Genetic Alterations in Intrahepatic Cholangiocarcinoma as revealed by Degenerate Oligonucleotide Primed PCR-Comparative Genomic Hybridization

Intrahepatic cholangiocarcinoma (ICC), a malignant neoplasm of the biliary epithelium, is usually fatal because of difficulty in early diagnosis and lack of availability of effective therapy. The genetic mechanisms involved in the development of ICC are not well understood and only a few cytogenetic studies of ICC have been published. Recently, technique of degenerate oligonucleotide primed (DOP)-PCR comparative genomic hybridization (CGH) permits genetic imbalance screening of the entire genome using only small amounts of tumor DNA. In this study chromosomal aberrations in 33 Korean ICC were investigated by DOP-PCR CGH. The common sites of copy number increases were 20q (67%), 17 (61%), 11q11-q13 (42%), 8p12-qter (39%), 18p (39%), 15q22-qter (36%), 16p (36%), 6p21 (30%), 3q25-qter (27%), 1q41-qter (24%), and 5p14-q11.2 (24%). DNA amplification was identified in 16 carcinomas (48%). The frequent sites of amplification were 20q, 17p, 17q23-qter, and 7p. The most frequent sites of copy number decreases were 1p32-pter (21%) and 4q (21%). The recurrent chromosomal aberrations identified in this study provide candidate regions involved in the tumorigenesis and progression of ICC.

Key Words : Cholangiocarcinoma, Chromosome Aberrations, Comparative Genomic Hybridization; Polymerase Chain Reaction

INTRODUCTION

Cytogenetic analysis plays an important role in the elucidation of the pathogenesis and prognosis of cancers. Several techniques are now available for genome-wide screening of alterations in copy number. Comparative genomic hybridization (CGH) is a molecular cytogenetic tool that allows the screening of copy number change, and has now become one of the most popular genome screening techniques (1). The degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) CGH technique permits genetic imbalance screening of the entire genome using only small amounts of tumor DNA. It has also been successfully applied to microdissected archival paraffin-embedded tissue by several investigators (2, 3).

Intrahepatic cholangiocarcinoma (ICC) is the second most common malignant tumor in the liver, arising from epithelial cells in the intrahepatic bile duct (4). Although the frequency of ICC is low, its incidence in Southeast Asian is higher than that in Europe and North America. ICC is refractory to conventional chemotherapy and radiation treatment and curative surgical resection is not effective due to the high recurrence rate (5). While risk factors including liver fluke infection, hepatolithiasis, congenital cysts, and sclerosing cholangitis have been reported (6), the genetic mechanisms involved in the development of ICC are not well understood and only a few cytogenetic studies of ICC have been published. Chromosomal gains of 5p, 7p, 8q, 13q, 15q, 17q, and 20q, and losses of 3p, 4q, 6q, 9p, 16q, 17p, and 18q were reported in ICC (7, 8). The structural abnormalities of chromosomes 1, 5, 7, and 12 were detected in ICC cell lines (9). Our previous study demonstrated the chromosomal aberrations in chromosomes 3, 6-8, 12, 14, 17, and 18 were analyzed by cross-species color banding (10). Loss of heterozygosity (LOH) studies of ICC has shown frequent allelic losses from various chromosomes including 1p, 1q, 3p, 6q, 8p, 9q, 14q, 17p, and 18q (11). Whereas specific genes responsible for the development of ICC, genetic alterations of APC, c-erbB2, K-ras, p53, and p16INK4A are proposed to be closely related to ICC progression (12-14).

In this study, the DNAs from 33 Korean ICC specimens were analyzed by DOP-PCR CGH with the aim of identifying the non-random chromosomal aberrations of Korean ICC.

MATERIALS AND METHODS

Tissue specimens

A total 33 formalin-fixed, paraffin-embedded specimens
Table 1. Clinical data and results of comparative genomic hybridization in 33 cholangiocarcinomas

| No. | Sex/age | Differentiation | Tumor size (cm) | DNA copy number changes                                                                 |
|-----|---------|-----------------|-----------------|----------------------------------------------------------------------------------------|
| 1   | F/36    | well            | 9.5             | 1q31-qter, 8p11.2-qter(q24.1-qter), 11p11.1-q13, 12p12-qter, 14q24-qter, 15q22-qter, 16q23-qter, 18p11.2-qter(q21-qter), 19p13-qter(20q) |
| 2   | F/36    | well            | 3.5             | X 1q21-q23, 3p14-p13, 4p15.3-p12, 4q27-q28, 5p15.1-qter, 6p21.1-q13, 7p14-q11.2, 8pter-q22(p11.2-q12), 9q13-q21, 12q14-q21, 13q14-qter, 18p11.3, 20q13.1-qter |
| 3   | F/64    | well            | 6.5             | X 1p22-q13, 5p14-q23, 6p21.1-q16, 8p12-qter, 13q21-q31 |
| 4   | F/51    | well            | 4               | 3p13-q12, 3q25-q26.3, 5p14-p12, 5q15-q23, 6p12-q22, 8pter-q22, 12q14-q21, 13q21-q31, 19p13.3-q12, 21q11.2-q21 |
| 5   | M/52    | well            | 1.1             | 19, 20q, 22q |
| 6   | M/64    | well            | 3.5             | 1p13-q32, 22q |
| 7   | M/64    | moderately      | 4.5             | 11p12-q13, 12q24.1-qter, 15q22-q24, 16p, 16q24, 17(p) 20p11.2-qter(q21-q22) |
| 8   | M/57    | moderately      | 3               | X 6p21.3-p21.1, 7p13-q11.2, 8p21-qter, 11p11.2-q13, 12q23-qter, 15q22-qter, 16, 17, 20q |
| 9   | M/64    | moderately      | 4.5             | 1pter-p35, 16pter-p13.1, 19q13.2-qter, 22q12-qter |
| 10  | F/77    | moderately      | 4               | 3p14-q11.2, 17p11.2-qter, 18p11.3, 19p, 20q13.1-qter |
| 11  | M/50    | moderately      | 3.9             | 1p41-qter, 12q23-qter, 14q, 15q22-q25, 16p, 16q22-qter, 17, 18, 20q |
| 12  | M/58    | moderately      | 3               | 1q, 3pter-p16, 6p21.3-p23, 9q, 15q22-qter, 16p, 17, 18pter-q11.2, 20p11.2-qter(q) |
| 13  | F/71    | moderately      | 7.5             | none |
| 14  | F/45    | moderately      | 4               | 3p25-qter, 5p13-q11.2, 7pter-q21, 11p11.2-q13, 17, 18p, 20q(q13) |
| 15  | F/68    | moderately      | 10              | Xq 3p12-qter, 4q31.1-qter, 5q11.2-q14, 6p21.2-q13, 8p12-qter, 14q, 15q24-qter, 16p12-q13 |
| 16  | F/60    | moderately      | 5               | 3p24-pter, 6q, 11q23-qter |
| 17  | F/54    | moderately      | 5               | 1p31-qter, 2q36-qter, 3q26.2-q28, 7pter-p22(p21-qter), 7q11.2-q21, 12pter-q12(p13), 13q33-qter, 17, 18pter-q11.2, 20q11.2-qter, 21q |
| 18  | M/62    | moderately      | 6               | 1p, 3p, 12q24.1-qter |
| 19  | M/58    | moderately      | 9               | none |
| 20  | M/61    | moderately      | 4               | none |
| 21  | M/62    | poorly          | 5               | none |
| 22  | M/64    | poorly          | 4.5             | none |
| 23  | M/47    | poorly          | 10              | none |
| 24  | M/69    | poorly          | 12              | none |
| 25  | M/53    | poorly          | 1.7             | none |
| 26  | M/45    | poorly          | 4               | none |
| 27  | M/65    | poorly          | 3               | none |
| 28  | M/56    | poorly          | 6.5             | 6pter-p21.3, 12q24, 17, 18pter-q11.2, 20q |
| 29  | M/56    | poorly          | 3               | none |
| 30  | M/38    | poorly          | 4               | 1p41-qter, 11p11.1-q13, 14q31-qter, 15q22-q25, 17(p12-q21), 20q(q) |
| 31  | M/42    | poorly          | 8               | 3p25-qter, 4p14-q13, 17 |
| 32  | M/69    | poorly          | 4.5             | 19q |
| 33  | M/43    | poorly          | 2               | 1q41-qter, 6pter-p21.1, 7q22-qter, 8q21.2-qter, 10q23-qter, 11p11.2-q13, 15q24-qter, 16pter-q22, 17, 20q, 22q, X(p11.4-q13) |

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of ICC were collected. Using a 4 μm hematoxylin and eosin stained adjacent section as a guide, tumor-containing areas were microdissected from sectioned 10 μm specimens with a sterile 25-gauge needle under a microscope. Normal DNA from human peripheral blood and tumor DNA from microdissected cells were prepared by standard protocols.

DOP-PCR and CGH

DOP-PCR was performed in two separate steps according to the published protocol with some modifications (15, 16). Briefly, in the preamplification step, the four initial cycles were performed at low stringency conditions (denaturation at 95 °C for 3 min followed by 4 cycles of 94 °C for 1 min; 25 °C for 1 min; 56 °C for 3 min; 74 °C for 2 min), followed by 35 cycles in high stringency conditions (at 94 °C for 1 min; 56 °C for 1 min; 72 °C for 2 min). Labellings of tumor DNA with biotin-16dUTP and control DNA with digoxigenin-11dUTP were performed by nick translation (Nick Translation System, Gibco/BRL, Rockville, MD, U.S.A.). Equal amounts of labeled normal control and tumor DNA including 40 μg of Cot-1 DNA (Gibco/BRL) and 1 μg salmon sperm DNA were precipitated with ethanol. Labeled DNA probes were mixed with hybridization buffer, denatured at 75 °C for 5 min and then applied onto the denatured metaphase slides. After incubation for 3 days, the slides were washed and the hybridization signal was detected using an avidin-fluorescin isothiocyanate (FITC, 1:400) and mouse anti-digoxigenin (1:100). Metaphase slides were counterstained with DAPI at a concentration of 0.1 μg/mL in an antifade solution (Oncor, Gaithersburg, MD, U.S.A.) for chromosomal identification. Mean ratio profiles were determined from analyses of 10-15 metaphase cells. Thresholds for gains and losses were defined as the theoretical value of 1.25 and 0.75, respectively. The following regions were excluded for the analyses: centromere, acrocentric short arms, telomere, and heterochromatin-rich region (17).

![Fig. 1. Summary for gains and losses of chromosomal regions in 33 cholangiocarcinomas. Gains are shown on the right side of the chromosome ideograms and losses on the left. Each line illustrates the affected region of the chromosome in a single tumor sample. Thick lines represent high-level amplifications.](image-url)
RESULTS

Thirty-three intrahepatic cholangiocarcinomas were analyzed using DOP-PCR CGH. The various chromosomal aberrations were detected in all tumors studied. All chromosomes were involved in imbalances and the mean number of imbalances per tumor specimen was 9 (range, 2-18). Table 1 summarizes clinical and chromosomal findings in the 33 entire cases. A schematic summary of copy number changes as detected by CGH analysis in 33 abnormal cases is shown in Fig. 1.

Among the 33 cases, the common sites of copy number increases, in order of frequency, were, 20q (67%), 17 (61%), 11q11-q13 (42%), 8p12-qter (39%), 18p (39%), 15q22-qter (36%), 16p (36%), 3q25-qter (27%), 1q41-qter (24%), and 5p14-q11.2 (24%) (Fig. 1). Sixteen tumors exhibited one or more sites of DNA amplification; the frequent sites of amplification were 20q13.2-qter (6 cases), 17p (3 cases), 17q23-qter (2 cases), and 7p (2 cases). The most frequent sites of copy number decreases were 1p32-pter (21%) and 4q (21%). Examples of these gains and losses are shown in Fig. 2. Gains of 3q25-qter were more frequently observed in moderately differentiated tumors. Gain of 3q25-qter was more frequently observed in moderately differentiated ICC.

DISCUSSION

The analysis of recurring chromosomal aberrations has become an integral part of the diagnostic and prognostic workup in many human cancers, and their molecular analyses have facilitated the identification of genes related to the pathogenesis of cancers. However, the characterization of complex chromosomal rearrangements was limited when conventional cytogenetic methods were used. In this study thirty-three ICC were successfully analyzed using DOP-PCR CGH.

In consistent with our result, a few alteration such as copy number increases of 5p, 8q, 15q, 17q, and 20q were previously reported in ICC (7, 8). The most common gain region was 20q, with eight tumors showing high amplification of 20q13-qter. Gains of 17q and 20q are common finding in several solid tumors including hepatocellular carcinoma, pancreas, and breast tumors (18-20). Chromosome arm 20q contains many genes such as E2F1 (20q11), AIB1 (20q12), BTAK/STK15 (20q13), and NABC14 (20q13.2), which are involved in several cancers (21-25). Gene amplification and protein expression of c-erbB-2 (HER-2/neu) on 17q21 were observed in ICC and breast cancer (26, 27).

In particular, gains of 3q26-qter, 6p21, 11q11.2-q13, 16p and 18p are frequently observed in this study (Fig. 2A, D, E). Chromosome region 3q26-qter harbors PIK3CA and ECT2 proto-oncogenes that are related to cell proliferation and carcinogenesis (28). The frequent over-represented PIK3CA has also been reported in ovarian, head and neck, and stomach cancers (29-31). Gain of 6p with a minimum overlapping region at 6p21 was frequently observed in this study. Among the genes mapped to 6p21, PIM1 was known as a proto-oncogene related to the progression of thymic lymphomas in transgenic mice, although the role of PIM1 in the other cancers is not elucidated (32). In addition, CCND3 on 6p21 shares considerable homology with proto-oncogene cyclin D1 and is involved in the cell cycle regulation at G1 to S phase transition (33). Gain of 11q13 is a common event in human cancers and is associated to poor prognosis (34, 35). Chromosomal band 11q13 contained several cancer related genes such as FGFR3, FGF4, and EMS1, and only CCND1 and EMS1 (36, 37).

Chromosomal loss was found to be less frequent in this study. Losses of 1p and 4q have been reported in ICC (7, 8), in consistent with our current result (Fig. 2B, E). Previous reports indicated that 1p loss is related to the early stage of hepatocarcinogenesis (38) and at least three or more tumor suppressor genes including the p73 gene are localized on 1p 36. The deletion of 1p36 was reported to be related to the progression of ICC without metastatic activity (39). Allelic loss from 4q appears to be a fundamental event in the development of HCC (40).

In conclusion, our CGH demonstrated the complexity of genetic aberrations in ICC. Although chromosomal loss was found to be less frequent in this study, we were able to identify several genetic changes, which were previously reported.
These findings suggest that the technical improvement in DOP-PCR-CGH technique may reliably enhance the sensitivity and accuracy of the analysis. The recurrent gains and losses of chromosomal regions identified in this study provide candidate regions containing oncogenes or tumor suppressor genes involved in ICC development and progression.

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