Prognostic Importance of p53 and c-*erb*B-2 Oncoproteins Overexpression in Patients with Breast Cancer

SUNANTA CHARIYALERTSAK, M.Sc.* SUWAT CHARIYALERTSAK, M.D., Ph.D.**,
ARKOM CHEIRSILPA, M.D.* KITI CHINDAVIJAK, M.D., F.A.C.S., F.R.C.S.T., F.I.C.S.*

Abstract

Using immunohistochemistry, 119 breast cancer tissues were examined for overexpression of p53 and c-*erb*B-2 oncogene proteins. In 46 (38.7%) of the cases p53 was overexpressed, while 35 (29.4%) demonstrated positive c-*erb*B-2 immunostaining. Expression of these two oncogene products was closely correlated ($p<0.01$). There was no significant association between p53 protein expression and age of the patients, clinical stage, tumor size, number of involved nodes or estrogen receptor status. However, we found significant correlation between p53 protein expression and 5-year disease-free survival ($p=0.0113$). In addition, the findings in this study clearly indicated that the co-overexpression of p53 and c-*erb*B-2 proteins was a powerful predictor for early recurrence in the patients with breast cancer.

The 20-kilobase p53 gene (TP53) is present on human chromosome 17p and encodes a 53-kilodalton (Kd) nuclear phosphoprotein[1-3]. Mutations in the p53 gene have been identified in a variety of human malignancies[3-6], including breast cancer[3,6]. Most mutations in the p53 gene are commonly associated with overexpression of mutated p53 protein which can be immunohistochemically detected in the nuclei of cancer cells [7-9]. Overexpression of this protein has also been suggested to be of prognostic value in breast cancer patients[10,11].

The c-*erb*B-2 (HER-2, *neu*) gene is carried on chromosome 17q and encodes a 185 Kd glycoprotein that is closely related to the epidermal growth factor receptor protein[12-14]. Both gene amplification and protein overexpression analyses have shown that elevated c-*erb*B-2 is a poor prognostic indicator in patients with breast cancer [15-17]. In our previous study we also found that overexpression of the c-*erb*B-2 protein detected by immunohistochemistry was an effective predictor for disease recurrence in the patients[18]. In this article, data of p53 expression correlating with cli-

* National Cancer Institute, Bangkok 10400,
** Department of Community Medicine, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand.

nical parameters and five-year disease-free survival were additive. Moreover, we also examined the effects of the two oncogene products coexpression on five-year disease-free survival which were included in the present study.

MATERIAL AND METHOD

Patients

This study included 119 primary tumors from women with breast cancer treated at the National Cancer Institute, Bangkok between 1987 and 1989. None of the patients had distant metastasis at the time of operation. All node-positive patients received six cycles of adjuvant chemotherapy containing cyclophosphamide, methotrexate and fluorouracil and local radiation (if the primary tumor was T3 and the patients had inadequate lymph node dissection). For the node-negative patients treatment varied according to T lesion, hormone receptor and age. After surgery, tissue samples were kept frozen at -70°C until use for biochemical determination of estrogen receptor (ER)[19]. Receptor concentration less than 10 fmol/mg was considered negative. A parallel sample was processed using routine techniques for histological examination and immunohistochemical study on paraffin sections. The mean patient follow-up period was more than five years.

Immunohistochemical staining

The method for detection of c-*erb*B-2 was performed as previously described[18]. To detect p53 in formalin-fixed, paraffin-embedded tissues, an antigen retrieval using microwave technique was employed. Four-micrometer thick paraffin embedded sections were deparaffinized in xylene, rehydrated through alcohol and then treated with 3 per cent hydrogen peroxide in deionized water for 5 min to block endogenous peroxidase activity. The sections were washed with phosphate-buffered saline (PBS) and placed in a plastic coplin jar containing distilled water. The jar was heated in a microwave oven (800 w) at the medium power setting for three 4-min cycles with an interval of one min between cycles to check on the water level in the jar. After heating, the coplin jar was removed from the oven and allowed to cool for 15 min. The slides were rinsed in PBS and preincubated with 1 per cent normal horse serum in PBS for 30 min. The sections were then incubated for 1 hour at room temperature with mouse monoclonal antibody against p53 (DO7, Novocastra Lab., New Castle, UK) at a dilution of 1:200 in PBS. After rinsing in PBS, the sections were incubated for 30 min at room temperature with a biotinylated anti-mouse immunoglobulin (Vector Lab., Burlingame, CA) at a dilution of 1:1000, then rinsed again with PBS. Antibody binding was visualized by incubation with avidin-biotin peroxidase complex (Vectastain ABC kit, Vector Lab., Burlingame, CA) for 30 min at room temperature. The sections were rinsed in PBS and immersed in Tris-buffered saline with a pH of 7.4 containing 3, 3'- diaminobenzidine and 0.5 per cent hydrogen peroxide for 5 min and counterstained with Mayer's hematoxylin for 5 min. Finally, they were rinsed in tap water, dehydrated in ethanol, cleared in xylene and mounted in permount.

Staining with 1 per cent normal horse serum as primary antibody was used as a negative control. As positive controls, we included in each run sections from a formalin -fixed, paraffin-embedded breast cancer which had been previously shown to contain mutated p53 protein. Obvious nuclear staining in any number of malignant cells was considered positive .

Statistical analysis

The correlation between the immunostaining of p53 oncoprotein and other clinical parameters including c-*erb*B-2 expression was evaluated by $\chi 2$ test. Five-year disease-free survival (DFS) curves were performed by the Kaplan-Meier method[20] and the difference between the curves was assessed using the log rank test[21].

RESULTS

Of the 119 breast carcinomas included in this study, 46 (38.7%) were positive for p53 and 35 (29.4%) were positive for c-*erb*B-2. The relationship between staining of p53 oncoprotein and clinocopathologic features including c-*erb*B-2 protein expression is shown in Table 1. Positivity of p53 protein was significantly associated with c-*erb*B-2 protein overexpression ($P<0.01$). No significant association was observed between expression of p53 protein and age, stage, tumor size, number of positive axillary nodes or ER status.

There was a significant difference in the 5-year disease-free survival curves between the patients with p53-positive tumors and the other cases with p53-negative tumors (Fig.1) ($p=0.0113$).

Table 1. Expression of p53 protein in relation to clinicopathologic parameters.

Parameters	Number of cases Staining of p53 protein				Total	P
	Negative		Positive			
Age at diagnosis (yrs)						
<40	23	(69.7)	10	(30.3)	33	
40-60	45	(59.2)	31	(40.8)	76	NS
>60	5	(50.0)	5	(50.0)	10	
Stage						
I	18	(78.3)	5	(21.7)	23	
II	36	(62.1)	22	(37.9)	58	
III	11	(52.4)	10	(47.6)	21	NS
IV	0	(0)	3	(100.0)	3	
Unknown	8		6		14	
Tumor size (cm)						
≤ 3	61	(66.3)	31	(33.7)	92	
> 3	10	(47.6)	11	(52.4)	21	NS
Unknown	2		4		6	
No. positive axillary nodes						
0	40	(70.2)	17	(29.8)	57	
1-3	17	(63.0)	10	(37.0)	27	NS
>3	16	(45.7)	19	(54.3)	35	
ER status						
-	37	(56.1)	29	(43.9)	66	NS
+	36	(67.9)	17	(32.1)	53	
*c-erb*B-2						
-	58	(69.0)	26	(31.0)	84	<0.01
+	15	(42.9)	20	(57.1)	35	

Five-year disease-free survival curves were also plotted for the combination of p53 and c-*erb*B-2 oncoproteins with four nonoverlapping categories: negative for both, p53-positive; c-*erb*B-2 negative, p53 negative; c-*erb*B-2 positive, and doubly positive as shown in Fig. 2. Patients with tumors overexpression either p53 or c-*erb*B-2 separately did worse than patients lacking both oncoproteins. Patients with tumors that were both p53 and c-*erb*B-2 positive had the worst prognosis (p= 0.0001).

DISCUSSION

Recently, several investigators have agreed that immunohistochemical methods used to detect expression of the p53 and c-*erb*B-2 gene products in paraffin-embedded archival samples is reliable in indicating p53 missense mutations and c-*erb*B-2 gene amplification[22-25]. The antibodies to p53 and c-*erb*B-2 proteins used for immunostaining in this study were clearly demonstrated to provide complete agreement between immunohistochemistry and molecular biology assays[26,27].

A wide range of experimental data has been published on p53 expression in breast cancer. Using different monoclonal antibodies and staining conditions, p53 overexpression has been detected in 15 per cent to 50 per cent of breast cancers[28]. In the present study, overexpression of p53 was found in 39 per cent of the tumors using th DO7 antibody on paraffin-embedded sections, being similar to the findings of a study by Jacquemier et al[29] who used DO1 antibody on paraffin-embedded sections.

The prognostic value of p53 expression in relation to other prognostic parameters in breast

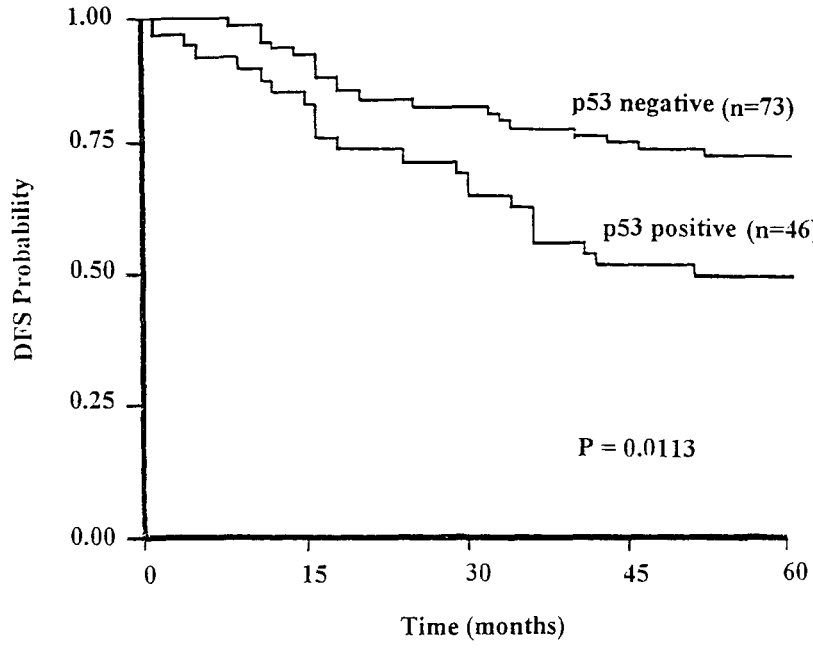

Fig. 1. Five-year disease-free survival curves for patients with breast cancer subdivided by p53 immunostaining.

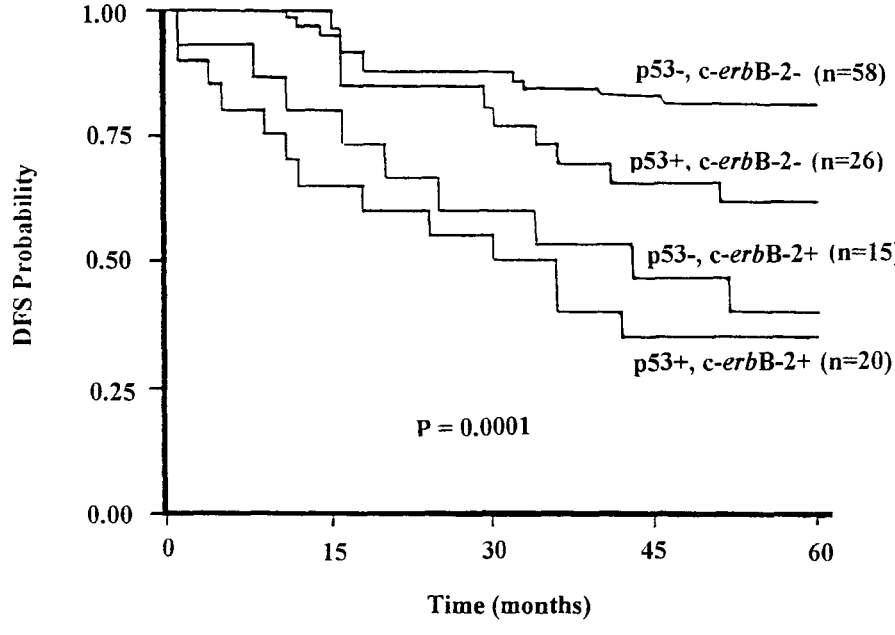

Fig. 2. Five-year disease-free survival curves for patients with breast cancer separated by staining for both p53 and c-*erb*B-2.

cancer is still undefinite. Some reports have suggested that p53 oncoprotein accumulation is associated with clinical stage[29,30,32], tumor size [31-33], nodal status[31,33], estrogen receptor[30, 32-35], and c-*erb*B-2 expression[29,33,35]. However, others indicate no correlation with age of the patients[31,32,34], clinical stage[36], tumor size [31,34], nodal status[32,34], estrogen receptor[37], or c-*erb*B-2 expression[28,30,32]. In the current study, we found correlation only with c-*erb*B-2 expression.

Thor et al[38] examined 253 patients with breast cancer of all stages and found that p53 immunostaining in paraffin-embedded samples was a prognostic factor for disease-free survival in both the node-negative and node-positive groups. Marks et al[32] found the significant prognostic effect on recurrence-free survival only in the node-negative subset. In our study, we did not examine the two subsets separately due to the small number of patients. However, our findings indicated that the overexpression of p53 oncoprotein was a prognostic predictor for 5-year disease-free survival in the patients, regardless of nodal status.

Since the study of p53 and c-*erb*B-2 oncoproteins can lead to improvements in the management of patients with breast cancer, either as a prognostic indicator or as a predictor of therapeutic responses[32-38], the combined study of these two oncogene products, therefore, could help to improve their clinical value. Our previous study clearly demonstrated that overexpression of c-*erb*B-2 protein was an effective prognostic predictor for 5-year disease-free survival in the patients[18]. In the current study, we also found the same prognostic effect in the overexpression of p53 protein. Moreover, when combining p53 and c-*erb*B-2 proteins expression, we observed that their coexpression had a more powerful effect on predicting 5-year disease-free survival than p53 protein expression alone. These results are consistent with the other studies[32,39]. In addition, one previous study suggested that overexpression of both these oncogenes in a single tumor had an additive effect on the prognosis[32]. From these findings and ours, it is suggested that double immunohistochemical detection of p53 and c-*erb*B-2 proteins overexpression is likely to help clinicians identify the patients with early recurrence more accurately and arrive at more rational treatment decisions.

ACKNOWLEDGEMENT

During this study the International Union Against Cancer provided an International Cancer Technology Transfer Fellowship to Ms. S. Chariyalertsak for a one-month training to study on an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections with Prof. Robert D. Cardiff at the Department of Pathology, University of California, Davis, U.S.A.

(Received for publication on February 24, 1997)

REFERENCES

1. Levine AJ. The p53 protein and its interactions with the oncogene products of the small DNA tumor virusses. Virology 1990; 177: 419-26.
2. Levine AJ, Momand J, Finlay CA. The p53 tumour suppressor gene. Nature 1991; 351: 453-6.
3. Hollstein M, Sidransky D, Vogelstein B, et al. p53 mutations in human cancers. Science 1991; 253: 49-53.
4. Sager R. Tumour suppressor genes. The puzzle and the promise. Science 1989; 246: 1406-12.
5. Weinberg RA. Tumor suppressor genes. Science 1991; 254: 1138-43.
6. Malkin D, Li FP, Strong LC, et al. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science 1990; 250: 1233-8.
7. Davidoff AM, Kerns BJM, Iglehart JD, Marks J. Maintenance of p53 alterations throughout breast cancer progression. Cancer Res 1991; 51: 2605-10.
8. Schneider J, Rubio MP, Barbazan MJ, Rodriguez-Escudero FJ, Seizinger BR, Castresana JS. P-glycoprotein, HER-2/neu and mutant p53 expression in human gynecologic tumors. J Natl Cancer Inst 1994; 86: 850-5.
9. Rodriguez NR, Rowan A, Smith MEF, et al. p53 mutations in colorectal cancer. Proc Natl Acad Sci USA 1990; 87: 7555-9.
10. Allred DC, Clark GM, Elledge R, et al. Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. J Natl Cancer Inst 1993; 85: 200-6.
11. Elledge RM, Fuqua SA, Clark GM, Pujol P, Allred DC, McGuire WL. Pognostic significance of p53 gene alterations in node-negative breast cancer. Breast Cancer Res Treat 1993; 26: 225-35.
12. Coussens L, Yang-Feng TL, Liao Y-C, et al. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science 1985; 230: 1132-9.
13. Bargmann CI, Hung MC, Weinberg RA. The neu oncogene encodes an epidermal growth factor receptor - related protein. Nature 1986; 319: 226-30.
14. Fukushige S, Matsubara K, Yoshida M, et al. Localization of a novel v-erbB related gene, c-erbB-2, on human chromosome 17 amd its amplification in a gastric cancer cell line. Mol Cell Biol 1986; 6: 955-8.
15. Tsuda H, Hirohashi S, Shimosato Y, et al. Correlation between histologic grade of malignancy and copy number of c-erbB-2 gene in breast carcinoma. Cancer 1990; 65: 1794-800.
16. Paterson MC, Dietrich KD, Danyluk J, et al. Correlation between c-erbB-2 amplification and risk of recurrent dissease in node-negative breast cancer. Cancer Res 1991; 51: 556-67.
17. Paik S, Hazan R, Fischer ER, et al. Pathologic findings from the National Surgical Adjuvant Breast and Bowel Project: Prognostic significance of c-erbB-2 protein overexpression in primary breast cancer. J Clin Oncol 1990; 8: 103-12.
18. Chariyalertsak S, Chariyalertsak S, Cheirsilpa A, Sugano K, Ohkura H. Immunohistochemical detection of c-erbB-2 oncoprotein in patients with breast cancer. J Med Assoc Thai 1996; 79: 715-21.
19. Chariyalertsak S, Trongtawsak A, Riganti M. Comparison of immunocytochemical and biochemical assays for estrogen receptor in human breast and liver tumors. Thai Cancer J 1990; 16: 130-4.
20. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. J Am Stat Assoc 1958; 53: 457-81.
21. Peto R, Pike MC, Armitage P. Design and analysis of randomized clinical trials requiring prolonged observation of each patient: II. Analysis and examples. Br J Cancer 1977; 35: 1-39.
22. Frebourg T, Friend SH. The importance of p53 gene alterations in human cancer: is there more than circumstantial evidence? J Natl Cancer Inst 1993; 85: 1554-7.
23. Kerns BJ, Jordan PA, Moore M-BH, et al. p53 overexpression in paraffin embedded tissue detected by immunohistochemistry. J Histochem Cytochem 1992; 40: 1047-52.
24. Kerns BJ, Jordan PA, Huper G, et al. Assessment of c-erbB-2 amplification by immunohistochemistry in paraffin embedded breast cancer. Mod Pathol 1993; 6: 673-8.
25. Berns EMJJ, Klijn JGM, Van Putten WLJ, Van Staveren IL, Portengen H, Foekens JA. c-myc amplification is a better prognostic factor than HER2/neu amplification in primary breast cancer. Cancer Res 1992; 52: 1107-13.
26. Lane DP, Benchimol S. p53: oncogene or anti-oncogene? Genes Dev 1990; 4: 1-8.
27. Tsuda H, Hirohashi S, Shinosato Y, et al. Immunohistochemical study on overexpression of c-erbB-2 protein in human breast cancer: its correlation with gene amplification and long-term survival of patients. Jpn J Cancer Res 1990; 81: 327-32.
28. Varley JM, Brammar WJ, Lane DP, Swallow ES, Dolan C, Walker RA. Loss of chromosome 17p13 sequences and mutation of p53 in human breast carcinomas. Oncogene 1991; 6: 413-21.
29. Iwaya K, Tsuda H, Hiraide H, et al. Nuclear p53 immunoreaction associated with poor prognosis of breast cancer. Jpn J Cancer Res 1991; 82: 835-40.

30. Davidoff AM, Herndon JE, Glover NS, et al. Relationship between p53 overexpression and established prognostic factors in breast cancer. Surgery 1991; 110: 259-64.
31. Noguchi M, Kitagawa H, Kinoshita K, et al. The relationship of p53 protein and lymph node metastases in invasive breast cancer. Surg Today 1994; 24: 512-7.
32. Marks JR, Humphrey PA, Wu K, et al. Overexpression of p53 and HER-2/neu proteins as prognostic markers in early stage breast cancer. Ann Surg 1994; 219: 332-41.
33. Nakopoulou LL, Alexiadou A, Theodoropoulos GE, Lazaris ACH, Tzonou A, Keramopoulos A. Prognostic significance of the co-expression of p53 and c-erbB-2 proteins in breast cancer. J Pathol 1996; 179: 31-8.
34. Jacquemier J, Penault-Llorca F, Viens P, et al. Breast cancer response to adjuvant chemotherapy in correlation with erbB2 and p53 expression. Anticancer Res 1994; 14: 2773-8.
35. Isola J, Visakorpi T, Holli K, Kallioniemi CP. Association of overexpression of tumor suppressor protein p53 with rapid cell proliferation and poor prognosis in node-negative breast cancer patients. J Natl Cancer Inst 1992; 84: 1109-14.
36. Koutselini H, Malliri A, Field JK, Spandidos DA. p53 expression in cytological specimens from benign and malignant breast lesions. Anticancer Res 1991; 11: 1415-20.
37. Spandidos DA, Karaiossifidi H, Malliri A, et al. Expression of Ras Rb1 and p53 proteins in human breast cancer. Anticancer Res 1992; 12: 81-90.
38. Thor AD, Moore DH, Edgerton SM, et al. Accumulation of p53 tumor suppressor gene protein : an independent marker of prognosis in breast cancers J Natl Cancer Inst 1992; 84: 845-55.
39. Albanell J, Bellmunt J, Molin R, et al. Node-negative breast cancers with p53(-)/HER2-neu(-) status may identify women with very good prognosis. Anticancer Res 1996; 16: 1027-32.

การใช้ p53 และ c-*erb*B-2 oncoproteins ช่วยพยากรณ์โรคในผู้ป่วยมะเร็งเต้านม

สุนันทา จริยาเลิศศักดิ์, วท.ม., สุวัฒน์ จริยาเลิศศักดิ์, พ.บ., Ph.D.**,*
อาคม เชียรศิลป์, พ.บ., กิติ จินดาวิจักษณ์, พ.บ., F.A.C.S., F.R.C.S.T., F.I.C.S.**

ผู้วิจัยได้ใช้วิธี immunohistochemistry ในการตรวจหา p53 และ c-*erb*B-2 proteins ในชิ้นเนื้อมะเร็งเต้านมของผู้ป่วยจำนวน 119 ราย พบ 46 ราย (ร้อยละ 38.7) มี p53 protein และ 35 ราย (ร้อยละ 29.4) มี c-*erb*B-2 protein การตรวจพบผลิตผลของยีนทั้ง 2 ชนิดนี้มีความสัมพันธ์กันอย่างมีนัยสำคัญทางสถิติ ($p<0.01$) การศึกษานี้ไม่พบความสัมพันธ์ระหว่าง p53 protein กับอายุของผู้ป่วย, clinical stage, tumor size, number of involved nodes และ estrogen receptor status แต่พบว่า p53 protein มีความสัมพันธ์กับอัตราการปลอดโรคในระยะ 5 ปี ($p=0.0113$) นอกจากนั้นผลจากการศึกษานี้ยังแสดงให้เห็นว่าการตรวจหาทั้ง p53 และ c-*erb*B-2 proteins จะเป็นตัวช่วยบอกการกลับคืนของโรคในผู้ป่วยมะเร็งเต้านมได้ดี

* สถาบันมะเร็งแห่งชาติ, กรุงเทพ ฯ 10400
** ภาควิชาเวชศาสตร์ชุมชน, คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่, จ.เชียงใหม่ 50200