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The relationship between p53, c-myc product expression and clinicopathological parameters in primary breast carcinomas

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Sixty-four primary breast carcinomas were analyzed by flow cytometry for their expression of p53 and c-myc proteins. c-myc protein expression was correlated with pathological infiltration ($p = 0.05$), but the other parameters (tumor size, lymph node status, menopausal status, steroid receptor status, and histological type) were not statistically correlated. On the other side, p53 protein expression was significantly correlated with large tumor size ($p = 0.0001$) and lymph node metastasis positive ($p = 0.001$), and weakly correlated with post menopausal status ($p < 0.01$) and pathological infiltration ($p = 0.01$). Although there was no correlation between only p53 protein oevr-expression and disease free survival. but when p53 protein over-expression and c-myc protein oevr-expression were simultaneously found in the same tumor-cell population, prognosis of these cases were poor. Namely simultaneous over-expression of p53 protein and c-myc protein was found in 22 cases, 7 of 22 (31.8%) have had a relapse. In other 2 cases relapse was found, but both these cases were medullary carcinoma and the level of expression of p53, and c-myc protein was low. This study indicates that plural gene product expression should be simultaneously analyzed when investigate the prognostic markers in breast carcinomas.

Introduction

In a point of view of proportional mortality rate of malignant disease, breast cancer is sixth on the list in Japanese woman. Since 1965, irrespective of advances in hormonal and pharmaceutic therapies, the proportional mortality rate has not been reduced[1]. And in twenty-one century suggested that proportional mortality rate of breast cancer in Japan may be top on the list.

But, the standardized factors affecting the prognosis in breast cancer are not proposed from now on.

Human breast cancer is a complex disease with regard to biological appearance and clinical behavior, and is characterized by a multiplicity of genetic alterations affecting both proto-oncogenes and tumor-suppressor genes[15) 18) 22)~24)]. Ane the development of cancer is regarded as a multi-step process of these genes. A crucial step in the development of many cancers is represented by changes in the p53 gene. Point mutations or deletions in this gene result in a loss of cantrol of cell proliferation, and may even exert a direct oncogenic pressure on the cells. A second frequent change in cancers is over-expression of the c-myc oncoprotein, with or without simultaneous amplification of the c-myc gene. The c-myc gene appears to be functionally involved in DNA synthesis, and over-expression of c-myc protein is associated with poor prognosis in breast cancer[3)~7)].

The purpose of this study was to investigate the p53, c-myc protein expression within the same breast cancer cell population by using flowcytometry, and to compare and correlate these findings to traditional clinicopathological parameters.

Materials and Methods

Samples

Fresh primary breast cancer tumor samples from sixty-four patients (24 solid-tubular, 14 papillo-tubular, 20 scirrhous, 3 invasive lobular, 2 medullary, 1 non-invasive) with no pre-operative chemotherapies were obteined at surgery between 1989 and 1993. As the negative control, non-malignant samples (12 normal breast, 4 fibroadnoma, and 8 dysplasia) were obteined. The tissue specimens were immediately frozen (one parallel specimens were embedded in Tissue-Tek □ Optimum Cutting Temperature compound (Miles Scientific, Naperville)) stored in a freezer at -80℃ for later use. As the positive control for c-myc protein expression, HL-60 cell line was used. (*Fig.1*) Histopathological classification was performed according to General Rules for Clinical and Pathological Recording of Breast Cancer (Japanese Breast Cancer Society, 11th edition). (*Table 1*)

Antibodies

MAb 1801, a human specific antibody which recognizes an exitope between amino-acids 32 and 79, and which reacts with both wild type and mutated form of p53 was used for p53 protein expression. 9E10, a mouse MAb reacting with residues 408-439 of the c-myc protein was used for c-myc protein expression. Both MAb were from Oncogene Science (Manhasset, N. Y.)

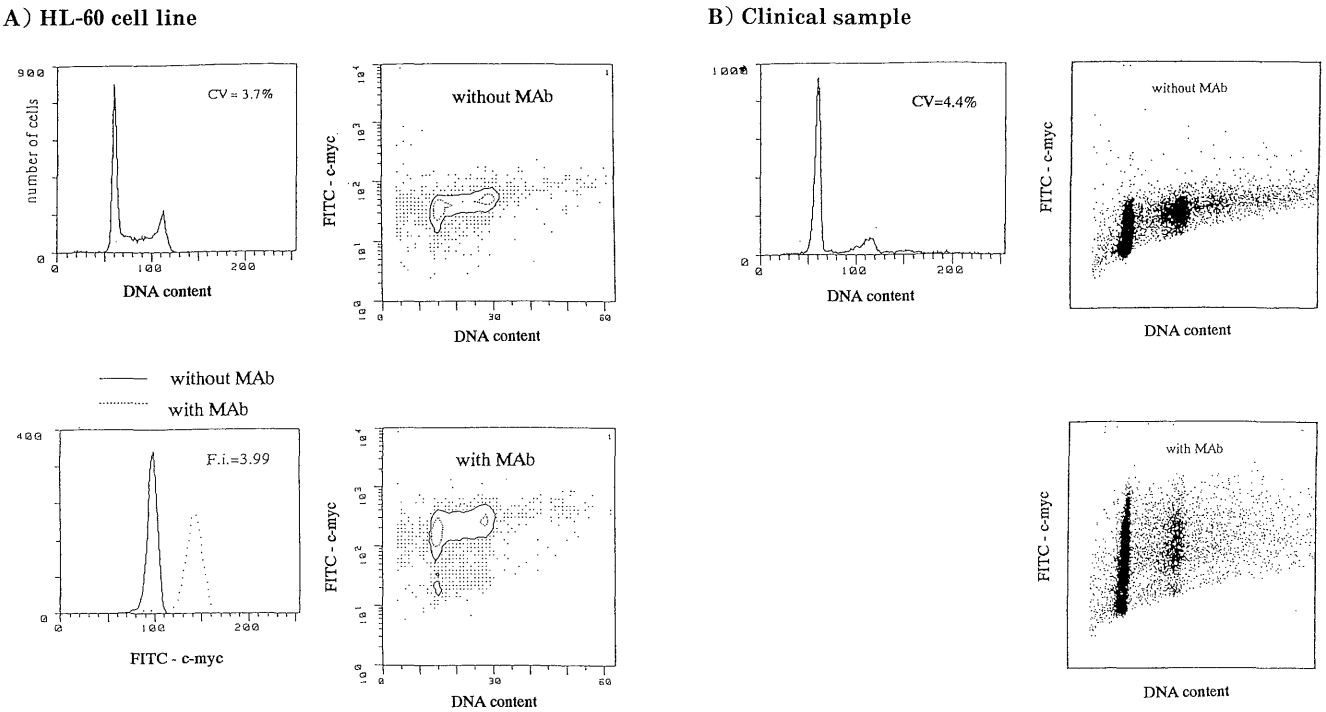

Fig.1 A) HL-60 cell line B) Clinical sample
HL-60 cell line was used for positive control. In clinical sample, area of fluorescence was not clearly separated among with MAb and without MAb.

Table 1. Clinicopathological features

1. Age	26~85	(mean age=58.1)
	pre-menopausal state	23 cases
	post-menopausal state	41 cases
2. Histological type		
	Solid-tubular	24 cases
	Papillo-tubular	14 cases
	Scirrhous	20 cases
	Invasive-lobular	3 cases
	Medullary	2 cases
	Non-invasive	1 cases
		64 cases
	Normal breast	12 cases
	Fibroadenoma	4 cases
	Mammary dysplasia	8 cases
3. Tumor size		
	< 2.0 cm	10 cases
	2.0~5.0 cm	34 cases
	> 5.0 cm	20 cases
4. Lymph node status		
	n0	22 cases
	n1	33 cases
	n2	7 cases
	n3	2 cases
5. Steroid Receptor status		
	ER(+)	28 cases
	ER(−)	26 cases
	PR(+)	30 cases
	PR(−)	17 cases

Immunohistochemical Staining

Three-μm-thick frozen sections were cut and mounted on poly-L-lysine coated slides, and air-dried at room temperature. These slides were fixed in 0.5% cold (4℃) paraformaldehyde for 20 min. For immunohistochemical demonstration of the p53 protein and c-myc protein, Labelled Strept-Avidin Biotin (LSAB kit, DAKO) method was used. The concentration of primary antibodies were 2 μg/ml, and incubated at room temperature for 1 hour. The colour was developed using diaminobenzidine (DAB). Control sections included omission of the primary antibodies. For each tumor and non-malignant tissue sample, the number of p53 and c-myc positive and negative tumor cells was evaluated in at least four randomely selected areas, using an eyepiece reticle disk inserted into the ocular of the light microscope.

Tissue preperation for innunofluorescence and DNA staining

Tissue samples approximaterly 50mg of each were thawed and minced with surgical blades in a small volume of phosphate buffer saline (PBS: NaCl 8.5g/l, Na_2HPO_4 9.76g/l, KH2PO4 1.64g/l, pH7.4). The samples were then flushed several times through a Pasteur pipette and filtered through a 50 μm nylon mesh. After washing in PBS and centrifugation, disdcarded the supernatant, the

cells were resusspended in 0.5% paraformaldehyde for 10min at 4℃. The cells were then washed once, centrifuged and incubated in 0.1% Triton X-100 for 5 min at 4℃ to permeabilize the cell membranes. After centrifugation, parallel samples were incubated with the following MAbs : anti-p53 (PAb1801, Oncogene Science), anti-c-myc (9E10, Oncogene Science). All antibodies were used at a concentration of 2 μ g/ml. For all antibodies, the cells were incubated at 4℃ for 60 min. The cels were then washed in PBS, centrifuged and incubated with a secondary FITC-conjugated goat anti-mouse IgG antibody (DAKO) at a dilution of 1:50, for 30 min at 4℃. After immunofluorescence staining, the cells were washed in PBS and centrifuged and resuspended in 20 μ g/ml Propidium Iodide (PI.) with 0.1% RNA ase (Sigma). The cells were stored protected from light for 20-40 min before flow-cytometry.

Flow cytometry

The cells were anaalyzed on a FACScan flow cytometer (Becton & Dickinson, San Jose, CA), equipped with an air-cooled argon laser. Excitation light wave-length was 488 nm to 15 m W.Green (FITC) and red (PI) fluorescence were separated by a 560 nm dichroic mirror. In addition, the green and red photomultiplier tubes were guarded by a 530 nm bandpass and a 650 nm logpass filter. For each sample, 10,000 events were collected and stored listmode for later analysis. A peak width *vs.* area cytogram was used to discriminate and gate out doublets from the analysis.

Stastical analysis

For the tumor samples, a fluorescence index (FI) was established for each antibodies, defined as the mean anti-body-associated fluorescence ((fluorescence of with MAb minus fluorescence of without MAb) divided by fluorescence of without MAb). Tumors with F1> 1.0 were classified as positive for a paticular antibodies.

The correlation between FI-value and clinicopathological parameters were evaluated using Wilcoxon signed-rank test, and survival curves (Kaplan-Meier survival curve) were evaluated with Generalized Wilcoxon test.

Result

Immunohistochemical Staining

Immunohistochemical staining was first applied in p53 product. The border line of a positive is dense staining in 50% of the nuclei. As a result, 25 (39%) out of 64 patients were positive. In contrast, no positive staining was seen in normal breast, fibroadenoma and dysplasia. One of dys-plasia showed a staining in 20% of nuclei. Faint staining of cytoplasma was observed in 2 with dense staining of nuclei. In an evaluation of immunohistochemical staining study on c-myc products, three with more than 8.0 of FI were positive. These results were driven from primary antibody at the concertration of 2 μ g/ml. At 4, 6, 10 μ g/ml of concentration, the same results were obteined.

The immunohistochemical staining is required for some levels of c-myc products. No positive gained in the control.

Flowcytometry

Great concern is how to set ur FI values in the study of flowcytometric and immunohistochemical staining. The expression of p53 products was evaluated in comparison with immunohistochemical staining. The maximum of FI corresponded to 20% of immunohistochemical staining in dysplasia (FI = 0.84) and the other control (FI <0.4). The FI of 1.0 as the expression of p53 was in accord with 32 out of 64 (50%). The FI was less than 1.0 in 36 out of 39 with negative immunohistochemical staining.

From the above results, positive of p53 products was regarded as FI of more than 1.0 in the flowcytometric analysis. In the analysis of the expression of c-myc products, it is difficult to compare the results of immunohistochemical staining and flowcytometry because there was no positive in cases of FI of 1.0 to 8.0 in immunohistochemical staining. But the three cases over 8.0 of FI of c-myc were positive in immunohistochemical staining. The criteria of determining positive was applied correspondingly for FI> 1.0 of the expression of p53 product.

The overexpression of c-myc products were 24 out of 64 (45.3%). There was no significant correlation with clinicopathological factors. In cases with small tumor size (t <5cm), the overexpression was frequently seen. As compared with the tumor sizes between less than and more than 5cm, the overexpression included in the tumor size of less than 5cm. This finding indicate that alteration of c-myc was occured in early stage of tumor progression. (*Fig. 2*)

The p53 products and clinicopathological factors correlated well with the tumor sizes ($p = 0.0001$), lymphnode metastasis ($p = 0.001$), histological infiltration ($p = 0.01$), post-menopausal status ($p = 0.001$). There was no correlation between p53 product expression and steroid receptor status, histological findings. Concerning the tumor size, whenever tumor has progressed p53 product has increased. (*Fig.3*) These result suggested that overexpression of p53 protein correlated with the usual prognostic factors as tumor size and lymph node status, and was thought to be a strong prognostic factor. But, in the investigation of disease free survival, difference was not found among p53 positive group and negative group.

Reccurence has occsured in nine cases among 64 (14%). Seven in reccurent cases except for 2 of medullary carcinoma expressed concurrently p53 and c-myc products.

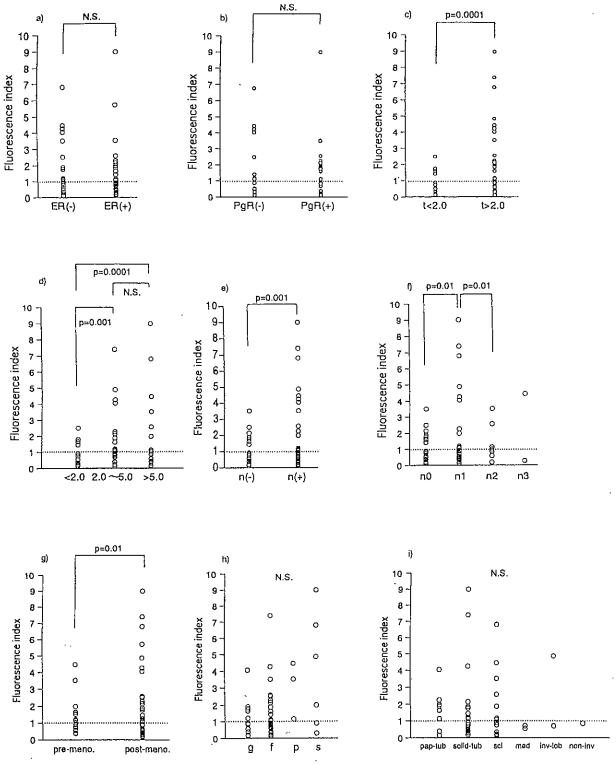

Fig.2 there was no correlation between c-myc protein expression and clinicopathological factors.

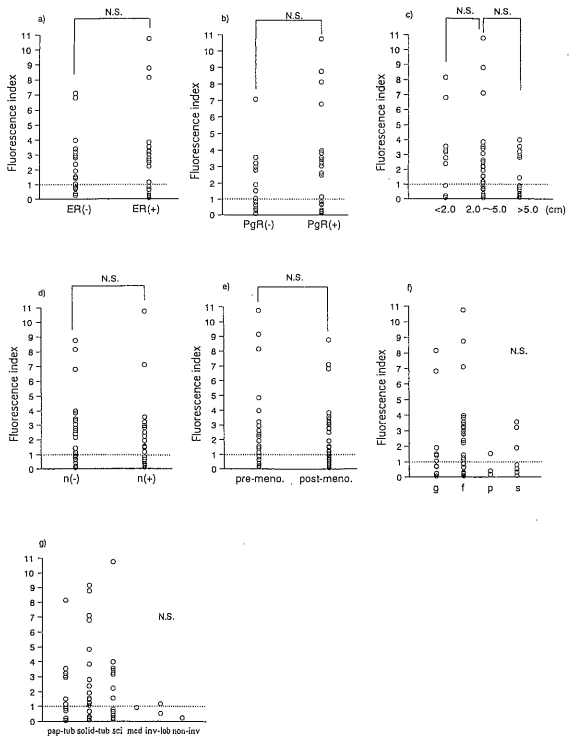

Fig.3 relationship wea found between p53 protein expression and tumor size(c), d)), lymph node status(e), f)), menopausal status(g)).

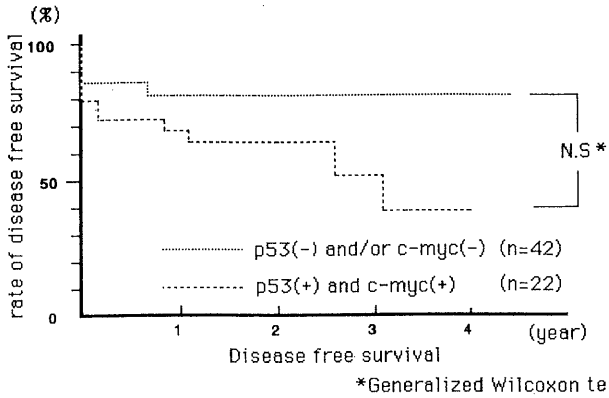

Fig.4 3-year disease free survival; group of p53(+) and c-myc(+) was 51.5%, p53(−) and/or c-myc(−) was 81.4%. Although statiatical significance was not found among these groups.

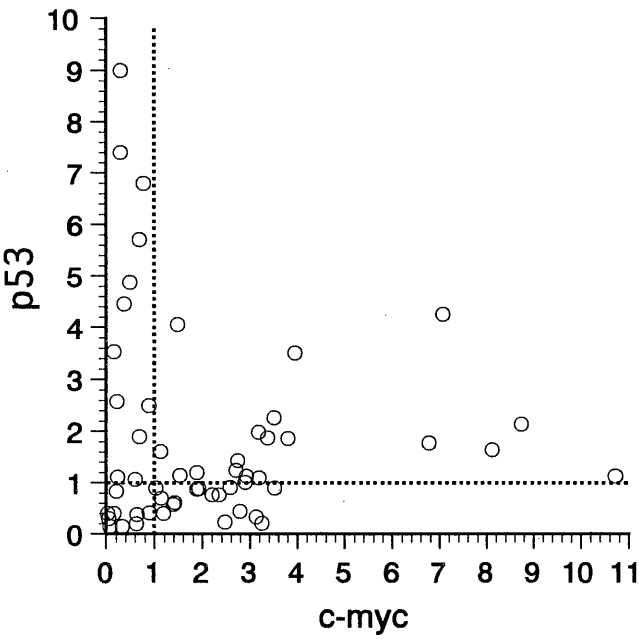

Fig.5 there was no correlation between p53 expression and c-myc expression.

Simultaneous expression of p53 and c-myc was seen in 22 in whom reccurence was seen in 7 (31.8%). The 3 year disease free survival was seen in 51.5% of simultaneous expression, and 81.4% of other expressions without statistical significance. It is concluded that the prognosis in cases with simultaneous expression is thought to be poor. Still more, there was no correlation with p53 and c-myc products expressions. (*Fig. 4, 5*)

Discussion

Cancer-associated oncogenes were detected and it is

defined that these are in normal cells. In cancer cells, these are activated by point mutation, amplification, deletion, and translocation. As a result, oncogene products ore excessively produced structural and functional analogue to oncogene product to give information in cells in association with carcinogenesis and automonous proliferation of carcinomas. On the other hand, the tumor suppressor genes plays a role in depression of tumor-genesis and deletion of chromosoms at the initiation of carcinoma and promotes mutated gene products.

The characteristics of the tumor is directly deter-mined by oncogenes and suppressor genes. In vitro study, the gene product expression of the tumor helps to assess the prognosis in comparison with clinicopathological factors.

The main drawback to immunohistochemical staining method is a subjective determination for the degree of staining, and is not necessarily objective[25)26)]. On the other hand, the advantage is a determinant of proceding site of the tumors and/or its location. The measurement by flowcytometry is characteristic of semi-quantative and objective with reproducible.

In this study, the expression of p53 and c-myc products were measured by flowcytometry[43)~49)51)52)]. p53 gene locates in the short arm of 17 chromosome[17)50)]. Deletion of this gene induces protein production which is recognized by PAb1801[27)], there are many reports about the mutated p53 in a solid tumor[16),29)~42)53)]. The investigation of mutated p53 in breast cancer by flowcytometry is scant[35)~42)]. The actual values correspond to values subtracted negative control from a total fluorescence in cultures cells. However, estimation by actual values includes in jeopardy from admixture of various clones in clinical materials. It is possible to exclude an error by small samples. In this study, FI was determined to know the dose of the relative expression. As compared between the control and carcinomas, the FI of 0.87 was observed in one of dysplasia. Thirty six (92%) out of 39 with p53 negative staining showed ; less than 1.0 of FI of p53. High FI was observed in benign dysplasia, suggesting that the process in repeated promotion provokes gene deletion to alter to carcinoma. Interesting enough, when obteining low FI values of p53 and c-myc products, there do not necessarily imply low biologic behavior in reflection of high incidence of node involvement as the tumor sizes increase. On the other hand, low FI in medurally carcinoma revealed aggressive biologic properties. Medullary carcinoma are known to be associated with a high growth rate, and are genellary recognized frequent alteration p53, and are regarded as a prognosticallyfavorable[19)36)37)]. Despite of these fact, our data exhibited low expression of p53, and prognostically poor in medullary carcinoma. It has been suggested that the diffuse lymphopasmacytic infiltrate, which characterized such a histotype, may represented a host reaction to tumor cell antigen. This study lacks an investigation of biologically poor prognosis despite study on the expression of p53 and c-myc products.

There was not correlation between the expression of c-myc product and clinicopathological factors. In breast cancers, the relationship between clinicopathological factors and the amplification of c-myc gene has been investigated but the reports concerning the expression of c-myc product in breast cancer were scant[53)54)]. Most alteration involving int-2/hst-1[2)] and HER-2[3)7)~14)33)] means an indicator of poor prognosis. Recently, alteration of c-myc gene represents more formidable prognosis than HER-2 and int-2/hst-1 gene alteration[2)19)20)21)25)56)57)]. c-myc gene alteration well correlates with tumor sizes, the number of metastatic nodes and steroid receptor status[4)7)55)].

On the other hand, it is assumed that c-myc gene alterations are seen in regenerative liver tissues in reflection of proliferative factors rather than cancer-associated oncogene. Recent study clarified that c-myc protein is a DNA binding, showing a structure of b-HLH-LZ success in isolation of Max and Mad proteins has been achieved. It is clarified that Mad-Myc-Mad promotes transcription, in contrast Mad-Max inversely depress it. Therefore, it is postulated that as Myc and Mad takes Max, competitive transcription enables cell proliferation to control. It is sure that the direction of cell proliferation should be determined by a FI assessment pf flow cytometry.

In this study, we investigated for two different gene product expression, and revealed the available prognostic marker, but the reports of multi-gene products expression have been scant[28)]. In a point of view of multi-step process of tumorgenesis, plural gene products should be examined as for the prognostic marker or indication of treatments.

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