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Invited Review

Alteration of cell cycle-related genes in hepatocarcinogenesis

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Summary. The mammalian cell cycle is controlled by regulators of the G1 to S transition such as tumor suppressor proteins, p53 and retinoblastoma (RB); cyclin D1 and cyclin-dependent kinase 4; and inhibitor of cyclin dependent kinase, $p16^{INK4A}$. Recently, aberrations of these cell cycle-related genes have been reported to contribute to the formation and development of cancer. In human hepatocellular carcinoma (HCC), high frequencies of aberration have been detected in the *p53* and *RB* genes. Loss of heterozygosity (LOH) of chromosome 13q was detected in 35% of HCC and LOH on chromosome 17p was detected in 49%. Mutation of the *p53* gene was also detected in 32%. The aberrations of these genes were observed more frequently in poorly differentiated and in advanced HCCs. On the other hand, genetic alterations of the *cyclin D1* and $p16^{INK4A}$ genes were not so frequent, but appeared to be associated with the aggressive behavior of the tumor, which suggests that disruption of the cell cycle-related genes results in the progression of HCC. Further study with a substantial number of cases is required to determine the actual frequency of the aberrations of the G1 controlling genes in hepatocarcinogenesis.

Key words: p53, RB, Cyclin D1, p16, Hepatocellular carcinoma

Introduction

With the progress in molecular genetic studies, various genetic abnormalities have been identified in human cancers. Activation of oncogenes and loss of function of tumor suppressor genes in each step of carcinogenesis may play an important role in tumor development. In hepatocellular carcinoma (HCC), aberrations of oncogenes, tumor suppressor genes, and loss of heterozygosity (LOH) on some chromosomal arms have been reported, and these multiple genetic alterations may contribute to the transformation of normal hepatocytes to cancer cells (Nishida et al., 1994a).

On the other hand, numerous cyclins and cyclin-dependent kinase inhibitors (CDKIs) have been identified, and studies on the cell cycle control mechanism have revealed that progression of eukaryotic cells through the cell cycle is controlled by the sequential formation, activation and inactivation of a series of cyclin-dependent kinase (CDK) complexes (Evans et al., 1983; Matsushime et al., 1992). Furthermore, overproduction of the cyclin and/or CDKs, which presumably act like oncogenes, has been reported in many types of cancers (Jiang et al., 1992; de Boer et al., 1993). In addition, cell cycle inhibitors might be like tumor suppressor genes the loss of function of which may lead to cancer (Serrano et al., 1995; Yang et al., 1995). From these perspectives, we discuss here about the alteration of the cell-cycle related genes in HCC and speculate on the role of the aberration of these genes in hepatocarcinogenesis.

Aberration of cell cycle-related genes in human HCC

(1) The p53 gene

The *p53* gene, which was at first considered an oncogene, acts as a tumor suppressor gene, and mutations of the *p53* gene are the most frequent genetic changes in a wide range of cancers in humans and experimental animals (Nigro et al., 1989). In human HCC, the loss of p53 function can occur through point mutations, rearrangements and intragenic deletions, and these alterations of the *p53* gene are closely associated with loss of heterozygosity (LOH) on chromosome 17p and vice versa (Murakami et al., 1991; Oda et al., 1992; Nishida et al., 1993). The wild type p53 has a specific DNA-binding function and activates the transcription of genes with p53 binding sequences (Farmer et al., 1992; Kern et al., 1992). The genes transcriptionally controlled by p53 are divided into three groups: (i) cell cycle-related genes such as the $p21^{WAF1}$ gene that is a universal inhibitor of cyclin kinase (El-Deiry et al.,

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1993; Harper et al., 1993); (ii) DNA replication and/or repair-related genes such as the *gadd45* gene (Kastan et al., 1992); and (iii) apoptosis-related genes such as the *bax* and *Fas* genes (Miyashita and Reed, 1995; Owen-Schaub et al., 1995). Loss of the normal function of the *p53* gene is associated with disruption of cell cycle control, DNA repair and apoptosis (Kinzler and Vogelstein, 1994). For this reason, it is important to discuss the aberration of the *p53* gene in HCC with special attention to the aberration of cell cycle-related genes.

Bressac et al. (1991) found a mutational hot spot at codon 249 of the *p53* gene in HCC in China and South Africa, which was considered to be associated with dietary aflatoxin B1 (AFB1) intake (Bressac et al., 1991; Hsu et al., 1991). In contrast, no such mutational hot spot has been found in other areas, suggesting the involvement of different etiological factors. Previously, we investigated the mutational profile of the *p53* gene in 53 Japanese HCC cases over the entire coding region, compared those mutations with several clinical parameters (Nishida et al., 1993), and also mapped the commonly deleted region of chromosome 17 by using 10 restriction fragment length polymorphism (RFLP) markers in the same tumor samples. We also analyzed the relationship between mutations of the *p53* gene and LOH on chromosome 17p. The overall frequency of the *p53* gene mutation in the HCC in our study was 32% and the frequency was higher in advanced tumors, which is consistent with previously reported values in Japanese patients (Murakami et al., 1991). These mutations were found in a wide region stretching from exon 4 to exon 10 without any single mutational hot spot; G:C to T:A transversions were predominant among the base substitutions observed. Recently, a variety of mutagenic heterocyclic amines, which are commonly found in cooked food, have been reported to produce DNA adducts in liver cells and to produce G:C to T:A transversions (Wakabayashi et al., 1992). Endogeneous mutagens caused by oxyradical damage can also produce G:C to T:A transversions (Harris, 1991). These mutagens may induce transversion mutations of the p53 gene in a subset of HCC cases. Our study also suggested an association between the allelic loss on chromosome 17p and tumor progression, indicating that structural aberration of the *p53* gene is a late event during hepatocarcinogenesis in Japan where infection of hepatitis virus, especially hepatitis C virus (HCV), is the major etiology of HCC (Nishida et al., 1993). On the contrary, Aguilar et al. (1994) reported that non-malignant human liver cells from Qidong, where AFB1 exposure is high, also exhibited the mutation of the *p53* gene at codon 249 (Aguilar et al., 1994). Therefore, mutation of the p53 gene may be an early event in AFB1-related hepatocarcinogenesis. In human HCC tissue with infection of hepatitis B virus (HBV), HBV X protein (HBx) appears to form a complex with p53 protein and inactivate the p53 (Feitelson et al., 1993). In a transgenic mouse model where expression of the HBx induces tumor development in the liver, HBx binds to normal p53 protein and may inactivate p53 without mutation of the *p53* gene (Ueda et al., 1995). Therefore, in the HBV-related hepatocarcinogenesis, an epigenetic change altering the function of the p53 may have occurred at an early stage (Henkler et al., 1995; Wang et al., 1995). Since the development of human HCC is a complex event associated with many agents, the role of inactivation of p53 in hepatocarcinogenesis may differ with the etiology (Unsal et al., 1994).

p21^{WAF1} has been shown to mediate p53-induced cell cycle arrest (El-Deiry et al., 1993), and to inhibit CDK activity (Harper et al., 1993) and proliferate cell nuclear antigen (PCNA)-dependent DNA replication (Waga et al., 1994). An increased p53 level induced an increased amount of the p21^{WAF1} protein in response to DNA damage. However, in human pancreatic carcinoma and melanoma, expression of *p21*^{WAF1} correlated neither with p53 mutational status nor with *p53* expression, although almost all mutations of the *p53* gene were in the specific DNA-binding domain (DiGiuseppe et al., 1995; Vidal et al., 1995). On the contrary, the expression of the *p21*^{WAF1} gene correlates with p53 status in human HCC (Hui et al., 1997).

Do the mutations of the *p53* gene affect the expression of other p53 inducible genes such as the *gadd45*, *bax* and *Fas* genes, and the efficiency of DNA repair and apoptosis in HCC? HBx has been reported to inhibit p53-mediated apoptosis and to contribute to an early stage of hepatocarcinogenesis (Wang et al., 1995). The clone with inactive p53 may escape from apoptosis and be selected by hypoxia during the expansion of HCC, and finally grow to a treatment-resistant tumor (Graeber et al., 1996). Hypoxia and cancer therapy, such as gamma radiation and anticancer drugs, may provide physiological selective pressure to tumors for the expansion of variants with the mutation of the *p53* gene that have lost the apoptotic potential (Lowe et al., 1994; Kinzler and Vogelstein, 1996). An important outcome of the inactivation of the p53 protein in hepatocarcinogenesis may be the inhibition of *p53*-dependent apoptosis during tumor development and induction of a tumor that is resistant to anticancer therapies.

(2) The RB gene

Loss of the restriction point (R-point) control in cell cycle is associated with malignant transformation and has been proposed to be one of the hallmarks of cancer (Strauss et al., 1995). Recently, the D-type cyclin has been suggested to act as an R-point protein (Baldin et al., 1993; Quelle et al.,1993), and the RB protein (pRB) to regulate the G1 to S transition (Dowdy et al., 1993). pRB prevents premature G1/S transition via physical sequestration of transcription factors the functions of which are required to activate S-phase genes. Phosphorylation of pRB at the R-point in G1 leads to release of the captured transcription factors from the complex with pRB (Chellappan et al.,1991). In addition

to its well established role as a cell cycle regulator at the G1 phase of the cell cycle, many recent studies have pointed to a role for pRB in differentiation and development (De Luca et al., 1996).

Previously, we analyzed the LOH at 8 loci on chromosome 13q and the structural alteration of the *RB* gene in 56 HCCs by Southern blot analysis. Allelic loss on chromosome 13q was detected in 35% of HCCs, whereas LOH was not detected on any loci in cirrhotic nodules. On chromosome 13q, the common deleted region was mapped to the region including the RB locus. Furthermore, one case with allelic loss on chromosome 13q had an interstitial deletion of the *RB* gene (Nishida et al., 1992). Zhang et al. (1994) also reported that the loss of the region of chromosome 13q including the RB locus significantly correlated with loss of RB protein in HCC by immunohistochemical analysis, indicating the involvement of inactivation of the *RB* gene in hepatocarcinogenesis (Zhang et al., 1994).

(3) The cyclin D1 and CDK4 genes

Many studies have revealed that the phospholylation of pRB in the G1 phase is carried out by the CDK4 and CDK6 complex with cyclins D1, D2 and D3 (Weinberg, 1995). In human cancer, the *cyclin D1* gene is rearranged in the parathyroid tumor and B-cell lymphoma and amplified in head and neck squamous cell carcinoma, breast cancer and esophageal carcinoma (Motokura et al., 1991; Jiang et al., 1992, 1993; Schuuring et al., 1992; de Boer et al., 1993). Stable overexpression of cyclin D1 in rat embryofibroblasts decreased the duration of G1 and induction of tumor formation in nude mice (Lovec et al., 1994). Furthermore, the transgenic mice with overexpression of the *cyclin D1* gene tend to develop breast cancer (Wang et al., 1994). These findings suggest that cyclin D1 is a critical component of the proliferation signal in G1 and its overexpression accelerates tumor cell growth. We found that the *cyclin D1* gene was amplified 3-16 fold in five of the 45 HCCs (11%) examined. We also analyzed the mRNA of cyclin D1 in four HCCs with gene amplification, and 6-10 fold overexpression was detected in all of them (Nishida et al., 1994b). The *cyclin D1* gene was amplified and overexpressed in 13% of HCC from Taiwan (Zhang et al., 1993). 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 the tumor (Nishida et al., 1994b). The *CDK4* gene, the product of which is a partner of cyclin D1 is amplified in glioma and osteosarcoma (Khatib et al., 1993; Schmidt et al., 1994). However, the *CDK4* gene was not amplified in our HCC cases (Kita et al., 1996).

4) The p16^{INK4A} gene

In addition to activation of the cyclin D/CDK and inactivation of the *RB* gene, the R-point control is often abrogated by loss of function of p16^{INK4A} (Nobori et al., 1994). p16^{INK4A} interacts with cyclin D1-CDK4 or cyclin D2-CDK4 and may act as an important factor in a regulatory feedback circuit with CDK4, D-type cyclins, and pRB (Lukas et al., 1995). p16^{INK4A} is considered as a candidate tumor suppressor gene (Nobori et al., 1994), since homologous deletion (Caldas et al., 1994; Kamb et al., 1994; Okamoto et al., 1994; Cairns et al., 1995), point mutation (Hayashi et al., 1994; Mori et al., 1994; Yoshida et al., 1995), or methylation of the 5'CpG island of the *p16*^{INK4A} gene (Gonzalez-Zulueta et al., 1995; Herman et al., 1995; Merlo et al., 1995), all of which result in loss of function of this protein, is reported in the majority of cell lines derived from various types of cancer and primary tumor tissue.

We examined the genetic status of the *p16*^{INK4A} gene and LOH on chromosome 9p in 62 human HCC. Although no sample showed the homologous deletion of the *p16*^{INK4A} gene, we detected intragenic mutation in 3 HCCs (5%), and 1 of 6 cell lines (17%), which were low values compared with those reported for other types of tumors such as melanoma, esophageal cancers, pancreatic adenocarcinomas, gliomas and lung cancers (Caldas et al., 1994; Kamb et al.,1994; Mori et al.,1994; Nobori et al., 1994; Schmidt et al.,1994). Sequencing revealed that 3 of these 4 samples had missense mutations and the remaining samples had a 2-base deletion causing a frameshift (Kita et al., 1996). LOH analysis on chromosome 9p revealed that 2 out of 3 samples with mutation of the *p16*^{INK4A} gene had LOH on chromosome 9p. Yang (1995) described the effect of point mutations of the *p16*^{INK4A} gene on the function of the protein and suggested that those point mutations in the conserved ankyrin affect the ability of p16^{INK4A} to bind CDK and to inhibit CDK activity (Yang et al., 1995). Since Ala (codon 94), which was mutated in our 2 primary HCC samples, is in the consensus sequence of ankyrin repeat and one case had a 2-base deletion causing a frameshift, these mutations may disrupt the function of the protein and play some role in hepatocarcinogenesis. As mentioned above, genetic aberration of the *p16*^{INK4A} gene is rare in HCC-derived cell line and human HCC (Okamoto et al., 1994; Kita et al., 1996), but several studies suggested that methylation of the 5'CpG island of the *p16*^{INK4A} gene is associated with the inactivation of p16^{INK4A} and that this aberrant methylation occurred frequently in colon cancer and breast cancer that rarely showed homozygous deletion of the *p16*^{INK4A} gene (Little and Wainwright, 1995; Merlo et al., 1995). Furthermore, Hui et al. (1996) reported that the p16^{INK4A} protein was absent from 11 of 32 primary HCCs (34%) and that neither homozygous deletion and mutation nor loss of p16^{INK4A} mRNA expression were detected. p16^{INK4A} may actually be inactivated more frequently by posttranscriptional regulation in hepatocarcinogenesis (Hui et al., 1996).

Table 1. Relationship between aberration of the *RB* gene or LOH on chromosome 13q and those of other cell cycle-related genes.

	No. of cases with amplification of the *cyclin D1* or mutation of the *p16*^{INK4A} gene or LOH on 9p	No. of the cases with mutation of the *p53* gene or LOH on 17p
No. of the case with aberration of the *RB* gene or LOH on 13q	1	7
No. of the cases without aberration of the *RB* gene or LOH on 13q	9	9
Total cases	10	16

Allelic loss on chromosomes 13q may associate with the disruption of the RB function (Nishida et al., 1992; Zhang et al., 1994). Therefore, we examined the LOH on chromosome 13q in samples where the *cyclin D1* gene was amplified. LOH on chromosome 13q was not detected in samples with the amplified *cyclin D1* gene, but detected in only one sample with mutation of the *p16*^{INK4A} gene or LOH on chromosome 9p. Aberration of the *cyclin D1* or *p16*^{INK4A} gene seems to be rare in samples with disruption of RB (p=0.031, Fisher's exact method). On the contrary, LOH on chromosome 13q was not correlated with the aberration of the *p53* gene (p=0.35, Fisher's exact method).

The relationship among the aberrations of cell cycle-related genes

Loss of pRB, overexpression of cyclin D1 and loss of p16^{INK4A} may have a similar stimulating effect on G1 progression (Lukas et al., 1995). Aberration is usually observed in only one of these genes in a single tumor (Okamoto et al., 1994), and the inverse correlation of pRB and p16^{INK4A} expression was also reported in non-small cell lung cancer (Kratzke et al., 1996). During HCC development, a component of the G1 control mechanism may also be deregulated. As mentioned above, allelic loss on chromosome 13q seems to be associated with the disruption of the RB function (Nishida et al., 1992; Zhang et al., 1994). We then examined the LOH on chromosome 13q in samples with the amplification of the *cyclin D1* gene with LOH on chromosome 9p where the *p16*^{INK4A} gene is located, or with aberration of the *p53* gene (Table 1). The LOH on chromosome 13q was not detected in samples with amplification of the *cyclin D1* gene (Nishida et al., 1994b). An inverse relationship was also observed between LOH on chromosome 13q and LOH on chromosome 9p, namely, tumor samples with LOH on chromosome 13q rarely showed LOH on chromosome 9p or mutation of the *p16*^{INK4A} gene (Kita et al., 1996). These findings indicate that alteration of any one of the G1 control components, pRB, p16^{INK4A}, or cyclin D1 is sufficient to disrupt the G1 checkpoint and contribute to hepatocarcinogenesis. On the other hand, LOH on chromosome 13q does not seem to correlate with the aberration of the *p53* gene, indicating that p53 participates in an independent pathway such as control of the G1 checkpoint in response to DNA damage, or induction to apoptosis (Dulic et al., 1994).

Progression of HCC and aberration of each cell cycle-related gene

Several reports indicated that the prognostic significance of the aberration of cell cycle-related gene in malignant tumor, such as esophageal cancer and non-small cell lung cancer (Kitagawa et al., 1991; Kratzke et

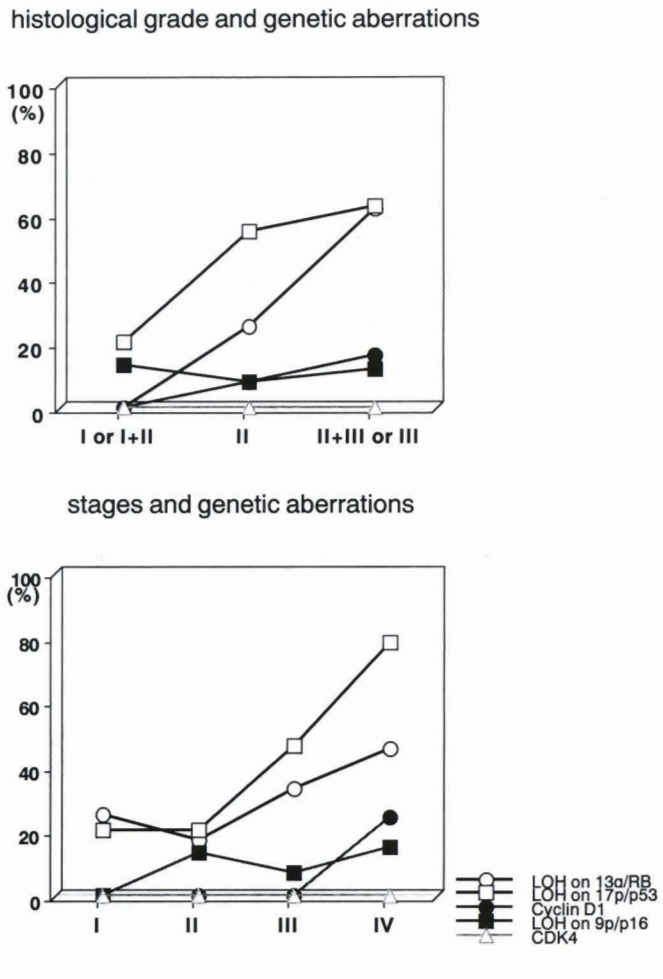

Fig. 1. Relationship between the aberration of each cell cycle-related gene and clinical parameters. The frequency of aberration was high in poorly differentiated tumors and in advanced clinical stages. The histological grade of each tumor was assigned according to the Edmondson and Steiner grading system (Edmondson et al., 1954), and the stage of tumor was determined according to the General Rules for the Clinical and Pathological Study of Primary Liver Cancer (Liver Cancer Study Group of Japan, 1992).

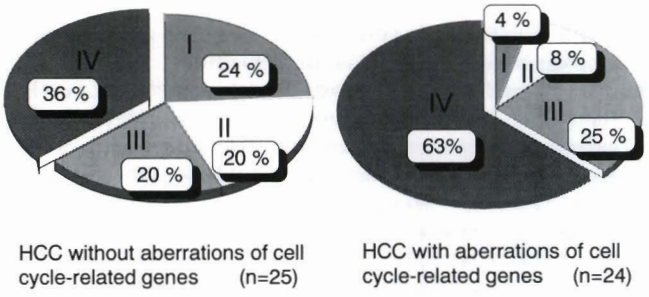

Fig. 2. HCC tumors were divided into 2 groups according to the presence or absence of the aberration in one of the cell cycle-related genes (the *p53*, *RB*, *cyclin D1* and *p16*^{INK4A} genes). In the groups with aberrations of the cell cycle-related genes, 63% of the tumors were in stage IV. In contrast, 36% of the tumors were in stage IV in the groups without any aberrations of cell cycle-related genes.

al., 1996). Therefore, we also analyzed the relationship between clinicopathological features and aberration of each cell cycle-related gene in HCC. Aberrations of the *RB*, *cyclin D1*, and *p53* genes were frequently observed in patients with more poorly differentiated tumor and advanced stages, suggesting that disruption of G1 control and check point genes are associated with dedifferentiation and progression of HCC (Fig. 1). Among the five patients that showed the amplification of the *cyclin D1* gene, two HCCs were followed up during the entire clinical course without surgical removal of the tumor because of rapid growth and poor liver function. In both HCCs, the tumor was less than 5 cm at initial diagnosis and spread to the entire liver within 1-2 months. The other three HCCs were classified as most advanced stage at the time of surgery (Nishida et al., 1994b). These finding also indicate that disruption of G1 control may be closely associated with a tumor progression and unfavorable clinical course of the disease (Fig. 2).

Conclusion

We have described the aberration of cell cycle-related genes in HCC. The relationship between each gene and frequency of aberration are schematically illustrated in Fig. 3. Mutation of the *p53* gene or LOH on chromosome 17p, and mutation of the *RB* gene or LOH on chromosome 13q was frequently observed in HCC. On the other hand, genetic aberrations of the cyclin D1 and *p16*^{INK4A} genes or LOH on chromosome 9p were not frequent, and amplification of the *CDK4* gene was not detected. However, aberrations of these cell cycle-related genes were frequently observed in advanced HCC (Fig. 2), and may be associated with poor prognosis. Therefore, further studies on the disruption of the G1 to S controlling mechanism should help to elucidate the mechanism of tumor progression and to develop an effective therapy of HCC, such as inhibition of the cell cycle progression from G1 to S in cancer cells.

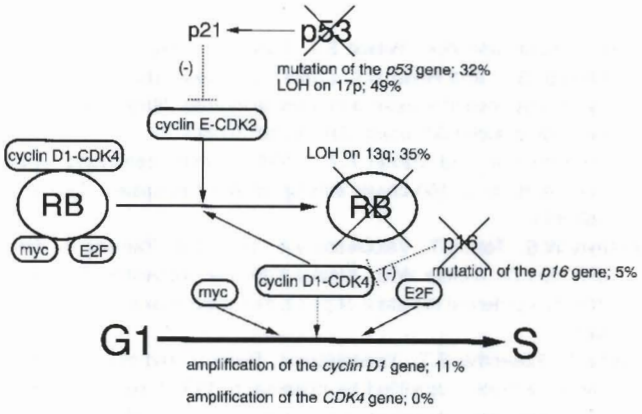

Fig. 3. Schematic expression of the relationship of the cell cycle-related molecules. Frequencies of the genetic aberrations in each genes in human HCC are also shown. Loss of p16^{INK4A} protein in HCC was reported by Hui et al., (1996).

References

Aguilar F., Harris C.C., Sun T., Hollstein M. and Cerutti P. (1994). Geographic variation of p53 mutational profile in nonmalignant human liver. Science 264, 1317-1319.

Baldin V., Lukas J., Marcote M.J., Pagano M. and Draetta G. (1993). Cyclin D1 is a nuclear protein required for cell cycle progression in G1. Genes Dev. 7, 812-821.

Bressac B., Kew M., Wands J. and Ozturk M. (1991). Selective G to T mutation of p53 gene in hepatocellular carcinoma from southern Afrifca. Nature 350, 429-431.

Cairns P., Polascik T.J., Eby Y., Tokino K., Califano J., Merlo A., Mao L., Herath J., Jenkins R., Westra W., Rutter J.L., Buckler A., Gabrielson E., Tockman M., Cho K.R., Hedrick L., Bova G.S., Isaacs W., Koch W., Schwab D. and Sidransky D. (1995). Frequency of homozygous deletion at p16/CDKN2 in primary human tumours. Nature Genet. 11, 210-212 .

Caldas C., Hahn S.A., Costa L.T., Redston M.S., Schutte M., Seymour A.B., Weinstein C.L., Hruban R.H., Yeo C.J. and Kern S.E. (1994). Frequent somatic mutations and homozygous deletions of the p16(MTS1) gene in pancreatic adenocarcinoma. Nature Genet. 8, 27-32.

Chellappan S.P., Hiebert S., Mudryj M., Horowitz J.M. and Nevins J.R. (1991). The E2F transcripition factor is a cellular target for the RB protein. Cell 65, 1053-1061.

de Boer C.J., Loyson S., Kluin P.M., Kluin-Nelemans H.C., Schuuring E. and van Krieken J.H. (1993). Multiple breakpoints within the BCL-1 locus in B-cell lymphoma: Rearrangements of the cyclin D1 gene. Cancer Res. 53, 4148-4152.

De Luca A., Esposito V., Baldi A. and Giordano A. (1996). The retinoblastoma gene family and its role in proliferation, differentiation and development. Histol. Histopathol. 11, 1029-1034.

DiGiuseppe J.A., Redston M.S., Yeo C. J., Kern S.E. and Hruban R.H. (1995). p53-independent expression of the cyclin-dependent kinase inhibitor p21 in pancreatic carcinoma. Am. J. Pathol. 147, 884-888.

Dowdy S.F., Hinds P.W., Louie K., Reed S.I., Arnold A. and Weinberg R.A. (1993). Physical interaction of the retinoblastoma protein with human D cyclins. Cell 73, 499-511.

Dulic V., Kaufmann W.K., Wilson S.J., Tlsty T.D., Lees E., Harper J.W., Elledge S.J. and Reed S.L. (1994). p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. Cell 76, 1013-1023.

Edmondson H.A. and Steiner P.E. (1954). Primary carcinoma of the liver. A study of 100 cases among 48,900 necropsies. Cancer 7, 462-503.

El-Deiry W.S., Tokino T., Velculescu V.E., Levy D.B., Parsons R., Trent J.M., Lin D., Mercer W.E., Kinzler K.W. and Vogelstein B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell 75, 817-825.

Evans T., Rosenthal E.T., Youngblom J., Distel D. and Hunt T. (1983). Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. Cell 33, 389-396.

Farmer G., Bargonetti J., Zhu H., Friedman P., Prywes R. and Prives C. (1992). Wild-type p53 activates transcription in vitro. Nature 358, 83-86.

Feitelson M.A., Zhu M., Duan L.X. and London W.T. (1993). Hepatitis BX antigen and p53 are associated in vitro and in liver tissues from patients with primary hepatocellular carcinoma. Oncogene 8, 1109-1117.

Gonzalez-Zulueta M., Bender C.M., Yang A.S., Nguyen T., Beart R.W., Tornout J.M.V. and Jones P.A. (1995). Methylation of the 5' CpG Island of the p16/CDKN2 tumor suppressor gene in normal and transformed human tissues correlates with gene silencing. Cancer Res. 55, 4531-4535.

Graeber T.G., Osmanian C., Jacks T., Housman D.E., Koch C.J., Lowe S.W. and Giaccia A.J. (1996). Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature 379, 88-91.

Harper J.W., Adami G.R., Wei N., Keyomarsi K. and Elledge S.J. (1993). The p21 cdk-interacting protein cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75, 805-816.

Harris C.C. (1991). Chemical and physical carcinogenesis: advances and perspectives for the 1990s. Cancer Res. 51 (Suppl.), 5023s-5044s.

Hayashi N., Sugimoto Y., Tsuchiya E., Ogawa M. and Nakamura Y. (1994). Somatic mutations of the MTS(multiple tumor suppressor)1/CDK4I(cyclin-dependent kinase-4 inhibitor) gene in human primary non-small cell lung carcinomas. BBRC 202, 1426-1430.

Henkler F., Waseem N., Golding M.H.C., Alison M.R. and Koshy R. (1995). Mutant p53 but not hepatitis B virus X protein is present in hepatitis B virus related human hepatocellular carcinoma. Cancer Res. 55, 6084-6091.

Herman J.G., Merlo A., Lapidus R.G., Issa J.P.J., Davidson N.E., Sidransky D. and Baylin S.B. (1995). Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Res. 55, 4525-4530.

Hsu I.C., Metcalf R.A., Sun T., Welsh J.A., Wang N.J. and Harris C.C. (1991). Mutational hotspot in the p53 gene in human hepatocellular carcinomas. Nature 350, 427-428.

Hui A.-M., Sakamoto M., Kanai Y., Ino Y., Gotoh M., Yokota J. and Hirohashi S. (1996). Inactivation of the p16INK4 in hepatocellular carcinoma. Hepatology 24, 575-579.

Hui A.-M., Kanai Y., Sakamoto M., Tsuda H. and Hirohashi S. (1997). Reduced $p21^{WAF1/CIP1}$ expression and p53 mutation in hepatocellular carcinoma. Hepatology 25, 575-579.

Jiang W., Kahn S.M., Tomita N., Zhang Y.J., Lu S.H. and Weinstein I.B. (1992). Amplification and expression of the human cyclin D gene in esophageal cancer. Cancer Res. 52, 2980-2983.

Jiang W., Zhang Y.J., Kahn S.M., Hollstein M.C., Santella R.M., Lu S.H., Harris C.C., Montesano R. and Weinstein I.B. (1993). Altered expression of the cyclin D1 and retinoblastoma genes in human esophageal cancer. Proc. Natl. Acad. Sci. USA 90, 9026-9030.

Kamb A., Gruis N.A., Weaver-Feldhaus J., Liu Q., Harshman K., Tavtigian S.V., Stockert E., Day R.S. III, Johnson B.E. and Skolnick M.H. (1994). A cell cycle regulator potentially involved in genesis of many tumor types. Science 264, 436-440.

Kastan M.B., Zhan Q., El-Deiry W. S., Carrier F., Jacks T., Walsh W.V., Plunkett B.S., Vogelstein B. and Fornace A.J. Jr. (1992). A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell 71, 587-597.

Kern S.E., Pietenpol J.A., Thiagalingam S., Seymour A., Kinzler K.W. and Vogelstein B. (1992). Oncogenic forms of p53 inhibit p53-regulated gene expression. Science 256, 827-830.

Khatib Z.A., Matsushime H., Valentine M., Shapiro D.N., Sherr C.J. and Look A.T. (1993). Coamplification of the CDK4 gene with MDM2 and GLI in human sarcomas. Cancer Res. 53, 5535-5541.

Kinzler K.W. and Vogelstein B. (1994). Cancer therapy meets p53. N. Engl. J. Med. 331, 49-50.

Kinzler K.W. and Vogelstein B. (1996). Life (and death) in a malignant tumour. Nature 379, 19-20.

Kita R., Nishida N., Fukuda Y., Azechi H., Matsuoka Y., Komeda T., Sando T., Nakao K. and Ishizaki K. (1996). Infrequent alterations of the p16 INK4A gene in liver cancer. Int. J. Cancer 67, 176-180.

Kitagawa Y., Ueda M., Ando N., Shinozawa Y., Shimizu N. and Abe O. (1991). Significance of int-2/hst-1 coamplification as a prognostic factor in patients with esophageal squamous carcinoma. Cancer Res. 51, 1504-1508.

Kratzke R.A., Greatens T.M., Rubins J.B., Maddaus M.A., Niewoehner D.E., Niehans G.A. and Geradts J. (1996) Rb and p16INK4A expression in resected non-small cell lung cancer. Cancer Res. 56, 3415-3420.

Little M. and Wainwright B. (1995). Methylation and p16: Suppressing the suppresor. Nature Med. 1, 633-634.

Liver Cancer Study Group of Japan (1992). The general rules for the clinical and pathological study of primary liver cancer, 3rd ed. Kanehara apress. Tokyo. pp 22-23.

Lovec H., Sewing A., Lucibello F.C., Müller R. and Möröy T. (1994). Oncogenic activity of cyclin D1 revealed through cooperation with Ha-ras: Link between cell cycle control and malignant trasformation. Oncogene 9, 323-326.

Lowe S.W., Bodis S., McClatchey A., Remington L., Ruley H.E., Fisher D.E., Housman D.E. and Jacks T. (1994). p53 Status and the efficacy of cancer therapy in Vivo. Science 266, 807-810.

Lukas J., Parry D., Aagaard L., Mann D.J., Bartkova J., Strauss M., Peters G. and Bartek J. (1995). Retinoblastoma-protein dependent cell-cycle inhibition by the tumor suppressor p16. Nature 375, 503-506.

Matsushime H., Ewen M.E., Strom D.K., Kato J.Y., Hanks S.K., Roussel M.F. and Sherr C.J. (1992). Identification and properies of an atypical catalytic subunit (p34PSK-J3/cdk4) for mammalian D type G1 cyclin. Cell 71, 323-334.

Merlo A., Herman J.G., Mao L., Lee D.J., Gabrielson E., Burger P.C., Baylin S.B. and Sidransky D. (1995). 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nature Med. 1, 686-692.

Miyashita T. and Reed J.C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 80, 293-299.

Mori T., Miura K., Aoki T., Nishimura T., Mori S. and Nakamura Y. (1994). Frequent somatic mutation of the MTS1/CDK4I (multiple tumor suppressor/cyclin-dependent kinase 4 inhibitor) gene in esophageal squamous cell carcinoma. Cancer Res. 54, 3396-3397.

Motokura T., Bloom T., Kim H.G., Juppner H., Ruderman J.V., Kronenberg H.M. and Arnold A. (1991). A novel cyclin encoded by a bcl1-linked candidate oncogene. Nature 350, 512-515.

Murakami Y. Hayashi K., Hirohashi S. and Sekiya T. (1991). Aberrations of the tumor suppressor p53 and retinoblastoma genes in human hepatocellular carcinomas. Cancer Res. 51, 5520-5525.

Nigro J.M., Baker S.J., Preisinger A.C., Jessup J.M., Hostetter R., Cleary K., Bigner S.H., Davidson N., Baylin S., Devilee P., Glover T., Collins F.S., Weston A., Modali R., Harris C.C. and Vogelstein B. (1989). Mutations in the p53 gene occur in diverse human tumor types. Nature 342, 705-708.

Nishida N., Fukuda Y., Kokuryu H., Sadamoto T., Isowa G., Honda K., Yamaoka Y., Ikenaga M., Imura H. and Ishizaki K (1992). Accumulation of allelic losses on arms of chromosomea 13q, 16q and 17p in the advanced stages of human hepatocellular carcinomas. Int. J. Cancer 5, 862-868.

Nishida N., Fukuda Y., Kokuryu H., Toguchida J., Yandell D.W., Ikenaga M., Imura H. and Ishizaki K. (1993). Role and mutational heterogeneity of the p53 gene in hepatocellular carcinoma. Cancer Res. 53, 368-372.

Nishida N., Fukuda Y. and Ishizaki K. (1994a). Molecular aspects of hepatocarcinogenesis and their clinical implications (Review). Int. J. Oncol. 4, 615-622.

Nishida N., Fukuda Y., Komeda T., Kita R., Sando T., Furukawa M., Amenomori M., Shibagaki I., Nakao K., Ikenaga M. and Ishizaki K. (1994b). Amplification and overexpression of the cyclin D1 gene in aggressive human hepatocellular carcinoma. Cancer Res. 54, 3107-3110.

Nobori T., Miura K., Wu D.J., Lois A., Takabayashi K. and Carson D.A. (1994). Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature 368, 753-756.

Oda T., Tsuda H., Scarpa A., Sakamoto M. and Hirohashi S. (1992). p53 gene mutatiom spectrum in hepatocellular carcinoma. Cancer Res. 52, 6358-6364.

Okamoto A., Demetrick D.J., Spillare E.A., Hagiwara K., Hussain S.P., Bennett W.P., Gerwin B., Serrano M., Beach D.H. and Harris C.C. (1994). Mutations and altered expression of p16INK4 in human cancer. Proc. Natl. Acad. Sci. USA 91, 11045-11049.

Owen-Schaub L.B., Zhang W., Cusack J.C., Angelo L.S., Santee S.M., Fujiwara T., Roth J.A., Deisseroth A.B., Zhang W.W., Kruzel E. and Radinsky R. (1995). Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. Mol. Cell. Biol. 15, 3032-3040.

Quelle D.E., Ashmun R.A., Shurtleff S.A., Kato J.Y., Bar-Sagi D., Roussel M.F. and Sherr C.J. (1993). Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. Genes Dev. 7, 1559-1571.

Schmidt E.E., Ichimura K., Reifenberger G. and Collins V.P. (1994). CDKN2(p16/MTS1) gene deletion or CDK4 amplification occurs in the majority of glioblastomas. Cancer Res. 54, 6321-6324.

Schuuring E., Verhoeven E., Mooi W.J. and Michalides R.J.A.M. (1992). Identification and cloning of two overexpressed genes, U21B31/PRAD1 and EMS1, within the amplified chromosome 11p13 region in human carcinomas. Oncogene 7, 355-361.

Serrano M., Gómez-Lahoz E., Depinho R. A., Beach D. and Bar-Sagi D. (1995). Inhibition of Ras-induced proliferation and cellular transformation by p16INK4. Science 267, 249-252.

Strauss M., Lukas J. and Bartek J. (1995). Unrestricted cell cycling and cancer. Nature Med. 1, 1245-1246.

Ueda H., Ullrich S.J., Gangemi J.D., Kappel C.A., Ngo L., Feitelson M.A. and Jay G. (1995). Functional inactivation but not structural mutation of p53 causes liver cancer. Nature Genet. 9, 41-47.

Unsal H., Yakicier C., Marçais C., Kew M., Volkmann M., Zentgraf H., Isselbacher K.J. and Ozturk M. (1994). Genetic heterogeneity of hepatocellular carcinoma. Proc. Natl. Acad. Sci. USA 91, 822-826.

Vidal M.J., Loganzo F. Jr., de-Oliveira A.R., Hayward N.K. and Albino A.P. (1995). Mutation and defective expression of the WAF1 p21 tumour-suppressor gene in malignant melanomas. Melanoma Res. 5, 243-250.

Waga S., Hannon G.J., Beach D. and Stillman B. (1994). The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature 369, 574-578.

Wakabayashi K., Nagao M., Esumi H. and Sugimura T. (1992). Food-derived mutagens and carcinogens. Cancer Res. 52 (Suppl.), 2092s-2098s.

Wang T.C., Cardiff R.D., Zukerberg L., Lees E., Arnold A. and Schmidt E.V. (1994). Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. Nature 369, 669-671.

Wang X.W., Gibson M.K., Vermeulen W., Yeh H., Forrester K., Stürzbecher H.W., Hoeijmakers J. H. J. and Harris C. C. (1995). Abrogation of p53-induced apoptosis by the hepatitis B virus X gene. Cancer Res. 55, 6012-6016.

Weinberg R.A. (1995). The retinoblastoma protein and cell cycle control. Cell. 81, 323-330.

Yang R., Gombart A.F., Serrano M. and Koeffler H.P. (1995). Mutational Effects on the p16INK4a tumor suppressor protein. Cancer Res. 55, 2503-2506.

Yang Z.-Y., Perkins N.D., Ohno T., Nabel E.G. and Nabel G.J. (1995). The p21 cyclin-dependent kinase inhibitor suppresses tumorigenicity in vivo. Nature Med.e 1, 1052-1056.

Yoshida S., Todoroki T., Ichikawa Y., Hanai S., Suzuki H., Hori M., Fukao K., Miwa M. and Uchida K. (1995). Mutations of p16INK4/CDKN2 and p15INK4B/MTS2 genes in biliary tract cancers. Cancer Res. 55, 2756-2760.

Zhang Y.J., Jiang W., Chen C.J., Lee C.S., Kahn S.M., Santella R.M. and Weinstein I.B. (1993). Amplification and overexpression of cyclin D1 in human hepatocellular carcinoma. BBRC 196, 1010-1016.

Zhang X., Xu H.J., Murakami Y., Sachse R., Yashima K., Hirohashi S., Hu S.X., Benedict W.F. and Sekiya T. (1994). Deletions of chromosome 13q, mutations in retinoblastoma 1, and retinoblastoma protein state in human hepatocellular carcinoma. Cancer Res. 54, 4177-4182.