

Allelotype Analysis of Intrahepatic Cholangiocarcinoma

Yun Kyung Kang, M.D., Yong Il Kim, M.D., Woo Ho Kim, M.D.

Department of Pathology, Inje University Seoul Paik Hospital (YKK), Cancer Research Institute (WHK), and Department of Pathology (YIK, WHK), Seoul National University College of Medicine, Seoul, Korea

To identify the chromosomal loci of allelic loss in intrahepatic cholangiocarcinoma (ICC), we performed an allelotype study of 36 ICCs using 55 genome-wide microsatellite markers. Loss of heterozygosity was found most frequently on 8p (65.6%), 17p (64.7%), and 9p (64.5%), followed by 18q (54.2%), 1p (48.5%), 3p (44.8%), 9q (42.1%), 14q (41.7%), 6q (41.7%), and 1q (40.6%). The fractional allelic loss (FAL) values ranged from 0 to 0.731 (mean, 0.322). Analysis of the relationship between FAL values and clinicopathologic parameters disclosed significantly higher FAL values in moderately to poorly differentiated ICCs than in well-differentiated ones ($P < .05$). In summary, this study defined for the first time the overall number of chromosomes having allelic loss and the chromosomal arms and/or regions potentially involved in the development of ICC.

KEY WORDS: Allelotype analysis, Cholangiocarcinoma, Fractional allelic loss, Loss of heterozygosity, Microsatellite, Tumor suppressor genes.

Mod Pathol 2000;13(6):627–631

Intrahepatic cholangiocarcinoma (ICC) is a malignant tumor that arises from epithelial cells in the intrahepatic bile duct. It is the second most common malignant tumor in the liver and accounts for 8.3 to 20% of primary liver cancer in Korea (1, 2).

The prognosis of ICC is extremely poor (3, 4), and the molecular events involved in the development of ICC are not well understood. The reported genetic alterations in ICC include *K-ras* mutations (0 to 100%), *p53* mutations (30 to 35%), *APC* loss of heterozygosity (0 to 23.5%), and *p16* mutations (approximately 33%) (5–12).

As in other human cancers, loss of tumor suppressor gene function might be a critical event in cholangiocarcinogenesis. The typical inactivation of many tumor suppressor genes is the mutation of one allele and the loss of the other allele (13). The loss of one allele can be detected as a loss of heterozygosity (LOH); therefore, LOH can be a landmark in chromosomal regions that may harbor tumor suppressor genes.

Genome-wide allelotyping, which assays the frequency and extent of lost regions on autosomal arms, has been performed for several tumor types. To our knowledge, there has been no report of genome-wide allelotype analysis in ICC. In this study, we performed an allelotype study of 36 ICCs using 55 microsatellite markers that cover 39 non-acrocentric chromosome arms and investigated the relationship between these genetic alterations and the clinicopathologic findings.

MATERIALS AND METHODS

Tissue Samples and DNA Extraction

The hepatic resection samples from 36 patients who had pathologically defined ICC at Seoul National University Hospital (29 cases) and Inje University Seoul Paik Hospital (7 cases) were analyzed. All of these patients had been included in our previous study (6). All samples were formalin fixed and paraffin embedded. Five 10- μ m-thick serial sections were made from paraffin-embedded tissue blocks. The sections were stained with hematoxylin and eosin, and normal liver or tumor portions were selectively microdissected excluding mesenchymal cells as possible. These microdissections enabled us to enrich the tumor cell populations by 50% to 90% in each tumor sample. After deparaffinization, DNA from tumor and matching non-neoplastic tissue samples was collected and prepared by standard phenol/chloroform methods (6).

Polymerase Chain Reaction–LOH Analysis

Fifty-five microsatellite markers covering all of the non-acrocentric chromosome arms were ob-

tained from Research Genetics (Huntsville, AL). Polymerase chain reaction (PCR) was carried out in a mixture of 20 μ l containing 50 ng DNA, 1 \times *Taq* polymerase buffer (Promega, Madison, WI), 1.5 mM MgCl₂, 0.4 pmol of each primer, 0.2 mM of each deoxynucleotide triphosphate, 1.5 μ Ci of [α -³²P]dCTP, and 1.0 unit *Taq* polymerase (Promega). With the use of a thermal cycler (version 2.0; Perkin Elmer Cetus, Norwalk, CT), the reaction mixtures were put through PCR; initial denaturation (94° C for 4 min), 29 to 32 amplification cycles of denaturation (95° C for 30 seconds), annealing (55° C to 60° C for 30 seconds), and extension (72° C for 30 seconds). This was followed by elongation at 72° C for 10 min. One microliter of the PCR product solution was mixed with 2 μ L gel loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), denatured at 95° C for 5 min, and then applied (3 μ L/lane) to denaturing 6% polyacrylamide gel containing 7 M urea for 2 hours using a sequencing-type apparatus (Kodak Biomax STS-45 i Sequencer; Kodak, Rochester, NY). The gel was dried on filter paper and exposed to x-ray film (Kodak XRP-1) at -78° C overnight. LOH was scored after visual estimation by two of the authors (YKK, WHK) independently if the band intensity was reduced by more than 50% by visual estimation in tumor DNA as compared with normal DNA.

RESULTS

Frequency of LOH in ICC

Thirty-six primary ICCs were screened for LOH with 55 microsatellite markers. LOH interpretation was not possible in one case because of widespread microsatellite instability. The average informativeness per marker and chromosomal arm was 64.3% (range, 20 to 85.7%) and 72.2% (range, 34.3 to 97.1%), respectively. LOH was observed on all chromosomal arms. Of the 35 interpreted cases, 34 (97.1%) demonstrated LOH on one or more microsatellite loci, whereas one case (2.9%) did not show LOH on any of the markers. The frequency of LOH at each chromosomal locus varied from 10.5 to 71.4% (Table 1). Representative examples of allelic losses are shown in Figure 1. A high frequency of allelic loss (>60%) was detected on chromosomes 8p (65.6%), 17p (64.7%), and 9p (64.5%). Relatively high frequency loss (40 to 60%) was found on 18q (54.2%), 1p (48.5%), 3p (44.8%), 9q (42.1%), 14q (41.7%), 6q (41.7%), and 1q (40.6%). Intermediate frequency loss (25 to 40%) was found on 2q (25%), 4q (30.8%), 5q (32.4%), 7p (25%), 7q (33.3%), 8q (35%), 11p (35.5%), 12q (35%), 13q (33.3%), 15q (36%), 16q (26.9%), 17q (37.5%), and 20q (28.6%). Other chromosomal arms had an LOH frequency of less than 25% (Fig. 2).

TABLE 1. Microsatellite Markers Used and Their Frequency of Loss of Heterozygosity in Intrahepatic Cholangiocarcinoma

Chromosome Arm and Locus	Location	Allelic Loss/ Informative Cases (%)	Chromosome Arm and Locus	Location	Allelic Loss/ Informative Cases (%)
1p	D1S186	1p35-p32	9p	D9S162	9p23-p22
	D1S162	1p31-p22	9q	D9S103	9q33-qter
1q	D1S237	1q	10p	D10S183	10p
	D1S484	1q	10q	D10S109	10q11-qter
2p	D2S119	2p16	11p	D11S875	11p15-p13
	D2S123	2p16-p21		D11S861	11p15.2
2q	D2S103	2q23-q33	11q	D11S912	11q25
	D2S104	2q33-q37	12p	D12S61	12p12-p11
3p	D3S1274	3p12	12q	D12S95	12q13-q24
	D3S4103	3p14.2	13q	D13S118	13q14.1
3q	D3S1209	3q21-q24	14q	D14S51	14q32
4p	D4S174	4p13-p11	15q	D15S87	15q25-qter
4q	D4S1554	4q	16p	D16S403	16p12.3
5p	D5S406	5p	16q	D16S289	16q23-q24
5q	D5S409	5q21	17p	TP53	17p13.1
	D5S107	5q11-q13		D17S520	17p13-p12
6p	IRF1	5q23-q31		D17S786	17pter-qter
	D6S271	6p21		D17S796	17pter-qter
6q	D6S264	6q25-q27	17q	D17S513	17q12
7p	D7S507	7p15-p21	18p	D18S59	18pter-p11
7q	D7S483	7q31-qter	18q	D18S34	18q12
8p	D8S254	8p22	19p	D19S177	19p13.3
	D8S261	8p22	19q	D19S416	19q13.1
	D8S264	8p23	20p	D20S66	20p12
8q	D8S555	8q23-q24	20q	D20S17	20q12-q13
9p	D9S165	9p21-q21	21q	D21S258	21q11
	D9S171	9p21	22q	IL2RB	22q13
	IFNA	9p22			

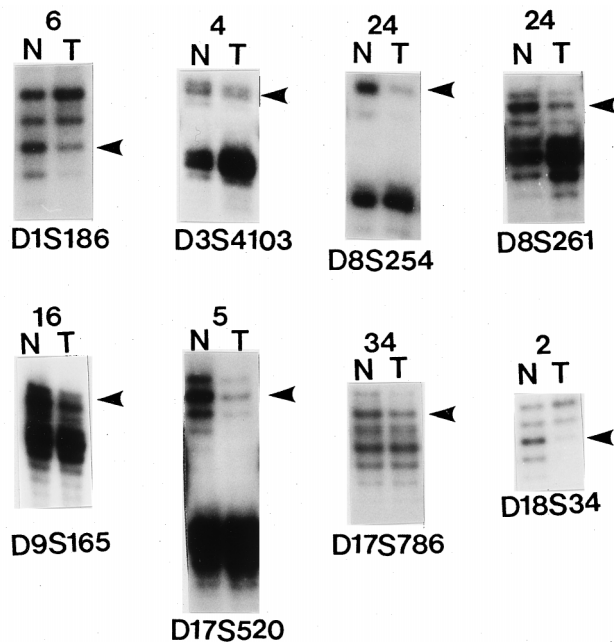


FIGURE 1. Representative examples of loss of heterozygosity in intrahepatic cholangiocarcinoma. Each non-neoplastic tissue (N) and its corresponding tumor (T) are shown with microsatellite markers indicated at the bottom. Case numbers are indicated on the top. The arrowheads indicate the alleles scored as lost.

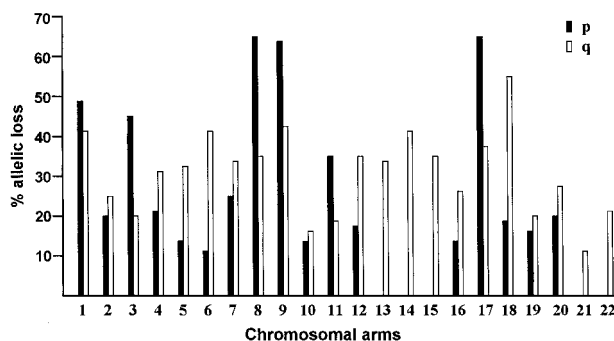


FIGURE 2. Frequency of loss of heterozygosity on each chromosome arm in 35 cases of intrahepatic cholangiocarcinoma. P and q denote short and long arms, respectively.

The three markers on 8p showed an overall high allelic loss with higher frequency at 8p22 (D8S254, D8S261) than at 8p23 (D8S264). The LOH on chromosome 17p was found in 22 of 34 cases (64.7%), and it was more frequent at locus D17S520 at 17p13-p12 than at *TP53* at 17p13.1. Four markers on 9p were located near the *p16* gene locus and revealed an overall high allelic loss. Other markers that showed high allelic loss were D3S4103 (12 of 22 [54.5%]) at fragile histidine triad (*FHIT*) locus (3p14.2) and D18S34 (13 of 24 [54.2%]) at 18q12. We also tried to find any correlation between LOH on different pairs of chromosome arms, and a significant association was noted between 1p and 9p. Among 29 cases of ICC that showed informativeness at both 1p and 9p, 13 cases demonstrated LOH on both arms, 8 cases demonstrated retention of

alleles in both arms, and the remaining 8 cases showed LOH on one arm (1p LOH in 2 cases and 9p LOH in 6 cases) ($P = .02$).

Fractional Allelic Loss in ICC

The fractional allelic loss (FAL) in a tumor was defined as the number of chromosomal arms on which allelic loss was observed divided by the number of chromosomal arms for which allelic markers were informative (14). The number of informative chromosomal arms was 23 or more in each case. The FAL values varied among the 35 cases, ranging from 0 to 0.731 with a median value of 0.273 and a mean of 0.322. Possible associations between FAL values and LOH at specific chromosome arms were investigated, and LOH at 1p, 3p, 8p, 9p, 14q, 17p, and 18q showed a significant association with high FAL values (Table 2).

We compared the FAL values between cases with or without *p53* mutation/overexpression, *K-ras* codon 12 mutation, and LOH on *APC* (6). Cases with *p53* mutation showed higher FAL value than cases without *p53* mutation (0.47 versus 0.26; $P = .0002$). Cases with LOH on *APC* also showed high FAL value (0.58 versus 0.25; $P = .0003$). In contrast, cases with *K-ras* code 12 mutation showed lower FAL value than cases without such mutation (0.17 versus 0.37; $P = .006$).

We also tried to determine the significance of FAL values in association with clinicopathologic parameters including age, sex, tumor size, histologic type, degree of differentiation, tumor location, and gross type. The only significant relationship found was with histologic differentiation. The moderately or

TABLE 2. Association Between the FAL Value and LOH on Specific Arms

Chromosome Arm	LOH	Mean FAL Value	p Value ^a
1p	+	0.391 ± 0.206	0.017
	-	0.238 ± 0.135	
1q	+	0.399 ± 0.209	NS
	-	0.280 ± 0.168	
3p	+	0.429 ± 0.205	0.012
	-	0.244 ± 0.165	
6q	+	0.367 ± 0.220	NS
	-	0.279 ± 0.107	
8p	+	0.393 ± 0.197	0.016
	-	0.217 ± 0.157	
9p	+	0.409 ± 0.200	0.002
	-	0.190 ± 0.108	
9q	+	0.316 ± 0.155	NS
	-	0.283 ± 0.189	
14q	+	0.468 ± 0.166	0.001
	-	0.240 ± 0.129	
17p	+	0.398 ± 0.188	0.001
	-	0.177 ± 0.124	
18q	+	0.421 ± 0.188	0.002
	-	0.200 ± 0.088	

FAL, fractional allelic loss; LOH, loss of heterozygosity; NS, not significant.

^a Based on one-way analysis of variance F test.

poorly differentiated ICCs showed significantly higher FAL values (mean, 0.358) than the well-differentiated ICCs (mean, 0.192) ($P = .047$) (Table 3). Although papillary and mucinous carcinoma showed lower FAL values (mean, 0.167 and 0.156, respectively) than tubular carcinoma (mean, 0.343), no significant association was demonstrated.

DISCUSSION

This paper represents the first study of genome-wide allelic loss in ICC. Ding *et al.* (5) first reported the LOH of 14 ICC cases using Southern analysis with 22 restriction fragment length polymorphism markers on 16 chromosomal arms. In their study, the informativeness was high (71.6%) but the LOH was found only on 7 of 16 arms (1p, 1q, 5q, 7p, 9q, 12q, 17p), and the mean LOH frequency was low (10.4%), ranging from 7.7 to 44.4%. In this study, we performed LOH on all 39 chromosomal arms by using a PCR with highly polymorphic microsatellite markers after careful microdissection, and we observed higher frequency of LOH (32.2%).

Most of the sites of allelic loss identified in this study corresponded to known or suspected tumor suppressor loci. LOH occurs most frequently on chromosomes 8p, 17p, and 9p. Frequent LOH on 8p has been found to occur in several human cancers, including colorectal, bladder, prostate, lung, ovary, breast, and liver cancers (14–19). Neither a consensus area of chromosomal loss nor a specific tumor suppressor gene has been recognized, yet the 8p21-p23 has been implicated most frequently (16, 19).

Our data showed frequent LOH of 8p with a possible localization at 8p22. This suggests a presence of candidate tumor suppressor gene in this locus. LOH of 17p has been reported in many malignant tumors and is thought to be associated with the inactivation of the *p53* gene. In ICC, 17p allelic loss of more than 40% has been reported (5). In our study, the frequency of allelic loss was also high. However, it was not localized only on the *p53* locus. An even higher frequency of loss was noted on markers around *p53* than on intragenic TP53 marker. It supported the presence of a second tumor suppressor gene on 17p, as suggested in other malignant tumors (20, 21). The high frequency of allelic loss of 9p around 9p21 in our study, which is the same as in previous reports, suggests that the *p16* gene might be involved in cholangiocarcinogenesis (12). Our study also showed frequent LOH on 18q12 near the *DCC* or *DPC4/SMAD4* tumor suppressor genes and 3p14.2 at the *FHIT* locus. The *FHIT* is a recently identified gene that spans the fragile site locus at 3p14.2. It has been proposed as a candidate tumor suppressor gene because of its frequent alteration in a variety of human tumors, including lung, renal, nasopharyngeal, breast, stomach, and pancreatic carcinomas (22–24). In the present study, the LOH of 3p was localized on the *FHIT* locus, which supports the suppressive role of *FHIT* implicated in cholangiocarcinogenesis. We also found associations between losses in 1p and 9p, indicating the possible cooperation of corresponding genes in the tumorigenesis of ICC.

The extent of allelic loss in a tumor can be estimated by FAL, and an increased FAL value has been reported to be related to aggressive tumor behavior in colorectal carcinoma (14). The calculated FAL value (0.322) in the 35 ICCs in our study was higher than the FAL value for colorectal (14), stomach (25), non-small cell lung (26), and hepatocellular carcinoma (18). This might partly explain the aggressive behavior of ICC. However, the large variations in FAL observed for each tumor type and the differences in various experimental settings preclude the conclusion that these differences reflect distinct mechanisms in tumor development.

We found that LOH of 1p, 3p, 8p, 9p, 14q, 17p, and 18q all showed a significant association with high FAL values. These findings suggest that allelic losses on these chromosome arms might play an important role in the development and progression of ICC. We also compared the FAL values with our previously reported data about *p53* mutation/overexpression, *K-ras* codon 12 mutation, and *APC* LOH in ICC (6). The significant correlation of high FAL values in cases with *p53* mutation and LOH on *APC* was as expected. However, there was an inverse relationship between FAL values and *K-ras* mutation. Because no difference in *K-ras* mutation be-

TABLE 3. Association Between the FAL Value and Clinicopathologic Parameters

Clinicopathologic Parameters	Mean FAL Value	<i>p</i> Value ^a
Sex		
Male (<i>n</i> = 29)	0.350 ± 0.209	NS
Female (<i>n</i> = 6)	0.186 ± 0.068	
Age		
>50 (<i>n</i> = 22)	0.280 ± 0.179	NS
≤50 (<i>n</i> = 13)	0.394 ± 0.207	
Size		
>5 cm (<i>n</i> = 17)	0.327 ± 0.203	NS
≤5 cm (<i>n</i> = 18)	0.322 ± 0.194	
Histology		
Tubular (<i>n</i> = 30)	0.343 ± 0.179	NS
Papillary (<i>n</i> = 2)	0.167 ± 0.152	
Mucinous (<i>n</i> = 3)	0.156 ± 0.146	
Differentiation		
Well (<i>n</i> = 8)	0.192 ± 0.141	0.047
Moderate or poor (<i>n</i> = 27)	0.358 ± 0.195	
Location		
Hilar (<i>n</i> = 10)	0.307 ± 0.238	NS
Peripheral (<i>n</i> = 25)	0.329 ± 0.180	
Gross type		
Mass forming (<i>n</i> = 9)	0.376 ± 0.218	NS
Spicula forming (<i>n</i> = 21)	0.322 ± 0.182	
Periductal (<i>n</i> = 5)	0.226 ± 0.209	

FAL, fractional allelic loss; NS, not significant.

^aBased on one-way analysis of variance F test.

tween low-FAL groups and high-FAL groups has been reported in colorectal carcinomas (14), it is unclear whether this inverse relationship accounts for distinctive pathways in cholangiocarcinogenesis. This warrants further study. The comparative analysis with clinicopathologic parameters disclosed a significant association between FAL values and tumor differentiation. This may partly explain the relatively favorable prognosis of well-differentiated ICCs, such as papillary adenocarcinoma.

In summary, the first comprehensive allelotype study of ICC using microsatellite markers defined chromosomal arms showing frequent LOH and FAL values. Further studies, using fine focusing to identify the locations of tumor suppressor genes, will be required in order to understand the molecular mechanisms of carcinogenesis in ICC.

Acknowledgment: We are grateful to Ms. Sun Hee Kim for her excellent technical assistance.

REFERENCES

- Kim YI, Park CK, Kim JR, Chang JJ. Primary malignant epithelial neoplasms of the liver. *Korean J Cancer Res* 1980;12:33-53.
- Shin HR, Lee CU, Park HJ, Seol SY, Chung JM, Choi HC, *et al.* Hepatitis B, and C virus, *Clonorchis sinensis* for the risk of liver cancer: a case-control study in Busan, Korea. *Int J Epidemiol* 1996;25:933-40.
- Ohashi K, Nakajima Y, Tsutsumi M, Kanehiro H, Fukuoka T, Hisanaga M, *et al.* Clinical characteristics and proliferating activity of intrahepatic cholangiocarcinoma. *J Gastroenterol Hepatol* 1994;9:442-6.
- The Liver Cancer Study Group of Japan. Primary liver cancer in Japan. Clinicopathologic features and results of surgical treatment. *Ann Surg* 1990;211:277-81.
- Ding SF, Delhanty JD, Bowles L, Dooley JS, Wood CB, Habib NA. Loss of constitutional heterozygosity on chromosomes 5 and 17 in cholangiocarcinoma. *Br J Cancer* 1993;67:1007-10.
- Kang YK, Kim WH, Lee HW, Lee HK, Kim YI. Mutation of p53 and K-ras, and loss of heterozygosity of APC in intrahepatic cholangiocarcinoma. *Lab Invest* 1999;79:477-83.
- Kiba T, Tsuda H, Pairojkul C, Inoue S, Sugimura T, Hirohashi S. Mutations of the p53 tumor suppressor gene and the ras gene family in intrahepatic cholangiocellular carcinomas in Japan and Thailand. *Mol Carcinog* 1993;8:312-8.
- Levi S, Urbano-Ispizua A, Gill R, Thomas DM, Gilbertson J, Foster C, *et al.* Multiple *K-ras* codon 12 mutations in cholangiocarcinomas demonstrated with a sensitive polymerase chain reaction technique. *Cancer Res* 1991;51:3497-502.
- Ohashi K, Nakajima Y, Kanehiro H, Tsutsumi M, Taki J, Aomatsu Y, *et al.* Ki-ras mutations and p53 protein expressions in intrahepatic cholangiocarcinomas: relation to gross tumor morphology. *Gastroenterology* 1995;109:1612-7.
- Tada M, Omata M, Ohto M. High incidence of ras gene mutation in intrahepatic cholangiocarcinoma. *Cancer* 1992;69:1115-8.
- Tsuda H, Satarug S, Bhudhisawasdi V, Kihana T, Sugimura T, Hirohashi S. Cholangiocarcinomas in Japanese and Thai patients: difference in etiology and incidence of point mutation of the c-Ki-ras proto-oncogene. *Mol Carcinog* 1992;6:266-9.
- Yoshida S, Todoroki T, Ichikawa Y, Hanai S, Suzuki H, Hori M, *et al.* Mutations of p16^{INK4}/CDKN2 and p15^{INK4B}/MTS2 genes in biliary tract cancers. *Cancer Res* 1995;55:2756-60.
- Knudson AG. Antioncogenes and human cancer. *Proc Natl Acad Sci U S A* 1993;90:10914-21.
- Vogelstein B, Fearon ER, Kern SE, Hamilton SR, Preisinger AC, Nakamura T, *et al.* Allelotype of colorectal carcinomas. *Science* 1989;244:217-21.
- Anbazhagan R, Fujii H, Gabrielson E. Allelic loss of chromosomal arm 8p in breast cancer progression. *Am J Pathol* 1998;152:815-9.
- Bova GS, Carter BS, Bussemakers MJ, Emi M, Fujiwara Y, Kyprianou N, *et al.* Homozygous deletion and frequent allelic loss of chromosome 8p22 loci in human prostate cancer. *Cancer Res* 1993;53:3869-73.
- Knowles MA, Shaw ME, Proctor AJ. Deletion mapping of chromosome 8 in cancers of the urinary bladder using restriction fragment polymorphisms and microsatellite polymorphisms. *Oncogene* 1993;8:1357-64.
- Piao Z, Park C, Park JH, Kim H. Allelotype analysis of hepatocellular carcinoma. *Int J Cancer* 1998;75:29-33.
- Wistuba II, Behrens C, Virmani AK, Milchgrub S, Syed S, Lam S, *et al.* Allelic losses at chromosome 8p21-23 are early and frequent events in the pathogenesis of lung cancer. *Cancer Res* 1999;59:1973-9.
- Dolan K, Garde J, Gosney J, Sissons M, Wright T, Kingsnorth AN, *et al.* Allelotype analysis of oesophageal adenocarcinoma: loss of heterozygosity occurs at multiple sites. *Br J Cancer* 1998;78:950-7.
- Kim CJ, Kim WH, Kim CW, Lee JB, Lee CK, Kim YI. Detection of 17p loss in gastric carcinoma using polymerase chain reaction. *Lab Invest* 1995;72:232-6.
- Le Beau MM, Drabkin H, Glover TW, Gemmill R, Rassool FV, McKeithan TW, *et al.* An *FHIT* tumor suppressor gene? *Genes Chromosomes Cancer* 1998;21:281-9.
- Ohta M, Inoue H, Cotticelli M, Kastury K, Baffa R, Palazzo J, *et al.* The *FHIT* gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma-associated t(3;8) breakpoint, is abnormal in digestive tract cancers. *Cell* 1996;84:587-97.
- Sorio C, Baron A, Orlandini S, Zamboni G, Pederzoli P, Huebner K, *et al.* The *FHIT* gene is expressed in pancreatic ductular cells and is altered in pancreatic cancers. *Cancer Res* 1999;59:1308-14.
- Choi SW, Park SW, Lee KY, Kim KM, Chung YJ, Rhyu MG. Fractional allelic loss in gastric carcinoma correlates with growth patterns. *Oncogene* 1998;17:2655-9.
- Tsuchiya E, Nakamura Y, Weng SY, Nakagawa K, Tsuchiya S, Sugano H, *et al.* Allelotype of non-small cell lung carcinoma—comparison between loss of heterozygosity in squamous cell carcinoma and adenocarcinoma. *Cancer Res* 1992;52:2478-81.