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Analysis of p53 Mutation and Cyclin D1 Expression in Breast Tumors

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P53 and cyclin D1 are interacting regulatory genes and both are frequently altered in breast cancer. We analysed p53 mutation by SSCP and sequencing methods as well as p53 protein accumulation immunohistochemically in 34 consecutively operated breast tumors. None of 4 fibroadenomas revealed p53 mutation or p53 protein accumulation. Mutation of p53 was present in 7 carcinomas. Immunohistochemistry revealed accumulation of p53 protein in 6 carcinomas and there was a signif-

icant correlation between p53 mutation and protein accumulation. Overexpression of cyclin D1 protein was observed in 11 carcinomas by immunohistochemistry and no correlation was observed between cyclin D1 overexpression and p53 mutation or accumulation. Our data support the concept that the p53-cyclin D1 signal pathway and the cyclin D1 cascade are disregulated in breast cancer. (Pathology Oncology Research Vol 5, No 2, 90–94, 1999)

Keywords: breast cancer; fibroadenoma; p53; mutation; cyclin D1; immunohistochemistry

Introduction

P53 and cyclin D1 have a basic role in the complex mechanism of the cell cycle. The multifunctional p53 protein is involved in the regulation of DNA synthesis and repair, progress of cell cycle and programmed cell death.^{11,27} Mutation of p53 is the most frequent known genetic alteration in human cancer and it occurs in 22% of breast cancers.¹⁰ Accumulation of the p53 protein has been observed in various tumors, and reflects the increased half life of the protein due to mutation or association with other proteins.¹⁶ The clinical significance of mutation or accumulation of p53 in breast cancer has been studied thoroughly, p53 alteration seems to be associated with worse prognosis.^{1,12,14,25}

Intrinsic or environmental DNA damages, through the increased synthesis of p53 protein change the transcriptional level of several cell cycle checkpoint related genes, including mdm,³⁰ GADD 45¹³ and p21 (WAF1/CIP1).⁶ p21

inhibits the activity of cyclin-dependent kinase (CDK) complexes,⁶ the progression of the cell cycle, the replication of DNA,²⁸ and represents the primary mediator of the effect of p53 on the cell cycle. In normal cells, cyclin D1 forms a multimer complex with CDK4, CDK6 and activates the CDK catalytic activity.²⁰ Cyclin D1 has a short half life, its expression is elevated in G1 phase of cell cycle⁵ and is highly influenced by growth factors.⁹ The primary role of cyclin D1 is the regulation of the cell cycle through the G1 phase. In some cases increased levels of cyclin D1 has been observed in arrested, senescent cells¹⁹ and may contribute to the induction of apoptosis in these cells as well.¹⁵ The chromosomal region (11q13) harbouring cyclin D1 gene is amplified in a variety of tumors including about 20% of breast cancers¹⁸ and commonly results in increased expression of the cyclin D1. Overexpression of cyclin D1, which can occur without amplification, has been observed in 25–50% of breast cancers by immunohistological methods.^{21,22} The biological consequence of 11q13 amplification and cyclin D1 overexpression in breast cancer is still discussed. Previous studies had indicated that amplification of 11q13 was associated with poor prognosis,^{7,24} while recent observations have shown that cyclin D1 overexpression was associated with increased or only minimally decreased relapse

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free interval.⁸ p21 mediates not only the biological activity of p53 by inhibition of cyclin dependent kinases, but also results in an increased expression of cyclin D1.⁴ However, little is known about how the mutation of p53 or overexpression of cyclin D1 affects this regulatory loop in mammary tumor cells. The aim of this study was to analyse the correlation between cyclin D1 expression and p53 mutation and/or accumulation in breast tumors.

Material and methods

Tumor specimens

34 consecutively operated women with breast tumor were included in this study. The frozen section was examined from each tumor and areas of normal tissue were separated. DNA was separately isolated from sections of frozen tumor and corresponding normal tissues by QIAamp kit (Qiagen). After formaldehyde fixation and paraffin-embedding, 5 µm thick tissue sections were stained with hematoxylin and eosin. Slides were coated for immunostaining with poly-L-lysine (Sigma).

Immunohistochemistry

Paraffin-embedded tissue sections were dewaxed, rehydrated, and autoclaved in 10 mM citrate buffer (pH 6.4) for 20 min in order to restore antigenicity.²⁶ Phosphate buffered saline (PBS) wash was used between each of the following steps. Unspecific protein binding of tissue section was blocked with 1% bovine serum albumin containing PBS for 30 min at 37°C, then the slides were incubated overnight with the primary antibodies p53 (CM-1 rabbit polyclonal in 1:20000 dilution and DO7 murine monoclonal 1:500 dilution, both from Medac, Hamburg, Germany) and cyclin D1 (HD11 mouse monoclonal in 1:400 dilution, Santa Cruz Biotech., Santa Cruz, USA), separately. Primary antibodies were revealed by a biotin-streptavidin-amplified detection system (Biogenex, Sun Ramon, USA) using naphthol phos-

phate and fast red as colour substrate. Slides were lightly counterstained with hematoxylin and mounted in Kaiser's glycerin-gelatine. Negative control was stained with the omission of the primary antibodies. Immunostaining was assessed by two of us independently. p53 staining was scored as positive or negative, while cyclin D1 staining was ranked as follows: negative (-), weak (+), moderate (++) , strong (+++), based on the intensity of nuclear staining in the majority of the cells. Discrepant scoring was re-evaluated and consensus reached.

Mutation analysis

Mutation analysis of exons 5–9 of p53 were performed by PCR-SSCP and direct sequencing methods. Exons were amplified individually, except 8 and 9, which were amplified in one fragment (*Table 1*). 25 µl PCR reaction mixture consisted of 10 nM Tris-HCl (pH 8.3), 1,75 mM MgCl₂, 50 mM KCl, 0,01% gelatine, 200 µM each of four dNTP, 0,25 µM of each primer, 0,5 U Taq DNA polymerase (Boehringer Mannheim Biochemica, Germany), and 200 ng DNA. The PCR reaction was carried out as follows: initial denaturation, 3 min at 94°C, then 30 cycles of 94°C 45 sec, 55°C 30 sec, 72°C 1 min. PCR products were diluted in 5 excess volumes of formamide dye solution (95% formamide, 0,05% bromophenol blue, 0,05% xylene cyanol, 20 mM EDTA, 10 mM NaOH) and were loaded on a 0,5x MDE polyacrilamid gel, immediately after heating for 2 min at 95°C and chilling on ice. Electrophoresis was performed at 15°C with 6 W constant power for 20 hours. DNA was then transferred to Hybond N membrane (Amersham, Germany) and fixed in an oven at 80°C for 2 hours. After hybridisation with digoxigenin end-labelled corresponding primers in Church buffer at 37°C for 1 hour, DNA was visualised by a digoxigenin detection system (Boehringer Mannheim Biochemicals, Germany) using BCIP/NBT substrates. Samples showing bands with altered mobility were subjected to sequencing. PCR products were purified from low melting temperature agarose gel and sequencing reaction was performed with PRISM dyedeoxy cycle sequencing reagent (Perkin-Elmer, USA) according to the manufacturer's instructions. Sequence was evaluated on an ABI-373 DNA analysis system (Perkin-Elmer, USA).

Table 1. Primers for PCR amplification of exons 5 to 9 of the p53 gene (31)

Exon	Sequence 5'-3'	Position ^x
5	GTCTCCTCCTCTCCTACAG	13034–13054
	TCTCCAGCCCCAGCTGCTCA	13259–13240
6	GAGACGACAGGGCTGGTT	13258–13275
	GAGACCCAGTTGCAAACC	12450–12433
7	CCAAGGCGCACTGGCCTC	13957–13974
	GAGGCAAGCAGAGGCTGG	14169–14152
8–9	CCTFACTGCCTCTTGCTTC	14413–14431
	CTGGAAACTTTCCACTTGAT	14796–14777

^x Positions are defined on sequence x54156.

Results

Four of the analysed tumors were benign fibroadenomas, 30 were carcinomas (*Table 2*). After antigen retrieval by autoclave treatment, all of the applied antibodies resulted in easily evaluable signals showing the expected nuclear localisation (*Figure 1*). Negative controls had no staining and there was no immunoreactivity for p53 in normal tissue, while sporadic weak staining was observed for cyclin D1. There was no substantial discrepancy between

the data obtained with the two p53 specific antibodies, and results were therefore combined. Six of 34 tumors (18%) had an increased amount of p53 protein, and none of the four fibroadenoma was in this group (Table 2). Cyclin D1 staining was displayed in all but one case intranuclear location (Table 2). The intensity of the reaction showed great variation. 11 of the 34 tumors, including 1 fibroadenoma had a significantly increased level of cyclin D1 relative to the normal mammary tissue (Table 2).

Table 2. Main clinico-pathologic data and results of p53 mutation as well as immunohistological analysis of p53 and cyclin D1 in 34 breast tumors

Histological diagnosis		pT	Grade malignancy	of p53 mutation	Immunohistochemistry	
					p53	cyclin D1
1	fibroadenoma			-	-	-
2	fibroadenoma			-	-	++
3	fibroadenoma			-	-	+
4	fibroadenoma			-	-	-
5	ductal carcinoma	2	G3	exon 6	-	+
6	ductal carcinoma	2	G2	-	-	++
7	ductal carcinoma	2	G2	-	-	-
8	ductal carcinoma	2	G3	-	-	++
9	ductal carcinoma	2	G3	-	+	-
10	ductal carcinoma	2	G2	exon 6	+	IC*
11	ductal carcinoma	1	G2	-	-	-
12	ductal carcinoma	2	G3	-	-	+++
13	ductal carcinoma	2	G2	-	-	++
14	ductal carcinoma	1	G2	-	-	-
15	ductal carcinoma	2	G3	-	-	+
16	ductal carcinoma	2	G2	-	-	+++
17	ductal carcinoma	2	G2	-	-	++
18	ductal carcinoma	2	G3	-	-	-
19	ductal carcinoma	1	G1	exon 5	-	++
20	ductal carcinoma	2	G2	-	-	-
21	ductal carcinoma	4	G3	exon 6	-	+++
22	ductal carcinoma	4	G3	-	-	++
23	ductal carcinoma	1	G2	-	-	+
24	ductal carcinoma	1	G3	-	-	+
25	ductal carcinoma	2	G2	-	-	-
26	ductal carcinoma	2	G2	-	-	-
27	ductal carcinoma	2	G3	-	+	+
28	ductal carcinoma	1	G1	-	-	+
29	ductal carcinoma	2	G3	exon 7	+	-
30	lobular carcinoma	3		-	-	-
31	lobular carcinoma	2		-	-	+
32	lobular carcinoma	2		exon 7	+	-
33	lobular carcinoma	2		exon 6	+	++
34	lobular carcinoma	2		-	-	+

* Intracytoplasmic staining

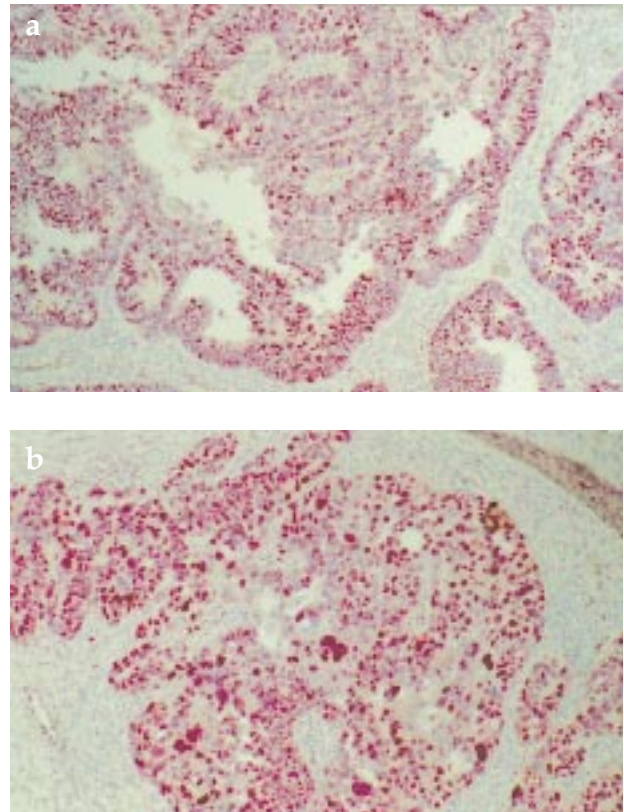


Figure 1. Immunohistochemical staining of primary breast cancer sections for p53 (a) and cyclin D1 (b). A strong nuclear reaction is present for both proteins.

PCR-SSCP analysis showed DNA fragments with altered mobility in 7 cases (Table 2). None of these tissues was a fibroadenoma. None of the mutations were present in the corresponding normal tissues. Mutation was confirmed in all of these cases by sequencing (Table 3). Three of the mutations resulted in the formation of a new stop codon, while all others in amino acid change. Four of the seven mutations were associated with accumulation of p53 protein. Two cases with increased level of p53 had no sign of mutation in the examined sequence. There was correlation between p53 mutation and p53 protein accumulation (χ^2 test $p < 0.01$). In contrast, no tendency of correlation was observable between p53 mutation and cyclin D1 expression (χ^2 test $p > 0.2$).

Discussion

Previous studies have identified p53 mutations in about 22% of breast cancers¹⁰ and recent reports indicated overexpression of cyclin D1 in 30–50% of the cases.^{21,22} In the present study we have identified in 23% and 33% of the carcinomas mutation of p53 and overexpression of cyclin D1, respectively. There was a significant correlation between p53 mutation and p53 protein

Table 3. Identified mutations of p53 gene in breast cancer

Patient N°	Exon	Codon	Nucleotide change	Consequence
5	6	192	CAGUTAG	Gln→Stop
10	6	222	CCGUACG	Pro→Thr
22	5	146	TGGUTAG	Trp→Stop
23	7	245	GGCUAGC	Gly→Ser
25	6	202	CGTUCCT	Arg→Pro
26	6	220	TATUTAA	Tyr→Stop
34	7	245	GGCUGAC	Asn→Thr

accumulation. On the other hand, no correlation was present between p53 mutation and cyclin D1 expression. In previous studies, association was observed in breast cancer between cyclin D1 overexpression and the presence of estrogen receptor.^{7,22} In contrast to the general expectation, increased cyclin D1 expression was not associated with increased rate of cells in S phase or with worse prognosis.^{8,22} This finding is in conformity with the generally better prognosis of estrogen receptor positive breast cancers but contradicts earlier studies which indicated that 11q13 amplification was relevant for poor prognosis.^{7,22} The frequent amplification of the chromosomal region of cyclin D1 in breast cancer and the increased susceptibility in mice for mammary cancer after cyclin D1 overexpression strongly indicate the substantial role of cyclin D1 in the pathogenesis of mammary cancer, although cyclin D1 does not seem to maintain the same crucial function in the tumor.²⁹ The major function of the cyclin-CDK complex is the phosphorylation of retinoblastoma protein (pRB) in normal cells.⁵ Various tumors such as retinoblastoma, small cell lung cancer, certain bladder cancer proliferate without functional retinoblastoma protein.^{2,23} The most probable explanation for the lack of correlation between cyclin D1 overexpression and prognosis in breast cancer is that during the tumor progression cancer cells loss, bypass or replace the regulatory role of pRB, and proliferate in the absence of pRB, as well. In normal cells there is a regulatory pathway between p53 and cyclin D1. p53, through the induction of p21 inhibits the CDK activity and induces cyclin D1 overexpression.⁴ It is known, that some of the p53 mutations abolish the inductive effect on p21.¹⁷ A recent immunohistological study found no correlation between p53 accumulation and cyclin D1 expression in mammary cancer.²² Our study, based on both mutation analysis of p53 and immunohistochemical assessment of p53 and cyclin D1 levels, reached the same conclusion, the lack of correlation between p53 mutation and cyclin D1 expression. This result is in agreement with the observations that p53 mutation may result in loss of the cell cycle effect of p53 and with the concept that the cyclin-CDK-pRB cascade is disregulated in breast cancer.²¹

References

- Andersen TI, Holm R, Nesland JM, et al: Prognostic significance of TP53 alterations in breast carcinoma. *Br J Cancer* 68:540-548, 1993.
- Bates S, Parry D, Bonetta L, et al: Absence of cyclin D/cdk complexes in cells lacking functional retinoblastoma protein. *Oncogene* 9:1633-1640, 1994.
- Bankfalvi A, Navabi H, Bier B, et al: Wet autoclave pretreatment for antigen retrieval in diagnostic immunohistochemistry. *J Pathol* 174:223-228, 1994.
- Chen X, Bargonetti J, Prives C, et al: P53, through p21 (WAF1/CIP1) induces cyclin D1 synthesis. *Cancer Res* 55:4257-6342, 1995.
- Cordon-Cardo C: Mutation of cell cycle regulators. *Am J Pathol* 147:545-560, 1995.
- El-Deiry WS, Tokino T, Velculescu V E, et al: WAF1, a potential mediator of p53 tumor suppression. *Cell* 75:817-825, 1993.
- Fantl V, Smith R, Brokes S, et al: Chromosome 11q13 abnormalities in human breast cancer. *Cancer Surv* 18:77-94, 1993.
- Gillett C, Smith P, Gregory W, et al: Cyclin D1 and prognosis in human breast cancer. *Int J Cancer* 69:92-99, 1996.
- Ggrana K, Reddy EP: Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). *Oncogene* 11:211-219, 1995.
- Greenblatt MS, Bennett WP, Hollstein M, et al: Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res* 54:4855-4878, 1994.
- Hollstein M, Sidransky D, Vogelstein B, et al: p53 mutation in human cancers. *Science* 253:49-53, 1991.
- Jacquemier J, Moles JP, Adelaide J, et al: P53 immunohistochemical analysis in breast cancer with four monoclonal antibodies: comparison of staining and PCR-SSCP results. *Br J Cancer* 89:846-852, 1994.
- Kastan MB, Zhan Q, El-Deiry WS, et al: A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-teleangiectasia. *Cell* 71:587-597, 1992.
- Kovach JS, Hartmann A, Blaszik H, et al: Mutation detection by highly sensitive method indicates that p53 gene mutations in breast cancer can have important prognostic value. *Proc Natl Acad Sci USA* 93:1093-1096, 1996.
- Kranenburg O, van der Eb AJ, Zanema A: Cyclin D1 is an essential mediator of apoptotic neuronal cell death. *EMBO J* 15:46-54, 1996.
- Lane DP: P53 guardian of genome. *Nature* 359:15-16, 1992.
- Li Y, Jenkins C, Nichols M, et al: Cell cycle expression and p53 regulation of the cyclin-dependent kinase inhibitor p21. *Oncogene* 9:2261-2268, 1994.
- Lidereau R, Mathieu D, Escot C, et al: Genetic variability of proto-oncogenes for breast cancer risk and prognosis. *Biochemie* 70:951-959, 1988.
- Lucibello FC, Sewing A, Brusselbach S, et al: Deregulation of cyclin D1 and E and suppression of cdk2 and cdk4 in senescent human fibroblasts. *J Cell Sci* 105:123-133, 1993.
- Matsumine H, Quelle DE, Shurtleff SA, et al: D-type cyclin-dependent kinase activity in mammalian cells. *Mol Cell Biol* 14:2066-2076, 1994.
- McIntosh GG, Anderson JJ, Milton I, et al: Determination of the prognostic value of cyclin D1 overexpression in breast cancer. *Oncogene* 11:885-891, 1995.
- Michalides R, Hageman P, Tinteren H, et al: A clinicopathological study on overexpression of cyclin D1 and p53 in a series

- of 248 patients with operable breast cancer. *Brit J Cancer* 73:728-734, 1996.
23. *Schauer IE, Siriwardana S, Langan TA, et al*: Cyclin D1 over-expression vs. retinoblastoma inactivation: implications for growth control evasion in non-small cell and small cell lung cancer. *Proc Natl Acad Sci USA* 91:7827-7831, 1994.
 24. *Shuurin E, Verhoeven E, van Tinteren H, et al*: Amplification of genes within the chromosome 11q13 region is indicative of poor prognosis in patients with operable breast cancer. *Cancer Res* 52:5229-5234, 1992.
 25. *Thor AD, Moore DH, Edgerton SM, et al*: Accumulation of p53 tumor suppressor gene protein: an independent marker of prognosis in breast cancer. *JNCI* 84:845-855, 1992.
 26. *Toguchida J, Yamaguchi T, Rithchie B, et al*: Mutation spectrum of the p53 gene in bone and soft tissue sarcomas. *Cancer Res* 52:6194-6199, 1992.
 27. *Vogelstein B, Kinzler KW*: P53 function and dysfunction. *Cell* 70:523-526, 1992.
 28. *Waga S, Hannon GJ, Beach D, et al*: The p21 inhibitor of cyclin dependent kinases controls DNA replication by interaction with PCNA. *Nature* 369:574-578, 1994.
 29. *Wang TC, Cardiff RD, Zukerberg L, et al*: Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. *Nature* 369:669-671, 1994.
 30. *Wu X, Bayle JH, Olson D, et al*: The p53-mdm2 autoregulatory feedback loop. *Genes Dev* 7:1126-1132, 1993.
 31. *Xiong Y, Hannon GJ, Zhang H, et al*: P21 is a universal inhibitor of cyclin kinases. *Nature* 366:701-704, 1993.