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Differential expression of proteins related to START checkpoint of the cell cycle in human stomach, lung, cervix and liver cancers

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Abbreviations : PCNA, proliferating cell nuclear antigen; CDK4, cyclin dependent kinase 4; pRb, retinoblastoma protein; CDIs, cyclin dependent kinase inhibitors; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphysm; CDKN2, cyclin dependent kinase inhibitor 2

Abstract

START (restriction) checkpoint in late G1 of the cell cycle is important in the regulation of cell proliferation. Many tumors have shown abnormalities partly in the components such as p53, proliferating cell nuclear antigen (PCNA), retinoblastoma protein (pRb), cyclin dependent kinase 4 (CDK4), cyclin D1 and cyclin dependent kinase inhibitors (CDIs) were involved in the START checkpoint. To determine the differential expression of p53, PCNA, CDK4 and pRb in most common human cancers in Korea, immunohistochemical analyses for these proteins were performed on each 20 formalin-fixed and paraffin-embedded tumor tissues of stomach adenocarcinomas, lung cancers, cervix cancers and liver cancers. Significant differences existed in the expression of the proteins among the cancers from different anatomic sites. Stomach adenocarcinomas (55%) and small cell lung cancers (SCLCs) (70%) had high rate of p53 overexpression. Overexpression of pRb was shown in 70% of stomach adenocarcinomas and 90% non small cell lung cancers (NSCLSs). SCLCs had the highest rate (80%) of pRb negativities. Cervix cancers showed the highest rate (60%) of CDK4 overexpression and lower rate (15%) of p53 overexpression. Liver cancers had the highest rate (90%) of PCNA overexpression and the lowest rate (10%) of p53 overexpression. Our results indicate that, at least, one of the abnormalities in p53, PCNA, CDK4 or pRb function occurs very commonly in these cancers, thereby, suggesting that components in restriction checkpoint play a critical role in the development of these cancers through functional inactivation of pRb.

Key words: START checkpoint, p53, PCNA, CDK4, pRb

Introduction

Many human cancers are in part characterized by abnormalities in one or more of the genes responsible for regulating the cell cycle (Levine, 1993). The cell cycle is composed of G1, S, G2 and M phase. The transitions between different phases are regulated at checkpoints; such as restriction (START) checkpoint in G1 to S, S phase checkpoint in S to G2 and mitotic checkpoint. These checkpoints are regulated by specific cyclins and cyclin-dependent kinases (CDKs). Especially, restriction checkpoint in late G1 is thought to be very important in control of the cell cycle. That is because, at this checkpoint, the cell commits itself to another round of DNA replication and many positive and negative regulators are integrated into the cell cycle (Hunter and Pines, 1994; Peter and Herskowitz, 1994). Many proteins such as cyclin D, CDK4 or CDK6, proliferating cell nuclear antigen (PCNA), cyclin-dependent kinase inhibitors (CDIs) and retinoblastoma protein (pRb) are involved in the regulation of the restriction checkpoint. In late G1, cyclin D1 is bound to CDK4 and activates it. In addition to CDK4, cyclin D1 can form quaternary complexes with PCNA and p21. Cyclin D1-CDK4 complex can phosphorylate pRb, which is underphosphorylated and arrests cells in G1 phase. Phosphorylation of pRb relieves G1 arrest and allows cells to enter into S phase (Weinberg, 1995). Recently, CDIs such as p16, p15, p18 and p21 are found to be involved in the regulation of the restriction checkpoint. p16 inhibits CDK4 or CDK6 by binding in competition with cyclin D (Serrano *et al*., 1993). p15 binds to and inhibits specifically CDK4 in TGFβ-treated cells (Hannon and Beach, 1994). p21 is a key mediator of p53-mediated growth suppression (El-Deiry *et al*., 1993) and high levels of p21 can inhibits the cyclin D-CDK4 complex (Zhang *et al*., 1994).

Some components in the restriction checkpoint are found to be implicated in the formation and progression of cancers through mutation or overexpression. An extremely high incidence of mutations and rearrangements of the p53 gene is observed in several human cancers, implicating it as the most significant tumor-suppressor gene to date (Hollstein *et al*., 1991). Aberrations of pRb, cyclin

D1, p16 or PCNA are also found in many human cancers (Horowitz *et al*., 1990; Jang *et al*., 1993; Kamb *et al*., 1994; Nobori *et al*., 1994; Shapiro *et al*., 1995). All these abnormalities found in cancers at the restriction checkpoint are integrated finally into the loss or suppression of pRb function. Therefore, it is reasonable to infer that multiple disruptions of the growth-regulatory mechanisms defined by these proteins would be redundant (He *et al*., 1995).

Stomach, lung, cervix and liver cancers are most common cancers in Korea. Various abnormalities in some components of restriction checkpoint have been partly observed in these cancers (Tamura *et al*., 1991; Jang *et al*., 1993; Hur and Hur, 1995; Wiethege *et al*., 1995; Kim *et al*., 1996). However, more systemic analysis may need to evaluate the important roles of these proteins in the formation and progression of cancers

In this study, we present differential expression of p53, PCNA, CDK4 and pRb in paraffin-embedded tumor tissues of stomach, lung, cervix and liver cancers detected using immunohistochemical analysis.

Materials and Methods

Tumor specimens

Formalin-fixed and paraffin-embedded tumor tissues of 20 stomach adenocarcinomas, 20 lung cancers (10 NSCLCs and 10 SCLCs), 20 cervix cancers and 20 liver cancers, were obtained at Yeungnam University Hospital, Daegu, Korea.

Materials

Mouse monoclonal antibodies, anti-p53 (DO-1), anticyclin-D1 (HD10) and anti-PCNA (PC10) and affinity-purified rabbit polyclonal antibodies, anti-p16 (M-156), anti-p21 (N-20), anti-pRb (C-15) and anti-CDK4 (C-22) antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal anti-human pRb (G3-245), anti-human cyclin D1 (G124-326) and polyclonal rabbit anti-human p16 antibody were obtained from Pharmingen (San Diego, CA).

Immunohistochemistry

The immunohistochemical assay for detecting p53, PCNA, pRb, CDK4, cyclin D1, p21 and p16 in formalin-fixed and paraffin embedded tumor tissues was carried out using DAKO LSAB (DAKO, Carpinteria, CA) kit according to the instructions supplied by the manufacturer with minor modifications. Briefly, five-μm of paraffin sections of tumors dried on slide glasses treated with poly-L-lysine, were dewaxed twice in 100% xylene for 7 min and re-hydrated through absolute, 95% and 70% ethanols. The sections were incubated in 3% hydrogen peroxide for 5 min and rinsed in 20 mM Tris/Cl, pH 7.5, 137 mM NaCl and 0.01% Tween 20 (TBS-T). Following boiling the sections in citrate buffer (pH 6.0) for 5 min using a microwave oven, they were incubated with blocking solutions for 10 min and then reacted with antibodies at a 1:50 dilution in TBS-T at 4˚C overnight. The incubation times with Link solution and Streptavidine peroxidase solution were lengthened to 30 min. Chromogen (3% 3-amino-9-ethylcarbazole in *N,N*-dimethylformamide, AEC) was used for color development of all antibody stains. Hematoxylin was used as counterstain. Nuclear and cytoplasmic reactivity in tumor cells and nonneoplastic cells were separately graded as: absent, weak and strong.

Results

To examine the role of proteins involved in restriction checkpoint of the cell cycle in most common cancer in Korea, we carried out immunohistochemical ananlysis for p53, PCNA, CDK4, pRb, p21, cyclin D1 and p16 with 80 formalin-fixed and paraffin embedded tumor tissues of stomach adenocarcinomas, lung cancers, cervix cancers and liver cancers.

Figure 1 shows typical overexpression of p53 in stomach adenocarcinoma (Figure 1A) and non-small cell lung cancer (Figure 1B). The strong immunoreactivity to

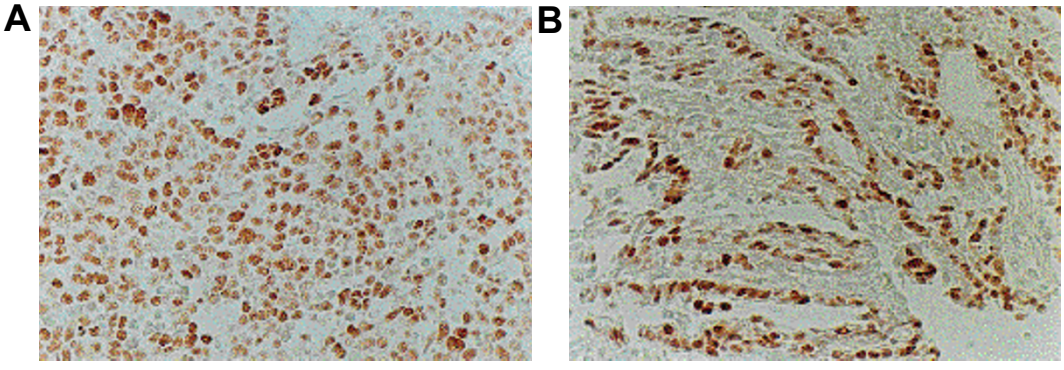

Figure 1. Typical p53 overexpression in stomach adenocarcinoma **(A)** and non-small cell lung cancer **(B)**. The strong immunoreactivity to p53 is heterogenous and clearly localized to nuclei of tumor cells (200x).

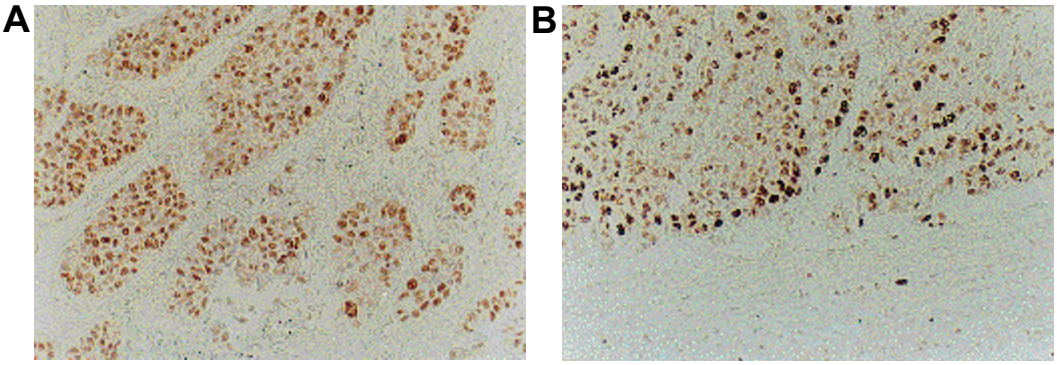

Figure 2. Typical PCNA overexpres-sion in non-small cell lung cancer **(A)** and liver cancer **(B)**. Nuclear staining for PCNA is observed in tumor cells and admixed nonneo-plastic cells, whereas tumor cells show stronger reactivity to PCNA than nonneo-plastic cells (100x).

p53 is heterogenous and clearly localized in nuclei of tumor cells.

Immunohistochemical analysis for p53 showed that 11 of the 20 (55%) stomach adenocarcinomas, 10 of the 20 (50%) lung cancers (in details, 3 of 10 (30%) NSCLCs and 7 of 10 (70%) SCLCs), 3 of the 20 (15%) invasive cervix cancers and 2 of the 20 (10%) liver cancers had clear nuclear staining only in the tumor cells, but not in nonneoplastic cells (Table 1).

In most of the 80 specimens exmained for PCNA, typical PCNA overexpression could be identified in nuclei both of tumor cells and nonneoplastic cells (Figure 2). However, nuclear reactivity to PCNA were different between tumor cells and admixed nonneoplastic cells. Overall, 10% (2/20) in stomach adenocarcinomas, 70% (7/10) in NSCLCs,

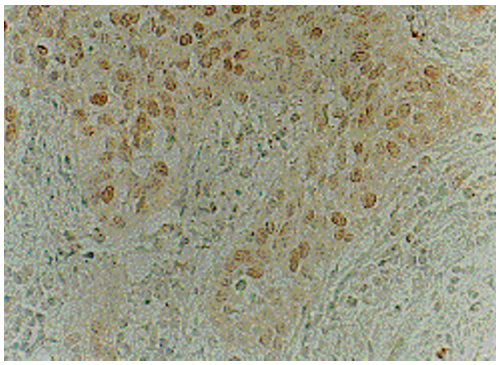

Figure 3. Typical CDK4 overexpression in cervix cancer. Tumor cells show strong nuclear and cytoplasmic staining and the reactivity is more intense in nuclei than in cytoplasm of tumor cells (200x).

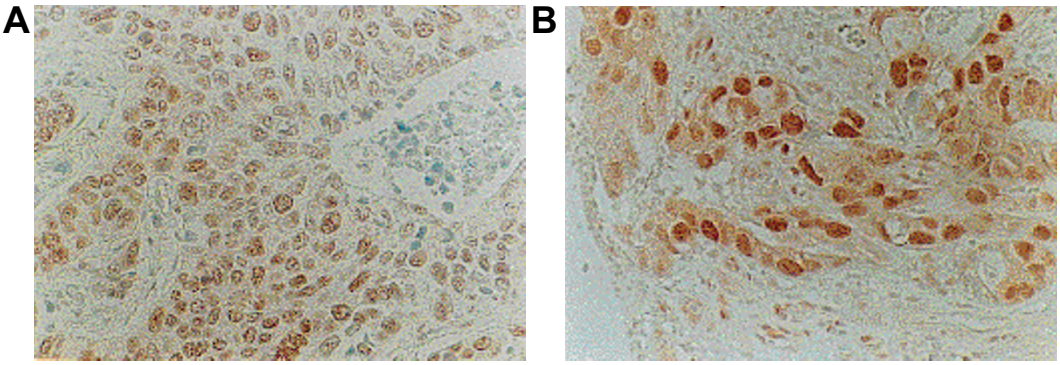

Figure 4. Typical pRb overexpression in non-small cell lung cancer **(A)** and stomach adenocarcinoma **(B)**. The immunoreactivity to pRb is stronger in tumor cells than in admixed nonneoplatic cells (200x).

Table 1. Differential overexpression of p53, PCNA, CDK4, and pRb in stomach, lung, cervix and liver cancers by immunohistochemistry

	No.	p53 overexpression	PCNA overexpression	CDK4 overexpression	pRb overexpression	pRb negativity
Stomach adenocarcinoma	20	11 (55%)	2 (10%)	3 (15%)	14 (70%)	0
Lung cancer						
NSCLC	10	3 (30%)	7 (70%)	3 (30%)	9 (90%)	0
SCLC	10	7 (70%)	3 (30%)	3 (30%)	1 (10%)	8 (80%)
Cervix cancer	20	3 (15%)	8 (40%)	12 (60%)	10 (50%)	0
Liver cancer	20	2 (10%)	18 (90%)	3 (15%)	6 (30%)	4 (20%)

30% (3/10) in SCLCs, 40% (8/20) in cervix cancers and 90% (18/20) in liver cancers showed more intense nuclear staining in tumor cells than in nonneoplastic cells in the same paraffin sections (Table 1).

In immunohistochemical staining for CDK4 (Figure 3), the nuclear and cytoplasmic reactivities were variable in 4 different tumor types. A tumor was considered to be CDK4 positive if there was nuclear staining in tumor cells, regardless of cytoplasmic staining. Fifteen percent (3/20) of stomach adenocarcinomas, 30% (6/20) of lung cancers, 60% (12/20) of cervix cancers and 15% (3/20) of liver cancers showed stronger nuclear CDK4 staining in tumor cells than in admixed nonneoplastic cells.

A tumor was considered to be pRb positive if there was nuclear staining in tumor cells and admixed nonneoplastic cells, regardless cytoplasmic staining, while pRb negative if there was no nuclear staining in tumor cells, whereas admixed nonneoplastic elements showed nuclear reactivity as a positive internal control. The nuclear reactivity for pRb was detected in all specimens. Typical pRb overexpression in stomach adenocarcinoma and NSCLC was shown in Figure 4. However, in 70% (14/20) of stomach adenocarcinomas and 50% (10/20) of cervix cancers, stronger nuclear staining was observed in tumor cells than in admixed nonneoplastic cells. Ninty percent (9/10) of NSCLCs showed the pRb overexpression, whereas 80% (8/10) of SCLCs were pRb negative. Twenty percent (4/20) of liver cancers was identified to be pRb negative.

Immunohistochemical analyses for p21, cyclin D1 and p16 were also performed in the 80 paraffin embeded tissues of 4 tumor types. However, we could not obtain any informative results.

Significant differences were observed in the expression of the proteins among the cancers from different anatomic sites (Table 1). Stomach adenocarcinomas had high rate of p53 (55%) and pRb (70%) overexpression and low rate of PCNA (10%) and CDK4 (15%) overexpression. Of interest, lung cancers showed clear differences in the expression of p53, PCNA and pRb between NSCLCs and SCLCs. NSCLCs showed a lower rate of p53 overexpression, a higher rate of PCNA overexpression and a higher rate in pRb overexpression than SCLCs, whereas SCLCs had a higher rate of p53 overexpression, a lower rate of PCNA overexpression and a higher rate in pRb negativities than NSCLCs.

Cervix cancers had the highest rate (60%) of CDK4 overexpression and low rate (15%) of p53 overexpression. Liver cancers had the highest rate (90%) of PCNA overexpression and the lowest rate (10%) of p53 overexpression.

Our results indicate that, at least, one of the abnormalities in p53, PCNA, CDK4 or pRb function occurs very commonly in tested four kinds of cancers, thereby, suggesting that components in restriction checkpoint play an critical role in the carcinogenesis and the progression of cancers.

Discussion

In immunohistochemical analysis for p53, the p53 overexpression was observed at a high rate in stomach adenocarcinomas (55%) and SCLCs (70%) and relatively at a low rate in NSCLCs (30%), cervix cancers (15%) and liver cancers (10%). Clear nuclear staining to p53 was detected only in tumor cells but not in nonneoplastic cells. The abnormalities of p53 observed in various tumors are its mutations (deletions or missense mutations) or the loss of function due to rapid degradation by binding of the transforming proteins such as HPV E6, SV40 large T and adenovirus E1B protein. The p53 overexpression was frequently observed in many tumor cells, which is identified to have p53 missense mutations in exons 5 to 8. The missense mutations in p53 were found to alter the conformation of protein and provide a longer half-life or greater stability in the cancer cells (Levine, 1993). Thus cancers with deletions, intronic mutations or missense mutations outside of exons 5 to 8 of p53 and cervix cancers infected with HPV 16 or 18 may show no nuclear staining in immunohistochemical analysis for p53. The p53 overexpression in stomach cancers were reported at the rate of 34% (Jang *et al*., 1993) and 58% (Kim *et al*., 1996). Our results of 55% in stomach adenocarcinomas might be comparable to the previous reports. Jang *et al*. (1993) and Kim *et al*. (1996) pointed out that no significant differences in the p53 expression were found in terms of the tumor size, differentiations, tumor invasion or metastasis of stomach cancers. PCR-SSCP analysis of p53 mutation in primary stomach cancer (Tamura *et al*., 1991) showed that 64% of aneuploid tumors had mutations in exons 4-8. The rate of p53 overexpression in lung cancers was variable from 39-67% in NSCLCs (Ebina *et al*., 1994; Fontanini *et al*., 1994; Wiethege *et al*., 1995) and 35% in SCLCs (Wiethege *et al*., 1995). In cervix cancers, the rate of p53 mutations was quite different between the results of either immunohistochemical (42%) and PCR-SSCP (13%) techniques (Kim and Kim, 1995). Our result in cervix cancers was comparable to that of PCR-SSCP analysis. Although we observed the lowest rate of p53 overexpression in liver cancers, the rate of p53 mutations in these cancers showed a significant discordance between the findings of immunohistochemical and molecular techniques. Henkler *et al*. (1995) showed a very high frequency (93%) of p53 mutation, as determined by using various antibodies and Hayashi *et al*. (1995) observed relatively low frequency (28%) of mutation by PCR-SSCP.

PCNA is an auxiliary protein of DNA polymerases δ and ε, required for DNA replication and repair. PCNA is physically associated with all the known CDK-cyclin complexes (Ruddon, 1995). The PCNA overexpression

was observed at the highest rate (90%) in liver cancers. Hur and Hur (1995) also showed that the PCNA labeling index (percentage of positive cells per 100 tumor cells) in liver cancers was 10 times higher in tumor tissues than in nonneoplastic tissues and significantly increased as the tumor grade was higher. Maeda *et al*. (1996) also showed a higher PCNA labeling index in stomach cancers with lymph node metastasis than in those without lymph node metastasis. Kaneko and Izutsu (1995) showed a higher PCNA labeling index in cervix tumors of high grade than in those of low grade. In NSCLCs, PCNA overexpression was detected in the majority of tumor specimens compared to normal lung (Horowitz *et al*., 1990). The rate of PCNA positivity in NSCLCs was reported to be high as 72% (Wiethege *et al*., 1995) to 98 % (Ebina *et al*., 1994), whereas SCLCs had a relatively low rate (51%) of PCNA positivity (Wiethege *et al*., 1995).

CDK4 overexpression was found the highest rate in cervix cancers, which showed relatively low rate of p53 overexpression and PCNA overexpression. There was no reported about the aberrations of CDK4 in cervix cancers and other three cancers. Some investigators reported that CDK4 amplification is an alternative mechanism to CDKN2 homozyous deletion in glioma cell lines (Nobori *et al*., 1994) and glioma tissues (Sonoda *et al*., 1995). Therefore, further studies on *CDK4* amplification in these cancers will be necessary.

pRb is a nuclear protein of 105 to 110 kDa and its locus is located on chromosome 13q14. Rb mutations or deletions has been seen at a high frequency in retinoblastoma, SCLCs, bladder and mammary carcinomas (Ruddon, 1995). The transforming proteins such as SV40 T, adenovirus E1A and HPV E7 were identified to affect carcinogenesis by binding to and inhibiting pRb. In our results, SCLCs had the highest rate of pRb negativities, which is comparable to results from other reports (Hagashima *et al*., 1994; Geradts *et al*., 1995). The pRb overexpression observed in stomach adenocarcinomas, cervix cancers and NSCLCs might result from the increase of the phosphorylated pRb. Our result of 20% of pRb negativities in liver cancer was the same as the results (20%) reported by Hsia *et al*. (1994) and Zhang *et al.* (1994). Recently the differential inactivation of pRb and CDKN2 were frequently identified in various tumors (Kelley *et al*., 1995; Ruddon, 1995).

We also carried out the immunohistochemical analyses for p21, cyclin D1 and p16 in the 80 paraffin embedded tissues of 4 tumor types. However, we could not obtain any informative results due to unresolved problems such as lack of the proper development of immunohistochemical methods for these proteins and differences in the specificity of commercially available antibodies.

Our results from PCR analysis for the *p16* gene (CDKN2) in the same tumor specimens showed that inactivations of CDKN2 due to mutations or hypermethylation were detected at a high frequency in these cancers. The overexpression of cyclin D1 expression was identified frequently in primary NSCLCs (56%) and NSCLC cell lines by Shapiro *et al*. (1995).

Lukas *et al*. (1995) suggested that cooperating aberrations within the cyclin D-CDK4-p16-pRb pathway are implicated in multistep oncogenesis and there are numerous candidate targets and potential oncogenic scenarios of deregulating the pathway. Four oncogenic scenarios have been documented as 1) loss of p16, 2) deregulated expression of cyclin D1, 3) cooperation of loss of p16 and overexpression of cyclin D1 and 4) lack of functional pRb. Our results suggests that the very high frequency of one or more abnormalities in p53, PCNA, CDK4 or pRb among these cancers indicate that functional inactivation of pRb may play a critical role in the development of most cancers and, therefore, there might be additional oncogenic scenarios involved other than aberrations of CDK4 , PCNA and p53.

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