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

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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

and denatured at 95°C. Fragmented DNA was hybridized with the codeset of 86 genes in the nCounter Cancer CN Assay Kit (Nanostring Technologies, Seattle, WA) for 18 hours at 65°C and processed according to the manufacturer's instructions. An nCounter Digital Analyzer was used to detect and tabulate the signals of the reporter probes. Average count numbers >3 were called and confirmed by immunohistochemistry (IHC), fluorescence *in situ* hybridization, or real-time polymerase chain reaction. Validation results for the nCounter assay were published previously [21].

Statistical Methods

Standard descriptive and analytical methods were used to describe the patient population and their baseline characteristics. *Progression-free survival* (PFS) was defined as the time from the date of surgery to the date of documented disease progression or death from any cause. Kaplan-Meier estimates were used to analyze the time-to-event variables, and the 95% confidence interval (CI) for the median time to event was computed. Comparisons of survival by univariate analysis were estimated by the log-rank test. Cox's proportional hazard model was used for multivariate analyses. $P < .05$ was considered statistically significant, and all P values correspond to two-sided significance tests. The statistical data were obtained using SPSS software version 18 (SPSS Inc., Chicago, IL)

Results

Clinical Characteristics of BTC Patients

From January 2012 to June 2015, 40 BTC patient samples were collected for this study (Table 1). 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. Twenty patients were initially diagnosed with stage IV disease, and 20 patients were stage I to III. At the time of analysis, however, 32 patients had recurrent or metastatic disease. In 24 patients, liquid biopsy samples were used as a source of cell culture to establish a PDC model (60.0%: 20 ascites [50.0%] and 4 pleural fluid [10.0%]); tissues from surgical resection (N = 8, 20.0%) or liver biopsy (N = 8, 20.0%) were also used for cell culture. The median CA 19-9 level at the time of PDC collection was 284 (range: 2.43 to >140,000). IHC for primary pathologic specimens was performed in 19 patients with variable markers including MUC1, MUC5AC, MUC6, and P53 (Table 2).

Table 1. Baseline Characteristics

Variables	Patients (N = 40)	%
Age (year)		
Median	61	
Range	31-78	
Gender		
Male	23	57.5%
Female	17	42.5%
Cancer types		
Extrahepatic cholangiocarcinoma	15	37.5%
Intrahepatic cholangiocarcinoma	10	25.0%
Gallbladder cancer/ampulla of Vater cancer	15	37.5%
Type of specimen		
Tissue by surgical resection	8	20.0%
Tissue by liver biopsy	8	20.0%
Ascites	20	50.0%
Pleural fluid	4	10.0%
Initial stage		
I-III	20	50.0%
IV	20	40.0%
Histologic grade		
Grade 1	4	10.0%
Grade 2	14	35.0%
Grade 3	16	40.0%
Grade 4	2	5.0%
Unknown	4	10.0%
Disease status at the time of analysis		
Recurrence and/or distant metastasis	32	80.0%
No evidence of disease	8	20.0%
CA 19-9 level at the time of PDC	N = 37	
Median	284	
Range	2.43->140,000	

Table 2. Results of IHC and Successful Rate of PDC Models

	Patients (N = 40)	
IHC of pathologic specimen	Total N = 19	0/1+/2+/3+
MUC1	N = 12	5/3/1/3
MUC5AC	N = 12	6/1/1/4
MUC6	N = 14	2/3/0/9
P53	N = 12	9/0/0/3
Successful rate of PDC models according to the type of specimen	27/40	67.5%
Body fluids (ascites or pleural fluid)	22/24	91.7%
Tissue by surgical resection	4/8	50.0%
Tissue by liver biopsy	1/8	12.5%

Genomic Analysis on BTC Tumor Specimens

We could successfully analyze the somatic mutation profiles in primary tumor tissues of 19 of 40 BTC patients because of the limited amount of tumor tissues or cells. The primary tumor site and the type of mutations included in the genome analysis were shown in Figure 1. We performed genomic profiling of primary tumor tissues from surgical resection or liver biopsy with various analytical methods. 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). In case 1, we identified CCND1 amplification, TP53 (C242Y), and CDKN2A (A128V) mutations as well. FGFR3 amplification with TP53 (E285K), ERBB2 (V777L), and PIK3CA (E545K) mutations was detected in case 3 (hilar cholangiocarcinoma). In cases 12 and 16, amplifications of ERBB2 and CCNE1 were identified, respectively, without any notable somatic mutations, and both showed strong HER2 protein overexpression (*data not shown*). Additionally, mutations of IDH1 (R132C), RB1 (S576L), and CTNBB1 (S45F) were accompanied by TP53 mutation. CCND1 amplification, ERBB2 amplification, and CCNE1 amplifications (Figure 1) were perfectly cross-validated by digital multiplexed gene analysis method (Figure 2).

Establishment of PDCs

PDC models were successfully established in 27 of 40 cases (67.5%). *Successful PDCs* were defined as those cells that were cytologically confirmed by a designated pathologist and maintained growth following two passages. In body fluids, PDC models were established in 22 of 24 samples (91.7%). However, the success rates of PDCs from pathologic specimens were inferior to those of body fluids: successful cell cultures were achieved in four of eight tissue samples from surgical resection (50.0%) and only one of eight liver

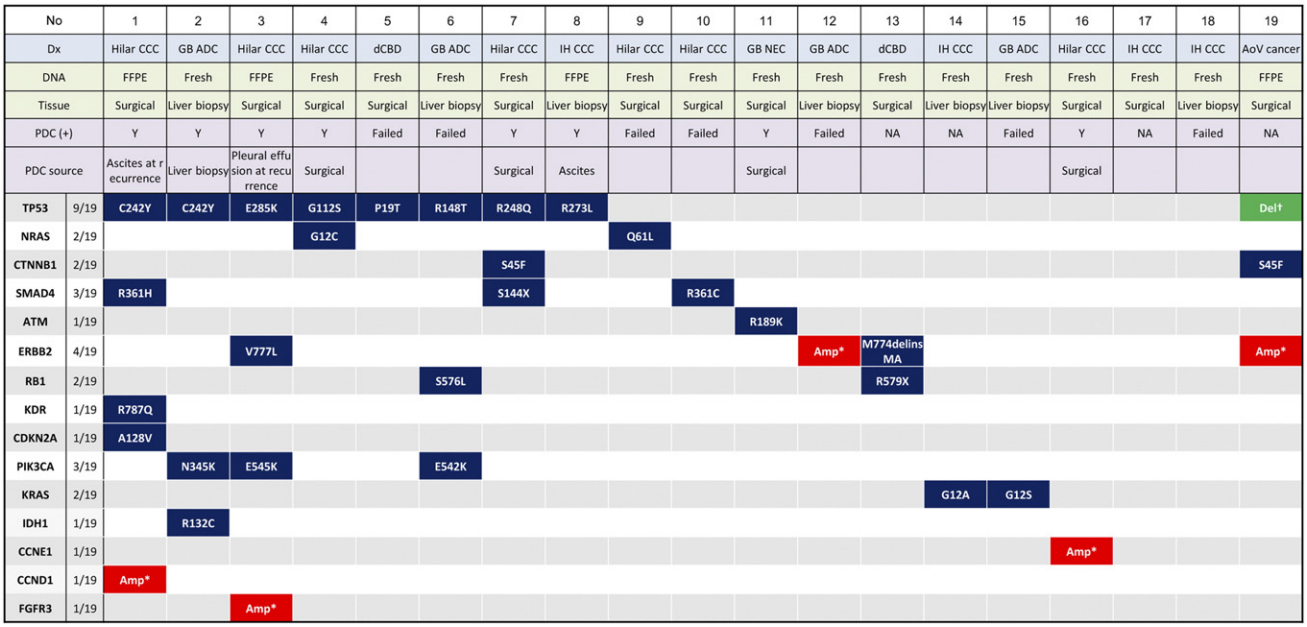

Figure 1. Genomic landscape of 19 BTC patients. Hilar CCC, hilar cholangiocarcinoma; IC CCC, intrahepatic cholangiocarcinoma; dCBD, distal common bile duct cancer; GB ADC, gallbladder adenocarcinoma; GB NEC, gallbladder neuroendocrine carcinoma; AoV, ampulla of Vater; FFPE, formalin-fixed paraffin-embedded; Amp*, amplification; Del †, deletion.

biopsy specimens (12.5%) (Table 2). We therefore analyzed variable factors for successful PDC establishment (Table 3). In univariate analysis, only type of specimen significantly affected success of PDC establishment (P = .009, hazard ratio = 60.3, 95% CI 2.74-1329.3). Primary tissue specimens and PDC lines were pathologically similar to that of pathologically resembled parental tumor. Immunohistochemical staining of CK7 and CK20 in PDCs was well correlated with that in primary tumors (Figure 3). Morphologically, the progeny PDCs resembled very well the primary adenocarcinoma of BTC.

Discussion

The current standard of care for metastatic BTC is gemcitabine plus cisplatin combination therapy (category 1) based on the phase II trial–demonstrated improvement of PFS compared with gemcitabine alone (8.0 vs 4.0 months) [8]. Except for 5-fluorouracil–based regimens, there is no evidence of effective treatment options for advanced BTC after first-line therapy [22]. For targeted therapy, erlotinib, an EGFR-tyrosine kinase inhibitor, showed interesting activity in combination with gemcitabine and oxaliplatin [23], demonstrating an objective response rate significantly superior to chemotherapy alone (30% vs 16%). However, this effect was not reflected in improvement of PFS and overall survival. Various subsequent studies with EGFR antibodies and inhibitors have been attempted in advanced BTC, and agents targeting the VEGF pathway such as bevacizumab, sorafenib, and sunitinib also have been tested alone or in combination with gemcitabine-based chemotherapy [24–26]. However, almost all trials demonstrated marginal efficacies in metastatic or advanced BTC patients, and no effective targeted therapies have been approved at present to our knowledge [11].

Since 2003, several retrospective mutational analyses of BTC samples have been reported; however, these studies yielded heterogeneous mutational frequencies influenced by small sample sizes, the inherent diversity of BTCs, and differences in sequencing methods [27–29]. Various targetable molecular alterations have been

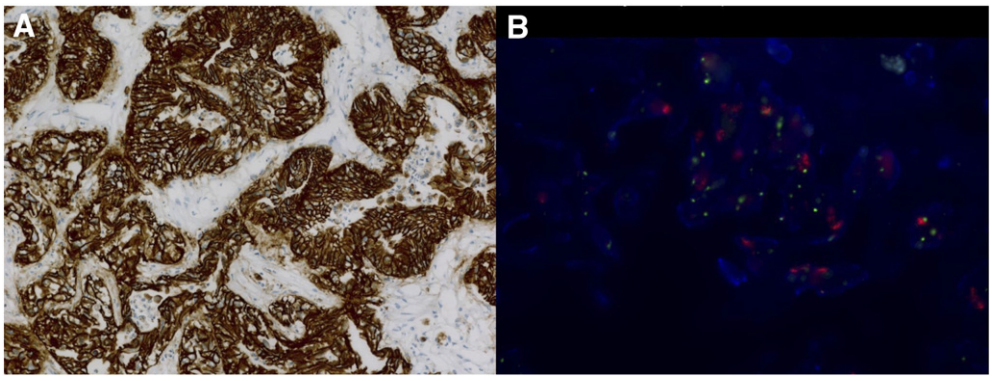

Figure 2. (A) Immunohistochemistry staining and (B) fluorescence *in situ* hybridization of HER2 in a patient with ampulla of Vater cancer (case 19 in Figure 1) (400× magnification).

Table 3. Univariate Analysis for Success of PDC Establishment

Variables	*P* Value	Harzard Ratio (95% CI)
Gender (male vs female)	.131	5.08 (0.62-41.7)
Age (≤60 vs >60)	.213	4.0 (0.45-35.4)
Location of primary tumor (GB cancer vs IH CCC vs EH CCC)	.743	0.58 (0.023-14.7)
Type of specimen (body fluids vs surgical specimen vs liver biopsy)	.009	60.3 (2.74-1329.3)
Stage (IV vs. I-III)	.506	0.26 (0.005-14.05)
Level of CA 19-9 (≤200 vs >200 U/ml)	.345	2.05 (0.46-9.0)
Histologic grade (G3-4 vs G1-2)	.164	0.36 (0.08-1.52)

GB, gallbladder; IH CCC, intrahepatic cholangiocarcinoma; EH CCC, extrahepatic cholangiocarcinoma.

identified by whole exome sequencing, and now clinical trials targeting PI3K/AKT, MEK/ERK, Hedgehog, and NOTCH pathways are being under way [11,30,31]. In addition, a variable degree of FGFR2 fusion/translocation was also detected in BTCs, and clinical trials involving an FGFR2 inhibitor are in progress [32]. More recently, analysis of the genomic spectra of 260 BTC patients in Japan by a combination of whole exome and transcriptome sequencing identified genetic alterations in nearly 40% of cases that may become therapeutic targets [10].

In our study, we observed diverse genomic alterations in 19 cases of BTC by targeted sequencing. The most commonly detected genomic alterations were in TP53 (C242Y, E285K, G112S, P19T, R148T, R248Q, and R273L), NRAS (G12C and Q61L), KRAS (G12A and G12S), ERBB2 (V777L and pM774delinsMA), and PIK3CA (N345K, E545K, and E521K). We also identified CCND1 amplification and concomitant TP53 (C242Y) and CDKN2A (A128V) mutations. There was one case with HER2 amplification (Figure 2); however, administration of a HER2 inhibitor, lapatinib, was not effective in inhibiting disease progression. Although a general conclusion cannot be drawn based on anecdotal experience, this clinical application based on identification of a genomic alteration emphasizes the need for clinical decisions to be established using an integrative approach combining genomic sequencing, pathology, tumor type, and, if feasible, a preclinical model.

A previous study of PDC models in metastatic cancer reported a high success rate of PDC establishment with histologic features, genomic profiles, and functional behaviors similar to those of real tumors [16]. In the current study, we developed PDC models from 39 BTC patients using body fluids, surgical specimens, or biopsy specimens. The success rate of PDC models was much higher for body fluids (N = 22/24, 91.7%) than tissue specimens (N = 5/16, 31.3%) and was particularly low for biopsy tissues (N = 1/8, 12.5%). Genomic sequencing of PDCs is in progress, and the genetic profiles of PDCs will be compared with sequencing data from primary tumors. Nevertheless, we have reported in our previous study using the same method that the genomic concordance rate between parental primary and PDC progeny was >80% [16].

There are several limitations in this study. Because a relatively small number of patients and diverse subtypes of BTC were enrolled, the results from genomic sequencing data could be interpreted restrictively. Only a limited number of IHC staining were performed; therefore, it was difficult to evaluate the correlations between protein expressions and changes at the molecular level. In conclusion, we describe a genomic landscape of the BTC cohort that identified potentially viable targets for treating this disease. In our future work, we will use genomically profiled patient-derived preclinical models to screen for drug sensitivity to an array of molecularly targeted agents to further optimize a genome-based targeted agent clinical trial for BTC.

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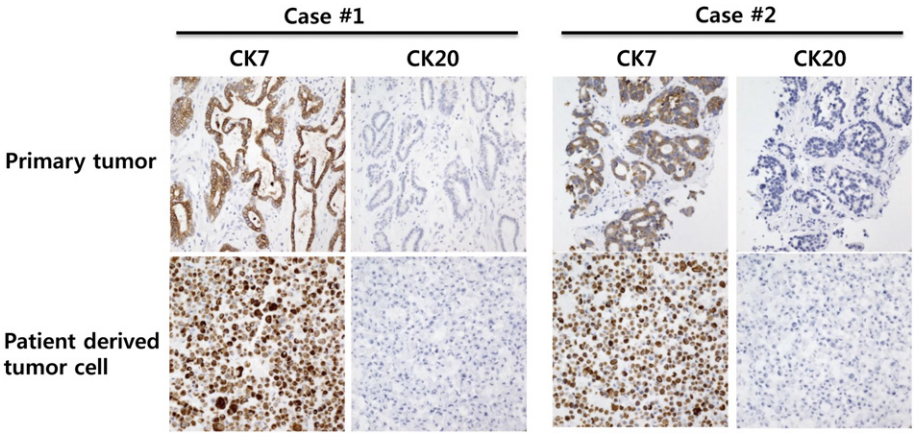

Figure 3. Immunohistochemical correlation between primary tumors and PDCs. (A) Primary cholangiocarcinoma (adenocarcinoma) (upper panel) and PDCs derived from malignant ascites (lower panel); (B) primary cholangiocarcinoma (adenocarcinoma) (upper panel) and PDCs derived from malignant pleural effusion (lower panel); (C) primary cholangiocarcinoma (adenocarcinoma) (upper panel) and PDCs derived from malignant ascites (lower panel).*Left and right sides represent immunohistochemical staining of CK7 and CK20, respectively (400× magnification).

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