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Localization of a Target Region of Allelic Loss to a 1-cM Interval on Chromosome 16p.13.13 in Hepatocellular Carcinoma

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To identify the location of the putative tumor suppressor gene on chromosome 16p that may be involved in hepatocellular carcinoma (HCC), we examined 96 primary HCCs and evaluated their patterns of allelic loss at 10 microsatellite marker loci distributed along this chromosome arm. Allelic loss at one or more loci was observed in 46 (48%) of these tumors. Through detailed deletion mapping of tumors having partial or interstitial deletions, we identified a commonly deleted region at a 1-cM interval, flanked by D16S519 and D16S3078 at 16p13.13, defining the location of a putative tumor suppressor gene for HCC. This region contains the gene for JAB (JAK-binding protein), which is responsible for negative-feedback regulation of the JAK-STAT pathway induced by cytokine stimulation, raising the possibility that inactivation of this gene may participate in hepatocarcinogenesis via genetic and/or epigenetic changes.

Key words: Hepatocellular carcinoma — Chromosome 16p — Loss of heterozygosity — Tumor suppressor gene

Hepatocellular carcinoma (HCC) is one of the most common human cancers throughout the world and particularly in certain areas of Africa and Asia. The majority of HCCs are associated with infection with HBV or/and HCV in Japan. It is well known clinicopathologically that persistent infection with HBV or/and HCV leads to liver cirrhosis (LC) and HCC.[1,2] However, the molecular mechanism of hepatocarcinogenesis still remains to be determined. It has been hypothesized that integration of HBV DNA causes an alteration of the host genome DNA, or X gene of HBV suppresses the function of *p53* gene.[3–5] On the other hand, integration of HCV DNA has not been reported, and to clarify the mechanism of the transformation caused by HCV, the interaction of cellular proteins and HCV-proteins needs to be analyzed. A common feature in chronic viral hepatitis and liver cirrhosis is long-lasting inflammation of the liver; as a consequence, chronic regenerative conditions might enhance the susceptibility of liver cells to genetic changes leading to the accelerated growth of the hepatocytes.

Genetic changes accumulate in the course of the development and subsequent progression of most human tumors. Several of these events involve activation of protooncogenes and inactivation of tumor suppressor genes.[6] Inactivation of tumor-suppressor function usually occurs as a consequence of mutation of one allele and loss of the other allele through aberrant somatic events.[7] Therefore, when allelic losses at specific chromosomal loci (loss of heterozygosity, LOH) are observed frequently in a particular type of human cancer, one can infer that under normal conditions putative tumor suppressor genes would present in the regions where the deletions were detected. In HCCs, LOH has been described on chromosome arms 1p, 4q, 5q, 6q, 8p, 10q, 11p, 16p, 16q, 17p and 22q.[8–18]

Chromosomal arm 16p has been analyzed in HCCs only with a single marker D16S407 in previous allelotype studies.[16,17] In the present study, we constructed a detailed deletion map of 16p in 96 HCCs by taking advantage of a high-resolution chromosome map of the region consisting of 10 marker loci, and examined possible correlation with several clinico-pathological parameters.

MATERIALS AND METHODS

Samples and DNA preparation Tumor samples and corresponding non-cancerous tissues were obtained from 96 patients with primary HCC who underwent surgery. Informed consent was obtained from all participating patients prior to surgery. Portions of the tissues were frozen immediately after surgery and stored at −80°C. DNAs were extracted according to procedures described previously.[19]

LOH analysis Ten microsatellite markers that had been described in the Genethon linkage map[20] covering the length of chromosome arm 16p were used for LOH analysis in the present study. The names of the polymorphic microsatellites, and their linear order based on the consen-

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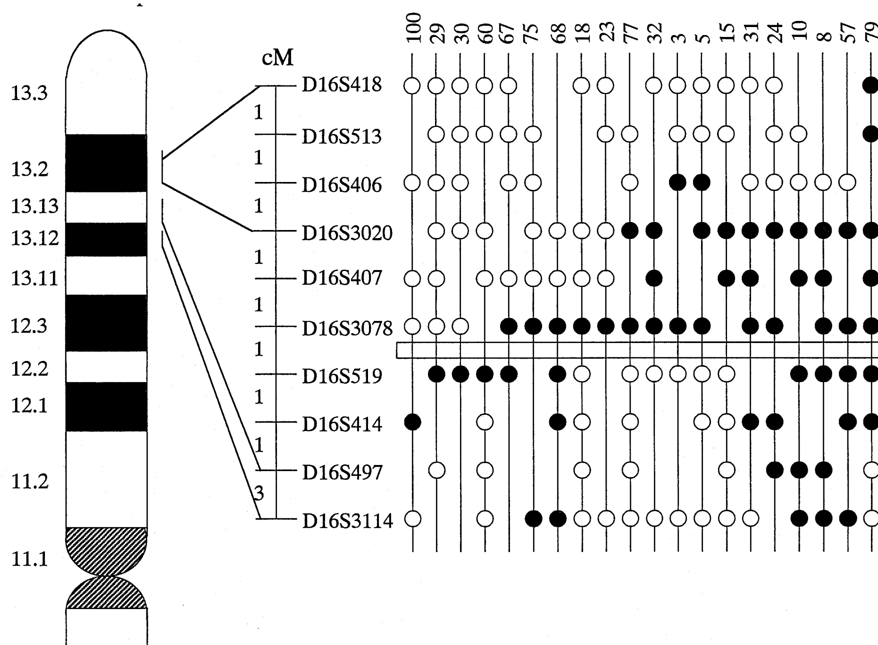

Fig. 1. Schematic representation of deletion mapping among 20 informative tumors that showed partial or interstitial deletions on 16p in HCCs. The karyograms show the location of each microsatellite marker used in the study. Numbers at the top of each column identify the tumors. A blank space indicates uninformativeness of the relevant marker in each case. The commonly deleted region is indicated by a rectangle superimposed on the map. Filled circles, LOH; open circles, retention of both alleles in the tumor; blank spaces, uninformative loci.

sus genetic and physical maps of 16p, are shown in Fig. 1. Microsatellite repeats were amplified by polymerase chain reaction (PCR) as previously described.[21] PCR products were electrophoresed, transferred to filters, dried at 80°C, and exposed to autoradiographic film as previously described.[22]

Definition of LOH Signal intensities of polymorphic alleles were quantified by a Hoefer GS-300 scanning densitometer; peak areas corresponding to each signal were calculated by electronic integration using a GS-370 electrophoresis data system (Hoefer Scientific Instruments, San Francisco, CA). When the signal intensities of alleles of tumor-tissue DNAs were compared with those of corresponding normal-tissue DNAs, a reduction in signal intensity >50% was interpreted as LOH.[22] We distinguished LOH from chromosome multiplication by normalizing each signal to the signal obtained when the same DNA was analyzed with markers for loci on other chromosomes.

Clinicopathological parameters The following parameters were studied: tumor stage, histologic type, tumor size, pathologic state of surrounding non-tumorous liver and serum markers for hepatitis virus (HBsAg or HCVAb). The tumor stage for each case was determined according to the TNM classification.[23] Histological grades of HCC were divided into three categories (well-differentiated, moderately differentiated, and poorly differentiated carcinoma), according to the typing scheme of the Japanese Liver Cancer Society. These three groups correspond to grades I, II, III+IV, respectively, of the Edmondson-Steiner classification.[24] The χ^2 test or Fisher's exact test was used for statistical analysis of the results. *P* values of less than 0.05 were considered statistically significant.

Table I. Frequencies of LOH at 10 Loci on Chromosome Arm 16p

Name	Total	Informative	LOH	LOH/informative (%)
D16S418	96	71	14	20
D16S513	96	50	8	16
D16S406	96	63	19	30
D16S3020	96	69	22	32
D16S407	96	68	20	29
D16S3078	96	64	25	39
D16S519	96	68	20	29
D16S414	96	47	12	26
D16S497	96	53	12	23
D16S3114	96	74	18	24
Total	96	96	46	48

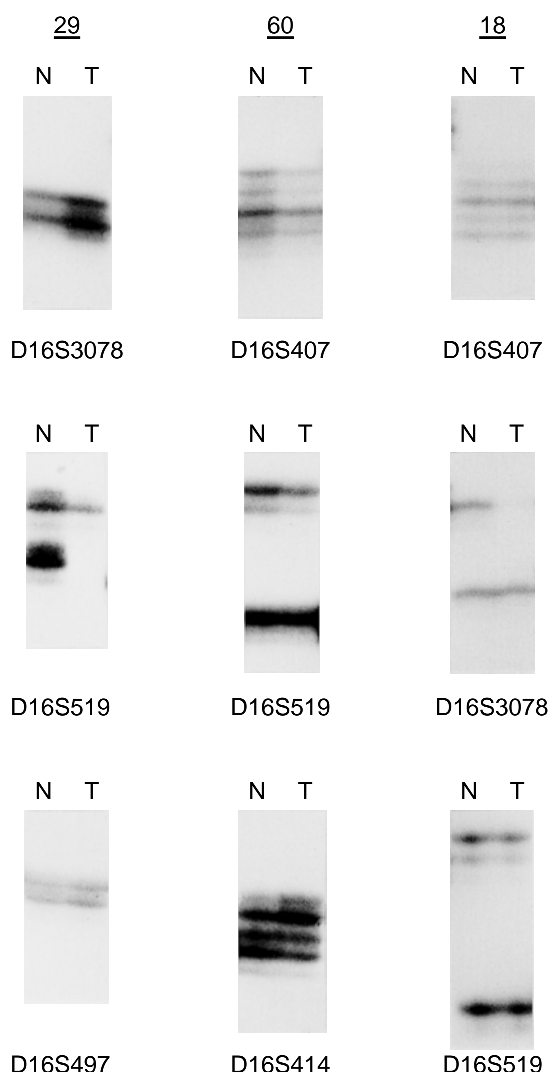

Fig. 2. Representative autoradiograms of normal and tumor genotypes that determined the commonly deleted regions at 16p13.13. Case numbers are shown on top of the panels. The microsatellite markers yielding these results are indicated beneath the respective autoradiographic images. N and T, matched DNA samples isolated from normal and tumor tissues, respectively.

RESULTS

Of the 96 HCCs analyzed, 46 tumors (48%) presented LOH with at least one of the 10 polymorphic microsatellite markers on chromosome 16p. The marker loci and frequencies are listed in Table I, in descending order from the telomere to centromere. The LOH frequencies ranged from 16% at D16S513 to 39% at D16S3078. The highest frequency of LOH (39%) was observed with marker D16S3078 at chromosomal band 16p13.13. Among the 46 tumors with LOH, 20 showed partial or interstitial deletions. The other 26 tumors showed LOH at all informative loci.

Representative autoradiograms of cases that revealed interstitial deletions of the 16p 13.13 region are presented in Fig. 2. Tumor 29 showed LOH at D16S519, but retained alleles at flanking loci, D16S3078 and D16S497. Tumor 60 showed LOH at D16S519, but retained heterozygosity for D16S407 and D16S414. Tumor 18 showed LOH at D16S3078, but retained both alleles of D16S407 and D16S519.

The results of deletion mapping with the 20 tumors that showed partial or interstitial deletions are summarized in Fig. 1. The proximal limit of the commonly deleted region was defined by D16S519, on the basis of observations in six tumors (3, 5, 15, 18, 32, and 77) that retained heterozygosity at the D16S519 locus while showing LOH at more distal loci. The distal limit was defined by D16S3078; three tumors (29, 30, and 100) retained heterozygosity at D16S3078 while showing LOH at more proximal loci. Hence, we defined the commonly deleted region on band 16p13.13, within a 1-cM interval flanked by D16S519 and D16S3078.

We compared LOH at 16p13.13 with clinicopathological parameters (tumor size, histologic types, staging, status of background liver disease) in all tumors for which these data were available (Table II). As shown in the table,

Table II. LOH on 16p13.13 and Clinicopathological Parameters[a)]

	LOH	Retention	Statistical significance
Tumor size			
2cm≥	2	9	
2cm<	25	37	NS
Histologic type[b)]			
Well diffe.	6	6	
Moderate diffe.	4	5	NS
Poor diffe.	5	10	(Well vs. moderate, poor)
Stage[c)]			
I	1	7	
II	13	17	NS
III	9	14	(Stage I, II vs. III, IV)
IV	2	2	
Liver state[d)]			
CH	10	17	
LC	16	26	NS

a) Of the total of 96 tumors, information on tumor size, histologic type, stage, and liver state was available in 73, 36, 65, and 69 cases, respectively.
b) Histologic type as defined by the Japanese Liver Cancer Society.
c) TNM classification.
d) CH, chronic hepatitis; LC, liver cirrhosis.

allelic loss at 16p13.13 was frequent in small, well-differentiated and less-invasive tumors as well as in tumors of more advanced phenotype. No significant difference in frequencies of LOH at 16p13.3 or 16p were observed when any of different groups of histologic types or stages were compared (e.g. well-differentiated vs. moderately or poorly differentiated histologic types, or, stage I, II vs. stage III, IV).

DISCUSSION

We present here the results of high-resolution deletion mapping of 96 HCCs using 10 microsatellite loci on chromosomal arm 16p. Allelic loss was observed in 48% of the tumors in our panel. We identified a novel target region of common deletion on 16p which was localized to the 1-cM interval between D16S519 and D16S3078 at 16p13.13. To our knowledge, detailed deletion mapping on chromosomal arm 16p has not yet been described in HCCs or other types of cancer. A comparative genomic hybridization study recently carried out by Marchio *et al.* demonstrated the presence of frequent loss of chromosome arm 16p in primary HCCs.[36] Their results support our finding that loss, not gain, is the major alteration in primary HCCs found on chromosome arm 16p.

Frequencies of LOH found on 16p in HCCs range from 6 to 83% among researchers who carried out allelotyping independently.[10, 11, 16, 17, 25–28] In the present study, we detected LOH in 46 of 96 HCCs (48%) by using 10 polymorphic markers. Nagai *et al.*[16] and Vale'rie *et al.*[17] detected LOH in 36% and 40% of HCCs, respectively, using microsatellite marker D16S407 located at 16p13.13. We observed LOH in 29% of HCCs with the same marker.

Several candidate genes involved in tumorigenesis or the regulation of the cell cycle have been localized on chromosomal arm 16p, such as TSC2 at 16p13.3,[29] UBE2I at 16p13.3,[30] XPF at 16p13.2–p13.1,[31] and GSPT1 at 16p13.1,[32] although no direct evidence has yet been demonstrated for a role of these genes in hepatocarcinogenesis. In addition, several genes or expressed sequence tags (ESTs) were mapped in the vicinity of the commonly deleted region defined in the present study; these includes pyrroline-5-carboxylate dehydrogenase, calcium-regulated heat-stable protein, N-methyl-D-aspartate receptor modulator, LPS-induced TNF-α factor, and epithelial membrane 2. Information on these or other ESTs are available in a New Gene Map of the Human Genome at http://www.ncbi.nlm.nih.gov/genemap. A prime candidate for a tumor suppressor gene, JAB, encoding an SH2-domain-containing protein named JAK-binding protein, was recently identified at 16p13.13, within the 1-cM interval described in the present study. The proliferation and differentiation of cells of many lineages is known to be regulated by various cytokines. Many cytokines exert their biological effect through a unique signalling cascade, the JAK-STAT pathway, which induces the activation of target genes in the cell nucleus. Interaction of JAB and members of the JAK family reduces the tyrosine-kinase activity of these products and suppresses the tyrosine-phosphorylation and activation of STATs.[33] JAB was recently shown to be responsible for negative-feedback regulation of the JAK-STAT pathway induced by cytokine stimulation.[34, 35] It would be intriguing to elucidate whether silencing of the JAB locus influences the process of uncontrolled cell proliferation that takes place during hepatocellular carcinogenesis; various genetic and epigenetic mechanisms of gene silencing need to be scrutinized, including gene deletions, rearrangements, point mutations, aberrant mRNA splicing or post-transcriptional processing, as well as inadequate methylation.

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REFERENCES

1) Poynard, T., Bedossa, P., Opolon, P., for the OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. Natural history of liver fibrosis progression in patients with chronic hepatitis C. *Lancet*, **349**, 825–832 (1997).
2) Okuda, K. Hepatocellular carcinoma: recent progress. *Hepatology*, **15**, 948–963 (1992).
3) Matsubara, K. and Tokino, T. Integration of hepatitis B virus DNA and its implications for hepatocarcinogenesis. *Mol. Biol. Med.*, **7**, 243–260 (1990).
4) Wang, X. W. Abnormal structure and expression of p53 gene in human hepatocellular carcinoma. *Proc. Natl. Acad. Sci. USA*, **91**, 2230–2234 (1994).
5) Takada, S. Cytoplasmic retention of p53 tumor suppressor gene product is observed in the hepatitis B virus X gene-transfected cells. *Oncogene*, **15**, 1895–1901 (1997).
6) Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smits, A. M. and Bos, J. L. Genetic alterations during colorectal-tumor development. *N. Engl. J. Med.*, **319**, 525–532 (1998).
7) Knudson, A. G. Antioncogenes and human cancer. *Proc. Natl. Acad. Sci. USA*, **90**, 10914–10921 (1993).

8) Wang, H. P. and Rogler, C. E. Deletions in human chromosome arms 11p and 13q in primary hepatocellular carcinomas. *Cytogenet. Cell Genet.*, **48**, 72–78 (1988).
9) Buetow, K. H., Murray, J., Israel, J., London, W., Smith, M., Kew, M., Blanquet, V., Brechot, C., Redeker, A. and Govindarajah, S. Loss of heterozygosity suggests tumor suppressor gene responsible for primary hepatocellular carcinomas. *Proc. Natl. Acad. Sci. USA*, **89**, 9622–9626 (1992).
10) Tsuda, H., Zang, W., Shimamoto, Y., Yokota, J., Terada, M., Sugimura, T., Miyamura, T. and Hirohashi, S. Allele loss on chromosome 16 associated with progression of human hepatocellular carcinoma. *Proc. Natl. Acad. Sci. USA*, **87**, 6791–6794 (1990).
11) Fujimori, M., Tokino, T., Hino, O., Kitagawa, T., Imamura, T., Okamoto, E., Mitsunobu, M., Ishikawa, T., Nakagawa, H., Harada, H., Yagura, M., Matsubara, K. and Nakamura, Y. Allelotype study of primary hepatocellular carcinoma. *Cancer Res.*, **51**, 89–93 (1991).
12) Emi, M., Fuziwara, Y., Nakajima, T., Tsuchiya, E., Tsuda, H., Hirohashi, S., Maeda, Y., Tsuruta, K., Miyaki, M. and Nakamura, Y. Frequent loss of heterozygosity for loci on chromosome 8p in hepatocellular carcinoma, colorectal cancer, and lung cancer. *Cancer Res.*, **52**, 5368–5372 (1992).
13) Takahashi, K., Kudo, J., Ishibashi, H., Hirata, Y. and Niho, Y. Frequent loss of heterozygosity on chromosome 22 in hepatocellular carcinoma. *Hepatology*, **17**, 794–799 (1993).
14) Yeh, S. H., Chen, P. J., Chen, H. L., Lai, M. Y., Wang, C. C. and Chen, D. S. Frequent genetic alteration at the distal region of chromosome 1p in human hepatocellular carcinoma. *Cancer Res.*, **54**, 4188–4192 (1994).
15) De Souza, A. T., Hankins, G. R., Washington, M. K., Orton, T. C. and Jirtle, R. L. M6P/IGF2R gene is mutated in human hepatocellular carcinomas with loss of heterozygosity. *Nat. Genet.*, **11**, 447–449 (1995).
16) Nagai, H., Pineau, P., Tiollais, P., Buendia, M. A. and Dejean, A. Comprehensive allelotyping of human hepatocellular carcinoma. *Oncogene*, **14**, 2927–2933 (1997).
17) Vale'rie, B., Pierre, L. P., Pierre, F., Jean, F. F., Genevieve, M., Pierre, B., Paulette, B. S., Frederique, C., Anette, S., Sylviane, O. and Gilles, T. Concerted nonsyntenic allelic losses in hyperploid hepatocellular carcinoma as determined by a high-resolution allelotype. *Cancer Res.*, **15**, 1986–1990 (1997).
18) Piao, Z., Park, C., Park, J. H. and Kim, H. Allelotype analysis of hepatocellular carcinoma. *Int. J. Cancer*, **75**, 29–33 (1998).
19) Shirahama, S., Ogura, K., Takami, H., Ito, K., Tohsen, T., Miyauchi, A. and Nakamura, Y. Mutational analysis of the RET proto-oncogene in 71 Japanese patients with medullar thyroid carcinoma. *J. Hum. Genet.*, **43**, 101–106 (1998).
20) Dib, C., Faure, S., Fizames, C., Samson, D., Drouot, N., Vignal, A., Millasseau, P., Marc, S., Hazan, J., Seboun, E., Lathrop, M., Gyapay, G., Morissette, J. and Weissenbach, J. A comprehensive genetic map of the human genome based on 5,264 microsatellites. *Nature*, **380**, 152–154 (1996).
21) Tsukamoto, K., Haruta, K., Shiba, T. and Emi, M. Isolation and mapping of a polymorphic CA repeat sequence at human interleukin 6 locus. *J. Hum. Genet.*, **43**, 71–72 (1998).
22) Iida, A., Isobe, R., Yoshimoto, M., Kasumi, F. and Emi, M. Localization of a breast cancer tumor-suppressor gene to a 3-cM interval within chromosomal region 16q22. *Br. J. Cancer*, **75**, 264–267 (1997).
23) Hermanek, P., Sobin, L. A. and Sun, T. "TNM Classification of Malignant Tumors," 4th Ed., UICC (1987). Springer, Berlin.
24) Edmondson, H. and Steiner, P. Primary carcinoma of the liver. A study of 100 cases among 48,900 necropsies. *Cancer*, **7**, 462–503 (1954).
25) Zhang, W., Hirohashi, S., Tsuda, H., Shimosato, Y., Yokota, J., Terada, M. and Sugimura, T. Frequent loss of heterozygosity on chromosomes 16 and 4 in human hepatocellular carcinoma. *Jpn. J. Cancer Res.*, **81**, 108–111 (1990).
26) Slagle, B. L., Zhou, Y. Z., Birchmeier, W. and Scorsone, K. A. Deletion of the E-cadherin gene in hepatitis B virus-positive Chinese hepatocellular carcinomas. *Hepatology*, **18**, 757–762 (1993).
27) Slagle, B. L., Zhou, Y. Z. and Butel, J. S. Hepatitis B virus integration event in human chromosome 17p near the p53 gene identified the region of the chromosome commonly deleted in virus-positive hepatocellular carcinomas. *Cancer Res.*, **51**, 49–54 (1991).
28) Kanai, Y., Ushijima, S., Tsuda, H., Sakamoto, M., Sugimura, T. and Hirohashi, S. Aberrant DNA methylation on chromosome 16 is an early event in hepatocarcinogenesis. *Jpn. J. Cancer Res.*, **87**, 1210–1217 (1996).
29) Green, A. J., Smith, M. and Yates, J. R. Loss of heterozygosity on chromosome 16p13.3 in hamartomas from tuberous sclerosis patients. *Nat. Genet.*, **6**, 193–196 (1994).
30) Tachibana, M., Iwata, N., Watanabe, A., Nobukuni, Y., Ploplis, B. and Kajigaya, S. Assignment of the gene for a ubiquitin-conjugating enzyme (UBE2I) to human chromosome band 16p13.3 by *in situ* hybridization. *Cytogenet. Cell Genet.*, **75**, 222–223 (1996).
31) Sijbers, A. M., de Laat, W. L., Ariza, R. R., Biggerstaff, M., Wei, Y. F., Moggs, J. G., Carter, K. C., Shell, B. K., Evans, E., De Jong, M. C., Rademakers, S., De Rooij, J., Taspers, N. G. J., Hoeijmakers, J. H. J. and Wood, R. D. Xeroderma pigmentosum group F caused by a defect in a structure-specific DNA repair endonuclease. *Cell*, **86**, 811–822 (1996).
32) Ozawa, K., Murakami, Y., Yokoyama, K., Soeda, E., Hoshino, S., Ui, M. and Hanaoka, F. Mapping of the human GSPT1 gene, a human homolog of the yeast GST1 gene, to chromosomal band 16p13.1. *Somat. Cell Mol. Genet.*, **18**, 189–194 (1992).
33) Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Matsumoto, A., Tanimura, S., Ohtsubo, M., Misawa, H., Miyazaki, T., Leonor, N., Taniguchi, T., Fujita, T., Kanakura, Y., Komiya, S. and

Yoshimura, A. A new protein containing an SH2 domain that inhibits JAK kinases. *Nature*, **387**, 921–924 (1997).
34) Naka, T., Narazaki, M., Hirata, M., Matsumoto, T., Minamoto, S., Aono, A., Nishimoto, N., Kajita, T., Taga, T., Yoshizaki, K., Akira, S. and Kishimoto, T. Structure and function of a new STAT-induced STAT inhibitor. *Nature*, **387**, 924–929 (1997).
35) Kuroki, T., Fujiwara, Y., Tsuchiya, E., Nakamori, S., Imaoka, S., Kanematsu, T. and Nakamura, Y. Accumulation of genetic changes during development and progression of hepatocellular carcinoma: loss of heterozygosity on chromosome arm 1p occurs at early stage of hepatocarcinogenesis. *Genes Chromosom. Cancer*, **13**, 163–167 (1995).
36) Marchio, A., Mebbeb, M., Pineau, P., Danglot, G., Tiollais, P., Bernheim, A. and Dejean, A. Recurrent chromosomal abnormalities in hepatocellular carcinoma detected by comparative genomic hybridization. *Genes Chromosom. Cancer*, **18**, 59–65 (1997).