Aberrant expression of the PHF14 gene in biliary tract cancer cells

TAKAKO AKAZAWA, KOHICHIROH YASUI, YASUYUKI GEN, NOBUHISA YAMADA, AKIRA TOMIE, OSAMU DOHI, HIRONORI MITSUYOSHI, NOBUAKI YAGI, YOSHITO ITOH, YUJI NAITO and TOSHIKAZU YOSHIKAWA

Department of Molecular Gastroenterology and Hepatology, Graduate School of Medical science, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan

Received December 7, 2012; Accepted March 5, 2013

DOI: 10.3892/ol.2013.1278

Abstract. DNA copy number aberrations in human biliary tract cancer cell lines were investigated using a high-density oligonucleotide microarray. A novel homozygous deletion was detected at chromosomal region 7p21.3 in the OZ cell line. Further validation experiments using genomic PCR revealed a homozygous deletion of a single gene, plant homeodomain (PHD) finger protein 14 (PHF14). No PHF14 mRNA or protein expression was detected, thus demonstrating the absence of PHF14 expression in the OZ cell line. Although the PHD finger protein is considered to be involved in chromatin-mediated transcriptional regulation, little is known about the function of PHF14 in cancer. The present study observed that the knock down of PHF14 using small interfering RNA (siRNA) enhanced the growth of the BTC cells. These observations suggest that aberrant PHF14 expression may have a role in the tumorigenesis of BTC.

Introduction

Biliary tract cancers (BTCs) are a heterogeneous group of tumors arising from the epithelial cells of the intra- and extrahepatic bile ducts and gallbladder (1,2). Histologically, the majority of BTCs are adenocarcinomas and have a poor prognosis. The majority of BTC patients exhibit an unresectable disease at the time of diagnosis due to the advanced cancer stage. Although patients rarely have identical risk factors, it is clear that the disorders that cause chronic inflammation of the biliary tract, including primary sclerosing cholangitis, gallstones and bile duct stones, are associated with an increased incidence of BTC.

Little is known about the molecular pathogenesis of BTC (1,2). Although alterations in a number of cancer-associated genes, including p53 and KRAS, have been identified as potential risk factors, the frequency of these alterations is low. Interleukin 6 (IL-6), an inflammatory cytokine, appears to have a more definite role in the pathogenesis of BTC. The activation of EGFR, ERBB2 and HGF has also been reported in BTC (1,2).

Homozygous deletions have been useful in the positional cloning of a number of tumor suppressor genes. Using high resolution single nucleotide polymorphism (SNP) arrays, we previously detected novel regions of homozygous deletions and identified potential tumor suppressor genes in human cancers (3,4). In the present study, DNA copy number aberrations in human BTC cell lines were investigated using SNP arrays to identify the genes potentially involved in BTC. It was observed that a novel homozygous deletion at the chromosomal region 7p21.3 occurred in a BTC cell line and that the plant homeodomain (PHD) finger protein 14 (PHF14) gene, which lies within the 19p13.2 chromosomal region, was homozygously deleted. The present study also further examined whether defective PHF14 expression has a functional role in BTC cells.

Materials and methods

Cell lines. The following eight human BTC cell lines were studied: TFK1, HuCCT1, OCG1, NOZ, OZ, SSP25, HuH28, and TKKK. These cell lines were obtained from the Health Science Research Resources Bank (Osaka, Japan) and the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. This study was approved by the Ethics Committee of Kyoto Prefectural University of Medicine, Kyoto, Japan.

SNP array analysis. DNA copy number changes were analyzed using the GeneChip Mapping 250K Sty array (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions, as previously described (3-5). Briefly, 250 ng genomic DNA was digested with a restriction enzyme, then ligated to an adaptor and amplified by PCR. The amplified products were fragmented, biotinylated and hybridized to the microarrays. Hybridization was detected by incubation with a streptavidin-phycocerythrin conjugate and scanning of the array. Following the appropriate normalization of the mean array intensities, signal ratios were calculated between the
BTC cell lines and the anonymous normal references. Copy numbers were then inferred from the observed signal ratios based on the hidden Markov model using Copy Number Analyzer for Affymetrix GeneChip mapping arrays (CNAG) software (available at http://www.genome.umin.jp).

**PCR analysis.** Conventional PCR was performed using Ex Taq DNA polymerase (Takara, Otsu, Japan) according to the manufacturer's instructions. Genomic DNA and mRNA were quantified using the real-time fluorescence detection method, as described previously (5). The primers that were used for the PCR are shown in Table I. The endogenous controls for the mRNA and genomic DNA levels were GAPDH and long interspersed nuclear element-1 (LINE-1), respectively.

**Immunoblotting.** Immunoblots were prepared according to previously published methodology (5). Cell lysates (20 µg protein per sample) were separated via SDS-polyacrylamide gel electrophoresis using 10% acrylamide gels. The anti-PHF14 rabbit polyclonal antibody and the anti-β-actin mouse monoclonal antibody were purchased from Sigma-Aldrich (Tokyo, Japan). The anti-PHF14 and anti-β-actin antibodies were used for immunoblotting at dilutions of 1:400 and 1:5,000, respectively. The anti-mouse or anti-rabbit IgG (Amersham, Tokyo, Japan) used for secondary immunodetection was diluted to 1:5,000. Antibody binding was detected using an ECL system (Amersham).

**RNA interference (RNAi).** To knock down PHF14 expression in the cells, two small interfering RNA (siRNA) duplex oligonucleotides targeting PHF14 [PHF14 Stealth Select RNAi™ siRNA HSS114491 (siRNAa) and HSS114492 (siRNac)] and negative control siRNA duplexes were purchased from Invitrogen (Carlsbad, CA, USA). The siRNAs were delivered into OCUG1 cells using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's instructions. The cell viability was assessed by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (NacalaiTesque, Kyoto, Japan) dye absorbance (MTT assay), according to the manufacturer's instructions, at 24, 48 and 72 h after siRNA transfection.

**Statistical analysis.** Differences between the groups were evaluated using the Student’s t-test. The statistical analyses were performed on SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Overview of genomic changes in BTC cell lines.** To identify the genes involved in BTC, eight BTC cell lines were screened for DNA copy number aberrations using SNP array analysis. The genetic changes that were detected are shown in Fig. 1. Chromosomal regions frequently involved in the gain of DNA were identified at 5p and 17q (seven cases, 88%), as well as 8q (six cases, 75%). The chromosomal regions most frequently associated with DNA loss were identified at 4p and 4q (seven cases, 88%) and 6q (six cases, 75%). The homozygous deletions and chromosomal amplifications are shown in Table II. SNP array analyses successfully identified chromosomal amplification regions containing known oncogenes, including KRAS (12p12.1) and ERBB2 (17q12), as well as chromosomal homozousgous deletion regions containing known tumor suppressor genes, including FHIT (3p14.2), CDKN2A (9p21), CDKN2B (9p21) and WWOX (16q23.1; Table II). Of these chromosomal regions, the homozousgous deletion at 7p21.3 became the focus for further investigation as it was a novel alteration in BTC.

**Identification of homozousgous PHF14 gene deletion.** Among the eight cell lines screened, the OZ cell line (6) exhibited a homozousgous deletion at chromosomal region 7p21.3 (Fig. 2A). It was estimated that the region of deletion included five genes. Further validation experiments using genomic PCR revealed a homozousgous deletion of a single gene, PHF14. The extent of the homozousgous deletion was narrowed down to a location between exons 5 and 17 of the PHF14 gene (Fig. 2B).

**Copy number and expression of PHF14 gene in BTC cell lines.** The DNA copy numbers and expression levels of the PHF14 gene in the BTC cell lines and control normal lymphocytes or liver (Fig. 2C-E) were then analyzed. Real-time quantitative reverse transcription (RT)-PCR and immunoblot analyses did not detect PHF14 mRNA or protein expression, respectively (Fig. 2D and E), thus demonstrating the absence of the PHF14 gene from the OZ cell line.

**Table I. Primers used for PCR.**

| PHF14 | STS-marker | Forward primer | Reverse primer |
|-------|------------|----------------|----------------|
| Exon 4 |  | 5'-TTGAAAATCTATATAAATGTGTTAA-3' | 5'-AGGCCACAGTCAGCCATTTCT-3' |
| Exon 5 |  | 5'-TTCTTTTTTCTTGTGATTATGTA-3' | 5'-AGGGAAATCAAGCCAGAC-3' |
| Exon 6 |  | 5'-TTTGGTGTGTGTTGTTGGAAT-3' | 5'-GCCAGTAAACTCAAACTGTA-3' |
| Exon 7 |  | 5'-TGAAAATTTGTGATTTGAGAA-3' | 5'-GTTTTTCTGAGTCTGACTAGC-3' |
| Exon 17 |  | 5'-TGTTGCTGTCTTAAATATTGTTTGT-3' | 5'-GGGTGACTGTTAAAATGTTGTT-3' |
| Exon 18 |  | 5'-CATGATGGTCATTTACCTGCAA-3' | 5'-AAACTTTTAAAGGTCAGCTTTTG-3' |

PHF14, plant homeodomain finger protein 14; STS, sequence-tagged site.
Enhanced growth of BTC cells by PHF14-knockdown. To determine whether the defective expression of PHF14 had a functional role in the BTC cells, PHF14 expression was knocked down with two independent siRNA molecules (siRNAb and siRNAc) in OCUG1 cells (Fig. 3A). The PHF14-knockdown led to an upregulation of cell growth, as determined via the MTT assay 72 h after the transfection with siRNAb and siRNAc (Fig. 3B). These observations suggest that the defective expression of PHF14 may promote the proliferation of BTC cells.

Discussion

In the present study, a novel homozygous deletion at chromosomal region 7p21.3 was identified in the OZ cell line, a human BTC cell line that was established from the ascites of a patient with mucin-secreting BTC in the hepatic hilus (6). Subsequent detailed analyses revealed that the homozygous deletion was located between exons 5 and 17 of the PHF14 gene. Moreover, the present data suggest that the defective
expression of PHF14 may promote the proliferation of the BTC cells.

Based on the amino acid sequence homology, PHF14 is considered to be a PHD finger protein. The PHD finger protein is known to be involved in chromatin-mediated transcriptional regulation (7-9). The PHD finger domain recognizes the methylation status of histone lysine residues, including histone H3 trimethylated at lysine 4, which is associated with an ‘open’
chromatin structure and transcriptional activation. Mutations, deletions and chromosomal translocation in the genes encoding PHD finger proteins, such as the tumor suppressor ING1, have been associated with various types of cancer (8). A mutation in PHF14 was previously identified in a colon cancer cell line (10). However, the function of PHF14 has remained unknown. Phf14, a mouse homologue of PHF14, was identified as a novel transcriptional factor that acts as a negative regulator of platelet-derived growth factor receptor-α (PDGFRα) expression in mouse mesenchymal cells (11). Furthermore, Phf14-null mice exhibited interstitial pulmonary hyperplasia. Mesenchymal fibroblasts derived from the Phf14-null mice showed an increased proliferation rate, accompanied by the enhanced expression of PDGFRα (11). The increased growth of Phf14−/− mesenchymal cells supports the present observation that the knockdown of PHF14 enhances the growth of BTC cells. Although the mechanisms by which PHF14 functions in tumors remain to be elucidated, the present data suggest that alterations in the expression of PHF14 may be involved in the tumorigenesis of BTC.

References

1. Koti RS and Davidson BR: Malignant biliary diseases. In: Sherlock's Diseases of the Liver and Biliary System. Dooley JS, Lok ASF, Burroughs AK and Heathcote EJ (eds). 12th edition. Wiley-Blackwell, Oxford, pp.294-311, 2011.

2. Goodman ZD, Terracciano LM and Wee A: Tumours and tumour-like lesions of the liver. In: MacSween's Pathology of the Liver. Burt A, Portmann B and Ferrell L (eds). 6th edition. Churchill Livingstone, Philadelphia, pp.761-851, 2011.

3. Zen K, Yasui K, Gen Y, et al: Defective expression of polarity protein PAR-3 gene (PARD3) in esophageal squamous cell carcinoma. Oncogene 28: 2910-2918, 2009.

4. Endo M, Yasui K, Zen Y, et al: Alterations of the SW1/SNF chromatin remodelling subunit-BRG1 and BRM in hepatocellular carcinoma. Liver Int 33: 105-117, 2013.

5. Zen K, Yasui K, Nakajima T, et al: ERK5 is a target for gene amplification at 17p11 and promotes cell growth in hepatocellular carcinoma by regulating mitotic entry. Genes Chromosomes Cancer 48: 109-120, 2009.

6. Homma S, Nagamori S, Fujise K, et al: Human bile duct carcinoma cell line producing abundant mucin in vitro. Gastroenterol Jpn 22: 474-479, 1987.

7. Aasland R, Gibson TJ and Stewart AF: The PHD finger: implications for chromatin-mediated transcriptional regulation. Trends Biochem Sci 20: 56-59, 1995.

8. Baker LA, Allis CD and Wang GG: PHD fingers in human diseases: disorders arising from misinterpreting epigenetic marks. Mutat Res 647: 3-12, 2008.

9. Saiga S, Möller B, Watanabe-Taneda A, Abe M, Weijers D and Komeda Y: Control of embryonic meristem initiation in Arabidopsis by PHD-finger protein complexes. Development 139: 1391-1398, 2012.

10. Ivanov I, Lo KC, Hawthorn L, Cowell JK and Ionov Y: Identifying candidate colon cancer tumor suppressor genes using inhibition of nonsense-mediated mRNA decay in colon cancer cells. Oncogene 26: 2873-2884, 2007.

11. Kitagawa M, Takebe A, Ono Y, Imai T, Nakao K, Nishikawa S and Era T: Phf14, a novel regulator of mesenchyme growth via platelet-derived growth factor (PDGF) receptor-α. J Biol Chem 287: 27983-27996, 2012.