Cytologic Evaluation of p53, Cyclin D1, and Cathepsin D and Their Correlation with Histologic Sections in Primary Breast Carcinoma Original Paper

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유방암 세포검사에서 p53, Cyclin D1, Cathepsin D의 검색과 조직표본 검색 결과의 비교

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Purpose: Biologic characteristics of the tumors could be altered after chemotherapy. Accurate assessment of tumor biology before treatment is important for selecting treatment modalities. Current study was designed to investigate whether multiple molecular markers could be accurately assayed on the fine needle aspirates from the breast carcinoma.

Methods: Immunocytochemical assays (ICA) for p53, cyclin D1, and cathepsin D were performed on cytologic samples from 76 primary breast carcinomas, 37 ductal carcinoma in situ (DCIS), and 36 benign ductal hyperplasia. ICA for 3 molecules was also performed on the histologic sections from the matching tumor blocks, and the results were compared.

Results: Three molecular markers were successfully detected in cytologic samples from the breast carcinomas; p53 in 71.1% (54/76), cyclin D1 in 73.7% (56/76), and cathepsin D in 44.7% (34/76). Their expression was rarely observed in benign hyperplasia; p53 in 0/36, cyclin D1 in 7/36, and cathepsin D in 4/36. Increase of expression frequency of 3 molecules was apparent through the progress of the disease. Results of ICA for each molecules were well correlated between cytologic and histologic samples; concordance was 93.4% (71/76) for p53, 81.6% (62/76) for cyclin D1, and 73.7% (56/76) for cathepsin D. When 3 molecular markers were integrated to the preoperative diagnosis, positive predictive value was 90.6% for malignant breast disease.

Conclusion: Three molecular markers were successfully assayed on cytologic samples and well correlated with the results from the histologic sections. The results indicate that the biologic information from the fine needle aspirates can be reliably integrated to patients treatment. **(Journal of Korean Breast Cancer Society 2002;5:113-117)**

Key Words: Cathepsin D, Cyclin D1, Cytology, Diagnosis, p53, Treatment

중심 단어: 세포검사, 진단, 치료, Cathepsin D, Cyclin D1, p53

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INTRODUCTION

Risk assessment and therapeutic stratification for individual patient have become a key component for improving survival of the breast cancer patients. Wide acceptance of breast conservation therapy has increased the use of pre-operative chemotherapy to down-stage locally advanced breast cancer. Pre-operative chemotherapy is an attractive method for in vivo test of therapeutic efficacy of individual chemotherapeutic agent. Pre-operative assessment of tumor biology is mandatory for selecting therapeutic regimen because biological characteristics of the tumors could be altered after chemotherapy because of clonal selection. It would be possible to provide a more reasonable treatment to the patient while minimizing adverse toxic effects, if the biological characteristics of the tumor could be assessed accurately before treatment.

Molecular markers to be measured have to reflect diverse biological characteristics of the breast cancer.(*1*) We have already performed immunohistochemical assay for cathepsin D, cyclin D1 and p53 in the breast cancer tissues.(*2*) The results of our preliminary study indicated that combined assay of multiple biological markers could provide a useful information about biological property of the individual breast cancer. When

multiple molecular markers were merged together, we could prove a better predictive value for discriminating aggressive phenotype of breast cancer.(*2*) Increasing use of preoperative chemotherapy prompted us to begin the present study because of limited availability of cancer tissues before definitive surgery. Fine needle aspiration cytology is a well established diagnostic tool for the evaluation of breast lump.(*3*) There are an increasing number of patients not having surgery because of age or other medical condition. Although fine needle aspiration has been mainly used for diagnostic purpose in the evaluation of various breast lesions, development of molecular biologic techniques enables the accurate preoperative evaluation for biological nature of the cancers.(*4*)

This study was designed to investigate whether multiple molecular markers might be accurately detected in the tumor cells obtained by fine needle aspiration. The accuracy was validated by comparison to the results from the matching sections of surgical specimens.

MATERIALS AND METHODS

1) Materials

Immunocytochemical assays for p53, cyclin D1, and cathepsin D were performed in 76 primary breast carcinoma, 37 DCIS, and 36 benign hyperplasia using fine needle aspiration cytology samples. Mean age of the patients was 47 years (range: 19～82 years). There was one male patient with invasive ductal carcinoma. In cancer patients, 16 tumors were less than 2 cm in their size, 35 tumors were between 2～5 cm, and 11 tumors were larger than 5 cm (Table 1). Fourteen cases of DCIS were not measurable their size because of diffuse involvement of satellite lesions.

2) Immunocytochemical and immunohistochemical assay

Fine needle aspiration samples were taken from studied patients prior to surgery or preoperative chemotherapy, using 23 gauge needle and a 10 ml syringe. Multiple aspirates slides were fixed in 10% formalin phosphate buffered saline (PBS) for 15 to 30 minutes at -4°C and rinsed with pH 7.0 PBS. Slides were fixed in methanol for 5 minutes at -20°C and rinsed with PBS. Slides were placed in acetone for 2 minutes at 20°C and rinsed with PBS. Immunocytochemical assay was subsequently performed on these samples. Fine needle aspiration slides and 5㎛ tissue sections were microwaved in 10 mM ethylenediamine-tetra-acetic acid (EDTA) for 25 minutes in a 600 W microwave oven for pretreatment before immunostaining. These samples were examined for expression of mutant p53, cyclin D1, and cathepsin D protein using the avidin-biotin complex (ABC) immunoperoxidase method. Fine needle aspiration slides and 5㎛ tissue sections were incubated in commercially available monoclonal antibodies: DAKO-p53, DO-7 for mutant p53 protein assay (DAKO Co., Glostrup, Denmark); NCL-cyclin D1-GM (1 : 20) dilution for cyclin D1 assay (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK); DAKO polyclonal rabbit anti- cathepsin D for cathepsin D protein assay (DAKO Co.). The reaction was detected using diaminobenzidine (DAB) with hydrogen peroxide (H_2O_2). Slides were counterstained with hematoxylin.

3) Slide review

Fine needle aspiration slides were reviewed by one of the authors (K.P.). Cellularity was regarded as adequate when there were more than 30 neoplastic cells per slide. Histologic sections were also reviewed without knowledge of the original cytologic diagnosis. Three separate blocks containing breast carcinoma were stained and scored by calculating the stained cells from 500 observed cancer cells as a percentage. Sections of breast cancer observed to express homogenous and intense nuclear immunostaining for 3 molecular markers in more than 5% of cells in the observed field were considered to be positive. This cut-off level in the current study was determined because an aim of this study was to clarify the diagnostic utility of studied markers on fine needle aspirates of the breast cancer. Thus, any evident staining for studied markers, irrespective of the heterogeneity of immunostaining in histologic sections, was considered as positive.(*5*)

Table 1. Distribution of tumors according to size and lymph node metastasis

	No. of patients	% of total
Size		
＜2 cm	16	21.1
2～5 cm	35	46.1
＞5 cm	11	14.5
Unknown	14	18.3
L/N metastasis		
0	28	36.8
1～3	18	23.7
4～9	13	17.1
≥10	6	7.4

Table 2. Results of immunocytochemical assay for molecular markers through the progress of the disease

Markers	Benign	DCIS	Invasive cancer (%)
P53+	0/36 (0)	24/37 (64.9)	54/76 (71.1)
Cyclin D1+	7/36 (19.4)	16/37 (43.2)	56/76 (73.7)
Cathepsin D+	4/36 (11.1)	14/37 (37.8)	34/76 (44.7)

RESULTS

Mutant p53 expression on cytologic samples was not observed in all 36 cases of benign breast disease. Its expression was demonstrated in 24 (64.9%) of 37 DCIS samples and 54 (71.1%) out of 76 invasive cancer samples (Table 2). Mutant p53 expression was significant to distinguish malignant tumor of the breast in cytologic samples. There was no difference in the frequency of mutant p53 expression between DCIS and invasive breast cancer (Table 2). Results of immunocytochemical assay on cytologic samples were compared with those of histologic sections. Of 54 cases expressing mutant p53 on cytologic samples, mutant p53 expression was observed in 49 cases on histologic sections. Concordance between cytology and histology was 93.4% (Table 3).

Cyclin D1 expression was increased through the progress of disease; 19.4% (7/36) in benign breast disease, 43.2% (16/37) in DCIS, 73.7% (56/76) in invasive breast cancer (Table 2). The frequency of cyclin D1 expression significantly increased in malignant tumor of the breast but cyclin D1 was expressed in a certain proportion of cytologic samples from the benign breast disease (Table 2). Cyclin D1 expression was observed in 46 on histologic sections whereas its expression was observed in 56 by cytologic assay. Concordance between cytology and histology was 81.6% (Table 3).

Cathepsin D expression was found in 34 (44.7%) of 76 invasive breast cancers (Table 2) whereas its expression was found 4 (11.1%) of 36 benign breast diseases. Cathepsin D expression in DCIS was observed in 14 (37.8%) of 37. Cathepsin D expression increased in its frequency through the progress of the breast disease (Table 2). In histologic study, cathepsin D expression was observed in 44 of 76 invasive breast cancers. Concordance between cytology and histology was 73.7% (Table 3).

Expression of 3 molecular markers was merged together and analyzed according to progress of the breast disease. In benign proliferative disease, 69.4% (25/36) was negative for 3 biological markers whereas only 8.1% (3/37) of DCIS was negative for all 3 biological markers on fine needle aspirates. The finding was more evident in invasive breast carcinomas in which only 4 (5.2%) cases out of 76 was negative for 3 biological markers. Three molecular markers were expressed alone or together in 117 of 149 cases including 11 benign breast disease. Immunocytochemical assay for 3 molecular markers had 90.6% of positive predictive value in the diagnosis of malignant breast disease (Table 4). Three biologic makers were successfully detected on fine needle aspirates as well as in histologic sections from DCIS and invasive carcinomas.

Table 3. Comparison of immunohistochemical assay of cytology and histology for mutant p53, cyclin D1, and cathepsin D in invasive breast cancer

Markers	Cytology	Histology		Concordance
		Positive	Negative	
P53	Positive	49	5	
	Negative	0	22	71/76=93.4%
Cyclin D1	Positive	44	12	
	Negative	2	18	62/76=81.6%
Cathepsin D	Positive	29	5	
	Negative	15	27	56/76=73.7%

Table 4. Positive rate of 3 molecular markers on cytologic evaluation

	All negative	1 marker+	2 markers+	3 markers+
Benign	25 (69.4)	11 (30.6)	0	0
DCIS	3 (8.1)	18 (48.7)	13 (35.1)	3 (8.1)
Invasive cancer	4 (5.2)	19 (25.0)	35 (46.1)	18 (23.7)

DISCUSSION

Three molecular markers measured in the current study are the widely investigated molecules in the breast carcinoma. They might be useful in risk assessment of individual patient and in planning more effective treatment after surgery. Expression of mutant p53 protein is one of the most frequent genetic changes observed in human cancers.(*6*) Mutations in the p53 gene lead to increased half-life and quantity of the p53 protein.(*7*) This protein over-expression can be successfully visualized by nuclear staining using immunohistochemistry in our previous study for p53 expression with another population of the patients.(*2*) In cytologic aspirates, mutant p53 expression was demonstrated in 71.1% of invasive breast cancer and 64.9% of

DCIS whereas its expression was not observed in benign proliferative disease. The finding well correlated with results from histologic sections. Assay of mutant p53 expression in cytologic aspirates seems to be a useful adjunct in the diagnosis of malignant breast disease.

An aim to analyze molecular markers in breast lesions is to distinguish malignant breast disease from benign lesions. Result of the current study is somewhat intriguing in respect of cyclin D1 expression. Cyclin D1 expression was observed in more than half of malignant breast diseases but its expression was also observed in a certain proportion (7/36) of benign breast lesions. Cyclin D1 could be expressed in benign proliferative cells because it is a molecule to mediate a cell proliferation signals.(*8*) Benign lesions included in the present study were all the benign ductal hyperplasia. Cyclin D1 is thought to be a proliferative markers rather than a marker for cancerous transformation.(*9*) Result of our recent study indicated that cyclin D1 expression was associated with ER expression and was closely linked to cellular differentiation in invasive breast cancer.(*10,11*) Cyclin D1 expression had a prevalence enough to discriminate breast cancer from benign breast disease, but we could not depend entirely upon its expression because it was expressed in a proportion of non-cancerous conditions.

Lysosomal protease, cathepsin D, has been thought to be involved in invasion and metastasis of the cancer cells. A number of studies demonstrated association of cathepsin D expression with lymph node metastasis.(*12-14*) Results of our earlier study also indicated its usefulness as a prognostic indicator in breast cancer patients.(*2*) The results of the current study showed that cathepsin D was expressed in a proportion of benign breast disease and DCIS. A recent study indicated that cathepsin D expression could be detected in histologic sections from benign breast disease and DCIS.(*15*) Cathepsin D-cDNA transfection increases tumor cell proliferation in vitro. Cathepsin D is activated as a protease following its activation at an acid pH but it can displace IGFII from the mannose-6-phosphate/IGFII receptor to the IGFI receptor at a neutral pH.(*16*) The nature of the cathepsin D activation mechanism involved in vivo may dependent upon the microenvironment of the cancer cells. Presence of cathepsin D expression in non-cancerous cells and DCIS indicates that cathepsin D has a role during tumor progression such as promoting cell growth and transformation other than escape of cancer cells through the basement membrane.(*17*)

In the current study, three representative biologic markers were successfully detected in cytologic aspirates from the breast cancers and well correlated with the results from histologic sections. Main utility of fine needle aspiration cytology is an accurate diagnosis of breast lesions. Although the cytologic diagnosis is well established, well differentiated breast carcinoma is sometimes difficult to distinguish from benign proliferative disease because of limited cellularity. Measurement of multiple markers provided a more accurate diagnosis of breast cancer from benign proliferative diseases and had high predictive value for the diagnosis in the results of the current study. These results indicate that more effective therapeutic regimens can be determined on the basis of the biological information from the fine needle aspirates.

CONCLUSIONS

Measurement of multiple molecular markers in cytologic aspirates from the primary breast cancers was successful. The reliability was confirmed by high concordance with the results obtained from matched histologic specimens. The results from the current study indicate that accurate biologic information of individual breast cancer can be determined on cytologic specimens.

REFERENCES

1) McGuire WL. Breast cancer prognostic factors: evaluation guideline. J Natl Cancer Inst 1991;83:154-5.
2) Han S, Yun IJ, Noh DY, Choe KJ, Song SY, Chi JG. Abnormal expression of four novel molecular markers represents a highly aggressive phenotype in breast cancer. Immunohistochemical assay of p53, nm23, erbB-2, and cathepsin D protein. J Surg Oncol 1997;65:22-7.
3) Layfield LJ, Chrischilles EA, Cohen MB, Bottles K. The palpable breast nodule: a cost-effective analysis of alternate diagnostic approaches. Cancer 1993;72:1642-51.
4) Powles TJ, Trott PA, Cherryman G, Clark S, Ashley S, Coombes RC, et al. Fine needle aspiration cytodiagnosis as a pre-requisite for primary medical treatment of breast cancer. Cytopathology 1991;2:7-12.
5) Park K, Han S, Kim HY, Koh IH. Cytologic evaluation of cyclin D1 expression in primary breast carcinoma. Cancer Cytopathol 2001;93:211-5.
6) Vogelstein B, Kinzler KW. p53 function and dysfunction. Cell 1992;70:523-6.
7) Greenblatt MS, Bennett WP, Hollstein M, Harris CC. Mutations in the p53 tumor-suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res 1994;54:4855-78.
8) Hartwell LH, Kastan MB. Cell cycle control and cancer. Science 1994;266:1821-3.
9) Baldin V, Lukas J, Marcote MJ, Pagano M, Draetta G. Cyclin D1 is a nuclear protein required for cell cycle progression in G1. Genes Develop 1993;7:812-21.

10) Gillett C, Smith P, Gregory W, Richards M, Millis R, Peters G, Barnes D. Cyclin D1 and prognosis in human breast cancer. Int J Cancer 1996;69:92-9.
11) Han S, Kim HY, Park K, Lee MS, Kim HJ, Kim YD. Expression of cyclin D1 protein in breast cancer and its correlation with prognosis. J Korean Surg Soc 1999;56:771-6.
12) Roger P, Montcourrier P, Maudelonde T, Brouillet JP, Pages A, Laffargue F, Rochefort H. Cathepsin D immunostaining in paraffin-embedded breast cancer cells and macrophages: correlation with cytosolic assay. Human Pathol 1994;25:863-71.
13) Kute TE, Shao L-M, Sugg K, Long RT, Russel GB, Case LD. Cathepsin D as a prognostic indicator for node-negative breast cancer patients using both immunoassays and enzymatic assays. Cancer Res 1992;52:1-6.
14) Ferrandina G, Scambia G, Bardelli F, Panici B, Mancuso S, Messori A. Relationship between cathepsin-D content and disease-free survival in node-negative breast cancer patients: a meta-analysis. Br J Cancer 1997;76:661-6.
15) Roger P, Daures JP, Maudelonde T, Pignodel C, Gleizes M, Chapelle J, et al. Dissociated overexpression of cathepsin D and estrogen receptor alpha in preinvasive mammary tumors. Hum Pathol 2000;31:593-600.
16) Rochefort H, Garcia M, Glondu M, Laurent V, Liaudet E, Rey JM, et al. Cathepsin D in breast cancer: mechanisms and clinical applications, 1999 overview. Clin Chim Acta 2000; 291:157-70.
17) Vignon F, Capony F, Chambon M, Freiss G, Garcia M, Rochefort H. Autocrine growth stimulation of the MCF7 breast cancer cells by the estrogen-regulated 52K protein. Endocrinology 1986;118:1537-45.