% of the total bases were aligned to the human complete genome (hg19). With normalization to 500,000 reads per specimen, an average coverage of $> 2500\times$ was achieved, with 99% of the target bases covered 500 times. A minimum coverage of $100\times$ was obtained in all cases. An average of $0.2\times$ coverage uniformity accounted for 97%.