

# Genetic alterations and growth pattern in biliary duct carcinomas: loss of heterozygosity at chromosome 5q bears a close relation with polypoid growth

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## Abstract

**Biliary duct carcinomas (BDCs) are relatively rare and the carcinogenic mechanisms underlying their induction are poorly understood. There are two growth patterns, polypoid and non-polypoid infiltrative type, but little information is available concerning the relation between growth pattern and genetic alterations. A comparative study was therefore conducted to clarify if differences in genetic changes, including loss of heterozygosity (LOH) at 5q, 9p, 17p, and 18q, and K-ras mutations exist between polypoid and non-polypoid infiltrative type BDCs. LOH analysis was performed using microsatellite markers and K-ras point mutations were analysed by dot blot hybridisation. The incidences of changes for polypoid and non-polypoid infiltrative types were 73% and 26% on 5q, 63% and 59% on 9p, 55% and 50% on 17p, and 20% and 18% on 18q, and 25% and 27% for K-ras mutations. Most importantly, we found the frequency of 5qLOH to be significantly higher with polypoid growth than in the non-polypoid infiltrative type ( $p < 0.05$ ), especially in extrahepatic duct carcinomas ( $p < 0.05$ ). The incidences of other genetic alterations (LOH at 9p, 17p, and 18q, and K-ras mutations) showed similar rates with both tumour types. The present data suggest that 5qLOH may have a close relation with polypoid growth in BDCs.**

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Keywords: biliary duct carcinoma; loss of heterozygosity; K-ras; chromosome 5q; growth pattern

Biliary duct carcinoma (BDC) is a relatively uncommon disease but the incidence in Japan is higher than in other countries.<sup>1</sup> Despite various clinical trials, its prognosis is still poor and an understanding of the carcinogenic mechanisms is required.

Many previous reports have indicated that tumours occur as a result of accumulation of alterations in tumour related genes such as *p53*, *p16*, *APC*, and *DPC4* tumour suppressor genes, and the *K-ras* oncogene which have been demonstrated to play an important role in the genesis of human malignancies.<sup>2–7</sup> Little has been reported on the molecular carcinogenesis of BDC, in particular on growth of extrahepatic duct carcinoma.<sup>8–10</sup> There are two types of

BDC, polypoid and non-polypoid infiltrative, and these may have different genetic alterations.

Hence in this study we focused on genetic changes and BDC growth pattern; we screened for loss of heterozygosity (LOH) at *p53*, *p16*, *APC*, and *DPC4* loci, and *K-ras* codon 12 point mutations in 34 cases of BDC with reference to the type of growth.

## Methods

### SAMPLE COLLECTION

Formalin fixed paraffin embedded tissue samples of tumours diagnosed as BDCs were obtained from the surgical pathology files of the Cancer Institute, Tokyo, Japan. A total of 34 BDCs were divided into two categories in terms of growth pattern: (1) polypoid type ( $n=12$ ) with papillary and polypoid growth into the intraductal spaces; (2) non-polypoid infiltrative type ( $n=22$ ) invading the ductal wall without polypoid formation. The two growth patterns are illustrated in fig 1. Clinicopathological data were recorded for each case and classification of the stage was made according to the World Health Organization criteria.<sup>11 12</sup>

### DNA EXTRACTION

Carcinoma and normal tissues were separately microdissected from 20  $\mu\text{m}$  formalin fixed paraffin embedded sections, as previously described.<sup>13</sup> The carcinoma tissues in polypoid and non-polypoid growth cases were taken from invasive portions where present. All microdissected tissues were deparaffinised with xylene three times, cleared with ethanol twice, completely dried, and digested with proteinase K. The resultant lysates were used directly for the polymerase chain reaction (PCR). As samples of clearly definable carcinoma tissues were limited, extracted DNA was low in quantity. Therefore, only limited genetic alterations described below could be analysed.

### LOH STUDY AND K-ras MUTATION ANALYSIS

PCR amplification of microsatellite markers was performed using fluorescent labelled primers for 5q (D5S346, D5S433), 9p (D9S157, D9S162, D9S165), 17p (D17S570, D17S786, D17S1176), and 18q (D18S474). These loci are linked with *APC*, *p16*, *p53*, and

**Abbreviations used in this paper:** BDC, biliary duct carcinoma; LOH, loss of heterozygosity; PCR, polymerase chain reaction; GBC, gall bladder carcinoma; ICC, intrahepatic cholangiocarcinoma.

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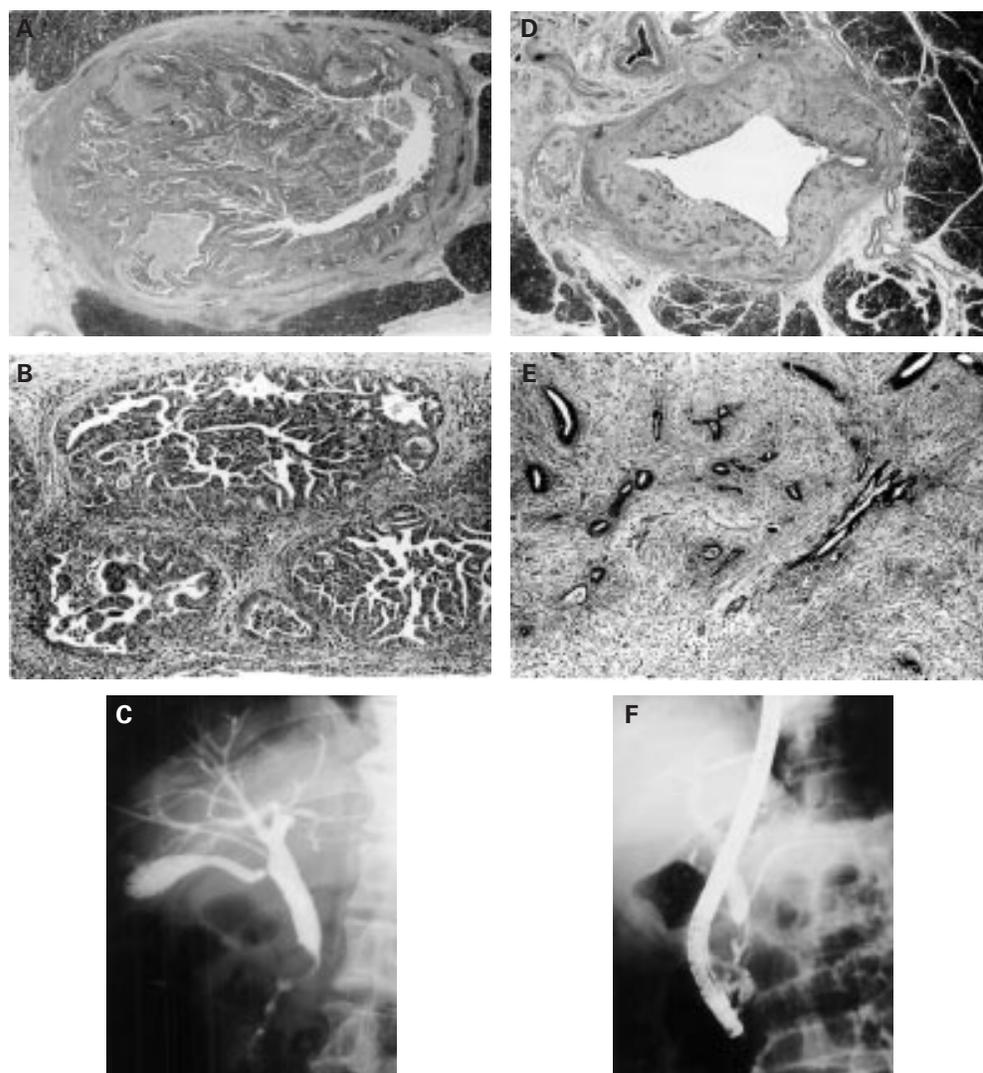


Figure 1 Examples of the two growth patterns. (A) Polypoid growth type (haematoxylin-eosin,  $\times 12.5$ ). (B) Invasive portion (haematoxylin-eosin,  $\times 100$ ) with cholangiography of the same case (C). (D) Non-polypoid infiltrative type (haematoxylin-eosin,  $\times 12.5$ ). (E) Invasive portion (haematoxylin-eosin,  $\times 100$ ) with cholangiography of the same case (F).

DPC4, respectively. The sequences of the primers used are shown in table 1. These were obtained from the Genome Database on National Center for Biotechnology Information. In chromosome 18q, as PCR amplification of DNA using a few microsatellite markers other than D18S474 proved difficult and the data derived from these PCR products were unreliable, 18qLOH could only be examined using one microsatellite marker (D18S474). The PCR products were electrophoresed in denatured 6% polyacrylamide gels and analysed for LOH with an ALFred Automatic sequencer (Pharmacia Biotech, Tokyo, Japan). LOH was defined by allelic signal reduction of more than 90% compared with the normal tissue signal (fig 2). The procedures for LOH analysis were repeated at least three times to confirm the results. We evaluated LOH for single chromosomes as detector of LOH for at least one microsatellite locus in the present study. For example, when LOH at the D5S346 locus was detected, but the D5S433 locus was not informative, we evaluated this finding as 5qLOH.

Point mutations at codon 12 of K-ras were analysed by dot blot hybridisation, as previously described.<sup>13</sup>

#### STATISTICAL ANALYSIS

Categorical variables were analysed using Fisher's exact probability test;  $p < 0.05$  was considered significant.

Table 1 Sequences of the primers used

D5S346	F: 5'-CACTCTAGTGATAAATCGGGA-3' R: 5'-GAATTGAGAGTTACAGGGTAC-3'
D5S433	F: 5'-TCTCTATCACCCATTGACAT-3' R: 5'-ATAAATTTTTGAGGCAGTAAATTC-3'
D9S157	F: 5'-ACACAAATAGATAAATGTGCATG-3' R: 5'-GGATGCCAGATAACTATATC-3'
D9S162	F: 5'-CATAACACATAGACACAGA-3' R: 5'-ACAACAAATCTCCTCACATA-3'
D9S165	F: 5'-CCCAGACAATACACAC-3' R: 5'-ATAGTGATAGATGAAATAAG-3'
D17S570	F: 5'-AGACCCATGAGGTATATTTAGT-3' R: 5'-GTTCTCAAGGGTGTAAATTTCT-3'
D17S786	F: 5'-TGTTTTCCAAATCACATGA-3' R: 5'-GAATTCTCTTTTATGGTTGT-3'
D17S1176	F: 5'-TCCTTCTGGGCCCTTTCAATGGA-3' R: 5'-CATATCACGTGCATTTTATACAC-3'
D18S474	F: 5'-CCACCCACTAGATGTGAC-3' R: 5'-AATGTCAGAAGGCATTTGTGAT-3'

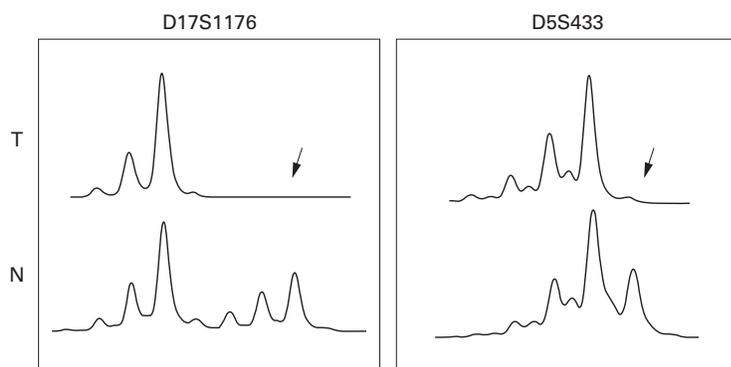


Figure 2 Illustration of examples of loss of heterozygosity (LOH). Polymerase chain reaction products for normal tissue (N) and tumour (T) DNA are shown. Microsatellite alleles are represented by two signals in the case of heterozygosity. LOH at one allele corresponds to almost complete loss of one of two signals (arrowheads).

Table 2 Clinicopathological characteristics of the two groups

	Tumour type	
	Polyploid (n=12)	Non-polyploid infiltrative (n=22)
Mean age (y)	44–73 (63.3)	43–80 (63.7)
Sex (M/F)	8/4	15/7
Location (n)		
Intrahepatic	2	6
Hilar	3	4
Upper	1	2
Middle	4	5
Lower	2	5
Size (n)		
>40 mm	7	9
≤40 mm	5	13
Histological grade (n)		
1	10	18
2	2	2
3	0	2
Stage (n)		
I, II	5	8
III, IV	7	14

## Results

Comparison of clinicopathological parameters between polyploid and non-polyploid infiltrative lesions is given in table 2. There were no significant differences between subgroups for any variable, including age, sex, location, size, histological grade, or stage.

The frequencies of LOH at 5q, 9p, 17p, and 18q, and mutations of *K-ras* overall and in the subgroups are shown in table 3. The number of informative cases at each locus was 30 cases at 5q, 25 cases at 9p and 17p, and 16 cases at 18q. The frequencies of polyploid and non-polyploid infiltrative type were: LOH at 5q, 73% (8/11) and 26% (5/19); 9p, 63% (5/8) and 59% (10/17); 17p, 55% (6/11) and 50% (7/14); and 18q, 20% (1/5) and 18% (2/11); and point

Table 3 Genetic alterations in biliary duct carcinoma

Genetic alterations	Type of carcinoma		
	Polyploid (n=12)	Non-polyploid infiltrative (n=22)	Total (n=34)
LOH			
5q	8/11 (73%)*	5/19 (26%)*	13/30 (43%)
9p	5/8 (63%)	10/17 (59%)	15/25 (60%)
17p	6/11 (55%)	7/14 (50%)	13/25 (52%)
18q	1/5 (20%)	2/11 (18%)	3/16 (19%)
<i>K-ras</i> mutation	3/12 (25%)	6/22 (27%)	9/34 (26%)

LOH, loss of heterozygosity.

\*p<0.05.

Table 4 Relationship between location and 5qLOH

Location	5qLOH	
	Polyploid (IC=11) (%)	Non-polyploid infiltrative (IC=19) (%)
Intrahepatic and hilar	2/4(50)	3/10(30)
Extrahepatic	6/7(86)*	2/9 (22)*

IC, informative case.

\*p<0.05.

mutations of *K-ras*, 25% (3/12) and 27% (6/22). The data for *K-ras* mutations were from our previous report.<sup>14</sup> Interestingly, 5qLOH was detected in 73% of polyploid growth carcinomas but in only 26% of the non-polyploid type (p<0.05) (table 3). This was especially noteworthy with extrahepatic duct carcinomas (p<0.05) (table 4). As for LOH at other chromosome loci and mutations of *K-ras*, no differences in their frequencies were found between groups.

## Discussion

Our present investigation revealed that LOH at 5q was prevalent in the polyploid type of BDCs, especially in extrahepatic duct carcinomas. Thus the results suggest that 5qLOH may have a close relation with polyploid growth.

There have been a number of reports concerning the relationship between LOH and growth pattern in various tumours but data for BDCs have not hitherto been reported. Regarding LOH at chromosome 5q, it is frequently detected in colorectal, oesophageal, gastric, ampullary, and duodenal carcinomas.<sup>15–21</sup> In colorectal and ampullary adenomas that have polyploid and papillary growth, 5qLOH is relatively common, in line with our findings for BDCs.<sup>15–16</sup> Thus 5qLOH may be an important genetic change determining polyploid growth because of the presence of tumour suppressor genes. For example, the *APC* gene located on chromosome 5q is connected with beta-catenin and associated with cell adhesion.<sup>22–24</sup> In future, analysis of the relationship between the *APC* gene and the polyploid growth pattern is warranted. However, regarding the character of non-polyploid infiltrating type tumours with 5qLOH compared with those without 5qLOH, no particular distinguishing characteristic in terms of clinicopathological or genetical findings was evident in the present study and further genetic studies in this area are required.

With regard to the overall incidence of LOH, in contrast with those for 5q, 9p, and 17p, the incidence of LOH at 18q was low in BDC. If we compare our present data with our previous results using the same primers and methods and Wistuba's findings for gall bladder carcinoma (GBC), the frequencies of 9p and 17pLOH are similar in both tumour types.<sup>25–27</sup> In contrast, 5qLOH in BDC was more frequent and 18qLOH in BDC less prevalent than in GBC. However, other investigators have reported that the frequencies of 5q, 9p, and 17pLOH are high in GBC, similar to our present data for BDC.<sup>27</sup> Further studies are

necessary to clarify the situation regarding the relation between BDC and GBC in terms of factors determining genetic alteration.

Recently, to explore the lost areas in chromosomes, analysis by comparative genomic hybridisation has been reported.<sup>28</sup> The single report for biliary tract carcinoma demonstrated copy number decreases of 6q, 18q, 4q, 5q, and 9p. The high frequencies of copy number decreases of 5q and 9p are in line with our present data. The number of cases however was small and investigation of the relationship between these genetic changes and morphological pattern was not included. In future, examination by this technique may allow clarification of which genes are most important for development of BDC.

With regard to *K-ras*, gene mutations occur more frequently in the intrahepatic cholangiocarcinoma (ICC) of periductal extension type than the mass forming type.<sup>29,30</sup> Moreover, in colorectal carcinomas, the frequency is higher in polypoid than ulcerative lesions.<sup>10</sup> In the present study, however, there was no relationship with growth pattern. Based on our results and previous reports, the relationship between *K-ras* point mutations and growth pattern in BDC may differ from those for ICC and colorectal carcinomas.

In summary, our data suggest that 5qLOH may have a close relation with polypoid growth of BDCs.

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