

Amplification and Overexpression of the Cyclin D1 Gene in Aggressive Human Hepatocellular Carcinoma¹

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Abstract

We analyzed the genetic alterations of the cyclin D1 and *INT-2* genes in hepatocellular carcinomas (HCCs) from 45 patients. Among these, expression of the cyclin D1 mRNA was also analyzed in 18 of them by Northern blotting. The cyclin D1 gene was amplified 3–16 fold in five HCCs (11%); among these, the *INT-2* gene was also amplified 2–10 fold in four HCCs. We analyzed the mRNA of cyclin D1 in four HCCs with gene amplifications, and 6–10 fold overexpressions were detected in all of them. Because the cyclin D1 gene was amplified in patients at an advanced stage of HCC with rapid tumor growth, it appeared to be associated with the aggressive behavior of tumors. Studies on loss of heterozygosity on chromosome 13q, where the retinoblastoma (*RB*) gene is located, indicated that all HCCs with an amplified cyclin D1 gene retained heterozygosity on chromosome 13q. These results suggest that amplification and overexpression of the cyclin D1 gene result in the rapid growth of a subset of HCC, even though the function of the *RB* gene is retained.

Introduction

In Asia and Africa, HCC³ is still one of the major causes of cancer death because of its high frequency and poor prognosis. Recent advances in molecular genetics have identified various genetic abnormalities related to hepatocarcinogenesis, which may be useful in elucidating the pathogenesis and development of HCC (1). A variety of oncogenes and tumor suppressor genes such as the *c-myc*, *RB*, and *p53* undergo changes, and gross abnormalities of karyotype or chromosomal subregions, such as a LOH and amplification of some chromosomal arms, have also been found in significant fractions of HCC (1–3).

Cyclins are thought to be essential proteins in cell cycle regulation because of their specific and periodic expression during cell cycle progression (4). Binding of the cyclin family with cyclin-dependent kinases regulates their activity and contributes to cell cycle regulation (5). In human cancers, the cyclin D1 gene is rearranged in parathyroid tumors and B-lymphocyte malignancies (6, 7) and amplified in head and neck squamous cell carcinoma, breast cancer, and esophageal carcinoma (8, 9). Furthermore, amplification of the cyclin D1 gene and overexpression of this protein was found in 13% of HCC from Taiwan (10). These findings suggest that alterations of cyclins, especially that of cyclin D1, promote cell cycle progression and play an important role in the formation and/or development of these malignancies.

From this evidence, we speculated that an alteration of the cyclin D1 gene is also involved in human HCC, especially related to tumor growth. In addition, a recent report has shown that pRB forms a

complex with cyclin D1 and may regulate its activity through binding (11). In esophageal cancer, the cyclin D1 gene is amplified and overexpressed especially in the group expressing normal pRB (12). Therefore, we analyzed amplification and overexpression of the cyclin D1 gene and also LOH on chromosome 13q in the same HCC samples and examined the relationship between the aberrant cyclin D1 and the loss of chromosome 13q.

Materials and Methods

Samples. Forty-five HCC tissues and their surrounding noncancerous regions were obtained from patients with their informed consent. The samples were resected at surgery or removed at autopsy at Kyoto University Hospital, and the stage of HCC was determined according to the classification of the General Rules for the Clinical and Pathological Study of Primary Liver Cancer (13). Among the specimens examined, 6 were in stage I, 7 in stage II, 14 in stage III, and 18 in stage IV. After resection, the tissues were stored at –80°C until isolation of DNA and RNA.

DNA Isolation and Southern Blots. High molecular weight DNA was extracted from the tumors and their corresponding noncancerous liver sections with phenol extraction (14). Genomic DNA (5 µg) was digested with a restriction endonuclease, electrophoresed on a 0.8% agarose gel, and transferred to a nylon membrane. Southern blot hybridization, labeling of the probes by nick-translation, and autoradiography were performed as described previously (2). The human cyclin D1 cDNA probe was provided by Dr. A. Arnold (6). After removing the human cyclin D1 probe, the same blots were then sequentially hybridized to the human *INT-2* gene probe, the human *c-Ha-ras* gene probe, and the β -actin probe, which were obtained from the Japanese Resources for Cancer Study (Gene Bank). The intensities of each band were determined by scanning autoradiograms of the blots on X-ray film with a Gelman densitometer (ACD-8). The intensities of the cyclin D1, *INT-2*, and *c-Ha-ras* bands on Southern blots were normalized by comparison with the β -actin gene, which is located on chromosome 7, as an internal control for each sample (15). The amplification was expressed as the ratio of the normalized intensity of the HCC tissue to that of the corresponding noncancerous tissue. The eight polymorphic probes used to determine the allelic status of chromosome 13q were pHU10, p123M1.8, p88R2.5, and p68RS2.0 provided by Dr. T. P. Drija and p7F-12, p9D11, p1E8, and p9A7 from Dr. W. K. Cavenee (2). The restriction enzymes and the loci detected by these polymorphic probes have been described previously (2).

RNA Isolation and Northern Blots. Total cellular RNA was prepared by guanidinium isothiocyanate solubilization (14). Fifteen µg of total cellular RNA were electrophoresed on a denaturing agarose gel (1% agarose gel containing 17% formaldehyde) and was transferred to a nylon membrane, hybridized, and autoradiographed as described above. The human cyclin D1 cDNA was used as the probe, and human β -actin mRNA was also analyzed as the internal control. After densitometric scanning, the degree of cyclin D1 expression was determined as described for Southern blotting.

Results

Coamplification of the Cyclin D1 and *INT-2* Genes in HCC. We analyzed 45 human HCC and adjacent noncancerous liver samples. Southern blots of the cyclin D1 gene revealed three *EcoRI* fragments of 4.0, 2.2, and 2.0 kilobases as described previously (Fig. 1; Ref. 8).

Received 3/7/94; accepted 5/4/94.

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¹ This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan.

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³ The abbreviations used are: HCC, hepatocellular carcinoma; RB, retinoblastoma; pRB, retinoblastoma protein; LOH, loss of heterozygosity; cDNA, complementary DNA; HBV, hepatitis B virus.

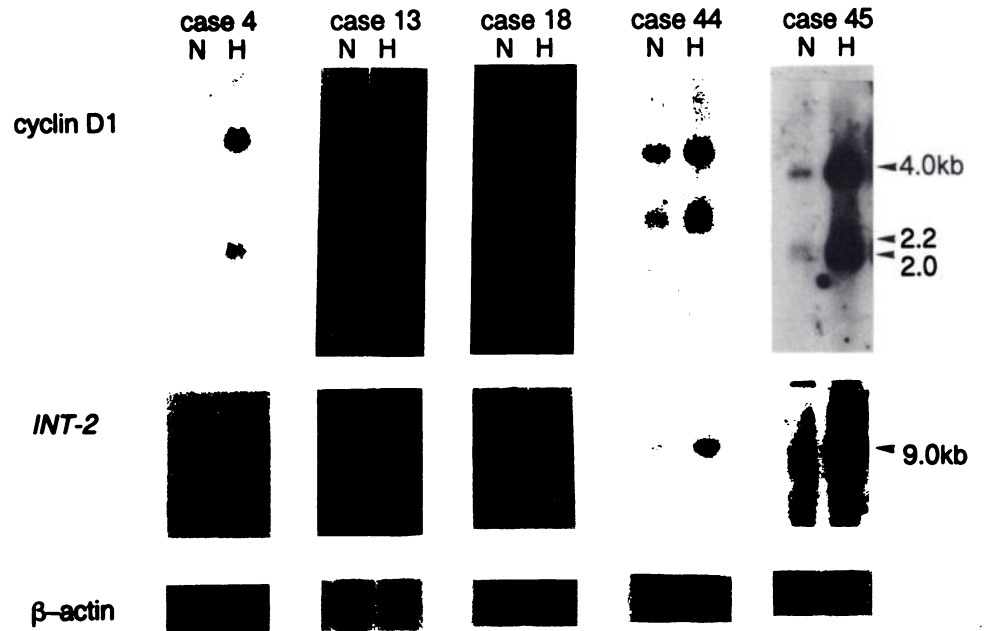


Fig. 1. Coamplification of the cyclin D1 and *INT-2* genes in human HCC detected by Southern blot hybridization. *N*, noncancerous liver; *H*, HCC. Five μg of *Eco*RI-digested DNAs were hybridized with cyclin D1 cDNA, *INT-2*, and β -actin gene probes as described in "Materials and Methods." The size of each band is indicated on the right. The degree of amplification in each is shown in Table 1.

Among 45 tumors, the cyclin D1 was amplified 3–16 fold in 5 (11%) of them, whereas no rearrangements were detectable. Among these 5, the *INT-2* gene was amplified 2–10 fold in 4 (Table 1). On the other hand, the intensities of the *c-Ha-ras* bands were the same as those of the noncancerous tissues in all tumors. This suggests that chromosome 11 was not aneuploid, since the *c-Ha-ras* gene is located on chromosome 11p and the cyclin D1 and *INT-2* genes are located on 11q. These amplifications seemed not to be exclusively associated with infection by the HBV or hepatitis C virus (Table 1).

All five samples in which the cyclin D1 gene was amplified were in the most advanced stage (stage IV), while only one-third of tumors without amplification (13 of 40) were in stage IV. Among these five, three were from surgical resections and two were obtained at autopsy. Of the three surgical specimens (patients 4, 18, and 44), the tumors occupied more than one lobe of the liver at the time of surgery, and in the other two cases (autopsies 13 and 45), the tumors had rapidly grown and occupied almost the entire region of the liver within 2 months of detection (Table 1).

Previously, we examined the allelic status of chromosome 13q in HCCs, and LOH on chromosome 13q was detected in 45% of HCC of stage IV (1, 2). However, all five specimens with the amplified cyclin D1 gene retained heterozygosity at all the informative polymorphic loci on chromosome 13q (data not shown).

Overexpression of the Cyclin D1 mRNA in HCC. Next, we examined whether amplification of the cyclin D1 gene was associated with overexpression of the mRNA. Transcripts of 4.7 and 1.8 kilobases were detected by Northern blotting with the cyclin D1 cDNA probe as described previously (Fig. 2; Ref. 8). Since mRNA could not

be isolated from all samples, we analyzed 18 of the 45 HCCs. Among these, cyclin D1 mRNA was overexpressed 6–10 fold in 4 of them (4 of 18; 22%; Table 1). Southern blots revealed that the cyclin D1 gene was amplified in these four specimens. These results showed that the amplified cyclin D1 gene in the amplicon of chromosome 11q13 was overexpressed in HCC. Although we also analyzed expression of the *INT-2* gene in these 18 HCCs, we could not detect *INT-2* transcripts in any tumor and normal RNA samples (data not shown). A summary of the clinical profiles, the degree of gene amplification, and expression level of cyclin D1 mRNA in these patients are shown in Table 1.

Discussion

Cyclins, which constitute an evolutionarily conserved gene family, are thought to control the cell cycle by regulating the activity of cyclin-dependent kinases (4, 5) and are altered in human malignancies. For example, HBV DNA has been found integrated into an intron of the cyclin A gene, which gave rise to a hybrid cDNA encoding an HBV-cyclin A fusion protein (16). Cyclin A is degraded through ubiquitin-dependent proteolysis, and this chimeric protein is resistant to degradation because the NH_2 -terminal region of cyclin A, including the signal for degradation, was replaced by a viral sequence (17). The cyclin D1 gene is rearranged in parathyroid tumor and B-cell lymphoma and amplified in breast, esophageal, and head and neck cancer (6–9). Furthermore, gene amplification of cyclin E was detected in breast cancer and in colorectal carcinoma, although this appears to be a rare occurrence since it had been seen in one cell line of each type

Table 1 Clinical profiles of the patients with an amplified cyclin D1 gene

Patient no.	Age/sex	Virus ^a	Size ^b (cm)	Stage ^c	Outcome	Gene amplification		Expression of cyclin D1 mRNA
						Cyclin D1	<i>INT-2</i>	
4	45/M ^d	HBV	5.0	IV	Operation	3×	1×	8×
13	58/F	HCV	≥10.0	IV	Autopsy	16×	10×	10×
18	47/F	HCV	5.5	IV	Operation	9×	7×	10×
44	48/M	HBV	8.0	IV	Operation	5×	2×	N.T.
45	53/M	HCV	≥10.0	IV	Autopsy	7×	7×	6×

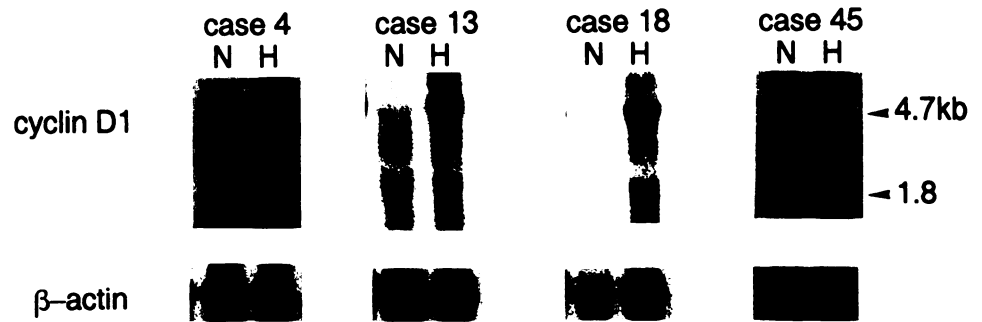
^a HBV and hepatitis C virus (HCV) infections were determined by the presence of serum HBV surface antigen and serum hepatitis C virus antibody, respectively.

^b Maximum diameter of the main tumor.

^c According to the classification of the Liver Cancer Study Group of Japan (13).

^d M, male; F, female; N.T., not tested.

Fig. 2. Expression of the cyclin D1 mRNA in human HCC analyzed by Northern hybridization. *N*, noncancerous tissue; *H*, HCC. Fifteen μ g of total RNAs were Northern hybridized. The size of each band is indicated on the right. The details are described in "Materials and Methods."



of tumor (18, 19). This evidence suggests the importance of cyclin gene aberrations in carcinogenesis.

The cyclin D1 gene has been mapped to the long arm of chromosome 11 close to the *INT-2* gene (6), and the chromosome 11q13 region is amplified in human HCC carrying the integrated HBV-DNA (20). And amplification of the cyclin D1 gene was shown in 4 of the 30 (13%) HCCs from Taiwan (10). From this perspective, we examined chromosome 11q13 amplifications using cyclin D1 and *INT-2* probes and expression of the cyclin D1 gene in Japanese HCCs.

We found that the cyclin D1 gene was amplified in 11% of HCC (5 of 45), which was an almost identical percentage in the previous report (10), and the *INT-2* gene was also coamplified in 4 of them. Therefore, the amplified copies of the cyclin D1 gene were contained within an amplicon of the chromosome 11q13 region. Cyclin mRNA was also overexpressed in all four samples with an amplified cyclin D1 gene, which were available for RNA analysis. Overexpression was observed in only these four tumors and in none of those without cyclin D1 amplification. This evidence suggests that the aberrant expression of cyclin D1 plays an important role in HCC, as it does in esophageal cancer where the cyclin D1 and *INT-2* genes are coamplified, but only the cyclin D1 gene is expressed (12, 21). A previous report shows that the integrated HBV lies in chromosome 11q13 and is coamplified with the *HST-1* gene in one case of HCC (20). However, integration of HBV in the chromosome 11q13 region does not seem to be a common event (22). In this study, the chromosome 11q13 region was amplified in both HBV- or hepatitis C virus-positive samples, suggesting that amplification of this region is not restricted to HBV-related hepatocarcinogenesis.

Among the five patients with an amplified cyclin D1 gene, two (cases 13 and 45) were followed up for the entire clinical course of HCC without surgical removal of the tumor because of its rapid growth and the decline of liver functions. In both patients, the tumor was less than 5 cm at initial diagnosis and had spread to the entire liver within 1–2 months. The other three patients (cases 4, 18, and 44) were classified as being at the most advanced stage at the time of surgery. In contrast, in the cases at earlier stages, the cyclin D1 gene was not amplified. Reportedly, amplification of chromosome 11q13, including the cyclin D1 gene, is associated with an unfavorable clinical course of disease in other cancers such as esophageal squamous cell carcinoma (21). Overexpression of G₁ cyclins, D1 and E, in fibroblasts *in vitro* shorten the duration of G₁ by accelerating G₁ progression and reducing the serum requirement for the transition from G₁ to S (23, 24). These observations indicate that the G₁ cyclins are rate-limiting for G₁ progression in mammalian cells. Furthermore, the introduction of cyclin D1, D3, or E genes with a pRB expression plasmid into SAOS-2 cells resulted in an increase of pRB-positive cells in S, G₂, and M, compared with those into which only a pRB-expressing plasmid was introduced (11). Microinjection with an anti-cyclin D1 antibody or an antisense cyclin D1 plasmid prevents both human and mouse fibroblasts from entering S (24, 25). This

evidence also seems to support the notion that cyclin D1 is a critical component of proliferative signals in G₁ and that its overexpression accelerates HCC tumor growth.

Previously, we examined the LOH on chromosome 13q and aberration of the *RB* gene in HCC, and allelic loss on chromosome 13q seems to be associated with the disruption of the *RB* function (2). We detected LOH on chromosome 13q in 45% of Japanese HCC in stage IV (1). Therefore, we also examined the LOH on chromosome 13q in samples where the cyclin D1 gene was amplified. However, the LOH on chromosome 13q was undetectable in samples with an amplified cyclin D1 gene, indicating that pRB function is retained, although all of these specimens were in stage IV. Recently, Dowdy *et al.* (11) have reported that cyclin D1 protein forms a complex with pRB and that its activity seemed to be down-regulated by association with pRB. In addition, the overexpression of cyclin D1 exclusively in human esophageal cancer which expresses normal pRB has also been reported (12). Stable overexpression of cyclin D1 in rat embryo fibroblasts gave rise to a decrease in the duration of G₁ and induction of tumor formation when injected into nude mice (26, 27). Therefore, we speculate that overexpression of cyclin D1 overrides a growth inhibition of pRB, enhances cell transformation, and results in the progression of HCC to a more malignant form.

The present study suggests that amplification and overexpression of the cyclin D1 gene play a role in multistep hepatocarcinogenesis, especially in the acceleration of tumor growth. Further studies on the cyclin gene family should help to elucidate hepatocarcinogenesis and to develop a gene therapy in HCC, such as the introduction of antisense cyclin cDNA into HCC that exhibit rapid growth and which is incurable by conventional therapy.

Acknowledgments

We thank Dr. A. Arnold for supplying the human cyclin D1 cDNA probe.

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