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MIB1-determined Proliferative Activity in Intraductal Components and Prognosis of Invasive Ductal Breast Carcinoma

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Intraductal components of breast carcinoma may have prognostic significance. In this study, we divided 181 invasive ductal breast carcinomas into comedo and non-comedo groups based on intraductal component morphology, and differences between the two groups in clinicopathological variables, including proliferative activity and survival, were assessed. Proliferative activity was evaluated by using MIB1 antibody, which reacts with the cell-proliferation-associated Ki-67 antigen, and was expressed as the number of MIB1-positive nuclei per 1000 cancer cells in intraductal components (MIB1 labeling index). We also investigated which variables had an impact on survival. The comedo group showed a significantly higher MIB1 labeling index than the non-comedo group ($P<0.0001$). The differences in disease-free and overall survival between the two groups were not significant ($P=0.2477$, $P=0.2069$). Multivariate analysis of the entire series showed the MIB1 labeling index to be an independent prognostic factor predicting both disease-free survival and overall survival ($P=0.0160$, $P=0.0035$). When multivariate analysis was repeated separately for the non-comedo and comedo groups, the MIB1 labeling index remained the most important variable predicting disease-free and overall survival in the non-comedo group ($P=0.0122$, $P=0.0040$). Moreover, non-comedo patients with a high MIB1 labeling index had significantly shorter disease-free and overall survival than those with a low MIB1 labeling index ($P=0.0040$, $P=0.0402$). These findings imply that MIB1-determined proliferative activity of intraductal components is an independent predictor of survival, and is the most important predictor in non-comedo cases.

Key words: Intraductal components — Comedo — Non-comedo — MIB1 — Proliferative activity

Invasive ductal breast carcinoma, which accounts for approximately 85% of the breast carcinomas in Japanese women, is pathologically composed of invasive foci and intraductal components. As a result of the widespread use of breast conserving therapy, the morphological and biological behavior of intraductal components has attracted a great deal of interest in recent years, because extensive intraductal components have been reported to show a significant association with local recurrence after breast conserving therapy.[1–7] However, the relationship between the characteristics of intraductal components and conventional clinicopathological variables has not yet been elucidated. Nor has there been adequate assessment of how intraductal components are related to postoperative survival in invasive ductal breast carcinoma. Various pathological classifications of intraductal components have been attempted to date, but the most common method has been simply to classify them into comedo and non-comedo types, and the morphological differences have largely depended on the proliferative activity of the malignant cells that constitute the intraductal components.

The main purpose of this study was to investigate whether the morphology of intraductal components as comedo or non-comedo has an impact on postoperative recurrence, and we decided that if it did not have an impact, we would seek some other optimal morphological classification of intraductal components to predict postoperative survival. For this purpose, we divided invasive ductal breast carcinomas into two groups, comedo and non-comedo, based on intraductal component morphology, and determined how clinicopathological variables differed between the two groups. In addition, we immunohistochemically investigated the proliferative activity of intraductal component cells using the monoclonal antibody MIB1 and compared the results in the above two groups. We also conducted a comparative assessment of disease-free survival and overall survival in the two groups. Furthermore, we assessed the impact of clinicopathological variables, including proliferative activity, on postoperative survival as a whole, and separately in the comedo and non-comedo groups. Based on these results, we have revised our previous morphological classification of intraductal components used to predict postoperative survival.

MATERIALS AND METHODS

Patients Two hundred and seventy-five women with UICC clinical Stage I or II primary unilateral invasive

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ductal breast carcinoma treated at Tokyo Women's Medical College Daini Hospital between 1987 and 1993 were reviewed. To minimize the possible confounding effects of potentially inadequate pathological examination, patients who had undergone preoperative excisional biopsy, and those for whom only a small number of pathology slides (10 or less) were available or who clinically had multiple tumors, were excluded from the following analysis. Carcinomas without intraductal components were also excluded (approximately 20% of invasive ductal carcinomas). The study population thus consisted of 181 patients. Tumors measuring 2.5 cm or less were treated by breast-conserving surgery (wide excision and axillary dissection) or mastectomy as decided individually after discussion with the patient, and larger tumors were treated by mastectomy. Based on this policy, 122 patients had undergone modified radical mastectomy and 59 patients had undergone breast-conserving surgery. All patients receiving breast-conserving surgery subsequently underwent radiotherapy.

Information on each patient was obtained from medical records and surgical pathology files, and the clinicopathological data are shown in Table I. The tumors were all less than 5 cm in diameter and were not fixed to either skin or muscle. Clinically, none of the patients showed signs of distant metastases at the time of surgery and all were followed up for 40–118 months postoperatively.

Table I. Distribution of Clinicopathological Variables in 181 Patients with Invasive Ductal Breast Carcinoma

Variable		Number of patients (%)
Age	≤40 years	25 (14)
	41–65	135 (74)
	>65	21 (12)
Menopausal status	premenopausal	100 (55)
	postmenopausal	81 (45)
Tumor size	≤20 mm	84 (46)
	>20	97 (54)
Axillary lymphnode status	negative	85 (47)
	positive	96 (53)
Peritumoral lymphatic vascular invasion	negative	131 (72)
	positive	50 (28)
Intraductal component subtype	non-comedo	140 (77)
	comedo	41 (23)
Estrogen receptor	negative	46 (25)
	positive	121 (67)
	unknown	14 (8)
Type of surgery	mastectomy	122 (67)
	wide excision	59 (33)
Adjuvant therapy	yes	138 (76)
	no	43 (24)
Recurrence	yes	34 (19)
	no	147 (81)

The median follow-up was 59 months. The follow-up was conducted at 1-month intervals during the first year, at 3-month intervals for the next 2 years and at 6-month intervals thereafter. Metastases were evaluated by physical examination and laboratory tests, including serum tumor markers (CEA and CA15-3), X-ray computed tomography, ultrasonography and radioisotope scanning. For the purpose of this analysis, a local recurrence was defined as any tumor recurrence within the treated breast and overlying skin occurring before or simultaneously with the diagnosis of distant metastases.

In patients, who underwent breast-conserving therapy and had positive lymphnode metastasis, postoperative adjuvant therapy consisted of combination chemotherapy with cyclophosphamide, methotrexate and fluorouracil (CMF) followed by oral fluorinated pyrimidine therapy. If the estrogen receptor (ER) status was positive or unknown, the patient also received tamoxifen therapy. When there was no lymphnode metastasis and the patient was diagnosed as being at low risk, no adjuvant therapy was performed. However, if the diagnosis was high risk, the patient received oral fluorinated pyrimidine therapy. Patients who underwent mastectomy and had four or more positive axillary lymphnodes received combination chemotherapy with CMF followed by oral fluorinated pyrimidine. If the ER status was positive or unknown, the patient also received tamoxifen therapy. When there were three or less positive lymphnodes, the patients received oral fluorinated pyrimidine therapy without combination chemotherapy or no chemotherapy. Node-negative patients received tamoxifen therapy or no treatment. Based on this protocol, two-thirds of the patients with positive axillary lymphnodes received adjuvant chemotherapy (66 of 96 patients [68.8%]), whereas only 24 patients (28.4%) with negative axillary lymphnodes were given adjuvant chemotherapy. Hormonal manipulation, primarily with tamoxifen, was employed in 99 cases (54.7% of the total patient group).

Among the mastectomy patients, 6 (4.9%) experienced local recurrence, 22 (18.0%) developed distant metastases, and 94 (77.1%) have remained disease-free to date. Among the patients receiving breast-conserving surgery, 2 (3.4%) experienced local recurrence, 4 (6.8%) developed distant metastases, and 53 (89.8%) have remained disease-free to date.

Histologic examination Tumor size was recorded as the maximum tumor diameter in a fresh specimen. After sampling for estrogen receptor assay, the specimen was immediately fixed in buffered 10% formalin and embedded in paraffin. All cases were divided into two groups according to tumor size: T1 group (less than 20 mm, 84 cases) and T2 group (21 mm or larger, 97 cases). In all cases, adequate numbers of permanent paraffin sections (11–78, average 26.8) were stained with hematoxylin and

eosin (HE). Every section was evaluated by two of the authors (H.I. and M.A.) without knowledge of either the immunohistochemical results or the patient's clinical outcome. Peritumoral lymphatic vascular invasion was considered to be present when groups of cancer cells were identified within endothelium-lined channels and conformed to the shape of the vessel, and all cases were classified into two groups according to the presence (ly^+) or absence (ly^-) of peritumoral lymphatic vascular invasion. All carcinomas had intraductal components within and/or extending beyond the invasive foci, though the amounts of these components were variable. The tumors were classified according to their predominant architectural pattern and the presence or absence of necrosis, based on published criteria.[8] They were typed morphologically as cribriform, papillary, low-papillary, solid or comedo, when such a pattern corresponded to more than 50% of the intraductal components. All types other than comedo were grouped as a whole into the non-comedo type. Forty-one patients were classified as having the comedo type, and 140 the non-comedo type.

All cases were examined for axillary lymphnode involvement and classified into two groups according to lymphnode status: node-negative (n^-) and node-positive (n^+).

Immunohistology After histologic examination, two representative blocks from each patient were selected. All selected blocks were confirmed to have intraductal components associated with invasive foci by histologic examination of HE-stained slides. Four-micrometer-thick sections mounted on triaminopropyltriethoxysilane-coated slides were dried overnight at 158°C. After removal of paraffin and dehydration, the sections were placed in 0.01 mol/liter citrate buffer at pH 6.0. In the present study, we employed an 800W Panasonic microwave oven for antigen retrieval. Sections were boiled five times at 95 to 100°C in 0.01 mol/liter citrate buffer, pH 6.0 for three minutes, and cooled at room temperature for one hour. They were then rinsed in phosphate-buffered saline (PBS), incubated for one hour at room temperature with MIB1 antibody (Immunotech S. A., Marseille, France) diluted at 1 : 100, and immunostained using the labelled streptavidin biotin method. The sections were washed in PBS between steps. The complex was visualized using diaminobenzidine, and nuclei were lightly counterstained with hematoxylin.

Cell counting The sections were scanned at low (×40), medium (×100) and high (×400) power, covering all fields. A cell was considered positive if any nuclear staining was present. For assessment of proliferative activity, the five most strongly stained ducts were selected from among intraductal components presenting the predominant subtype in each tissue section. The proliferative activity was expressed as the number of MIB1-positive nuclei per 1000 malignant cells viewed in high-power fields of these five ducts (MIB1 labeling index). Briefly, color photographs were taken from the five selected ducts and more than 1000 cancer cells were counted on the photographs to calculate the number of MIB1-positive cells. Neither necrotic areas nor the edges of the ducts were included in the counting so as to minimize the possibility of immunohistochemical false positives. Proliferative activity was jointly evaluated by two observers who had no information regarding the clinical outcomes of the patients.

ER assay Immediately after surgery, samples were extracted from the central portion of the carcinoma specimen for ER assay. They were snap-frozen in liquid nitrogen and stored at −70°C until analysis. The ER determination was carried out by the EIA method using an assay kit developed by Abbott Diagnostics (Chicago, IL). In this study, 13 fmol/mg was taken as the minimum value of ER positivity. One hundred and sixty-seven tumors were examined for ER status and classified accordingly into two groups: ER-negative (ER^-) and ER-positive (ER^+).

Statistical analysis The correlations of age, menopausal status, tumor size, axillary lymphnode status, peritumoral lymphatic vascular invasion, ER status, type of surgery, adjuvant therapy and MIB1 labeling index with the intraductal component subtype were evaluated using the Mann-Whitney U-test and the χ^2 test. The associations of MIB1 labeling index with various clinicopathological variables were evaluated using the Mann-Whitney U-test (two categories) and the Kruskal-Wallis test (three categories). A value of $P<0.05$ was considered statistically significant. Disease-free and overall survival rates for each group were estimated by the Kaplan-Meier method. Statistical significance was determined for the survival curves by use of the log-rank test. All survival times were calculated from the time of definitive breast surgery to the time of most recent follow-up. Multivariate survival analysis using Cox's proportional hazard regression model was carried out to assess the independent contribution of each variable to disease-free and overall survival. In this study, a computer program package (Stat View, Abacus Concepts, Inc., Berkeley, CA) was used for statistical testing and database management.

RESULTS

Relationship between intraductal component morphology and clinicopathological variables: Neither comedo nor non-comedo intraductal component morphology correlate with age ($P=0.2462$), menopausal status ($P=0.3420$), tumor size ($P=0.0928$), axillary lymphnode status ($P=0.1645$), peritumoral lymphatic vascular inva-

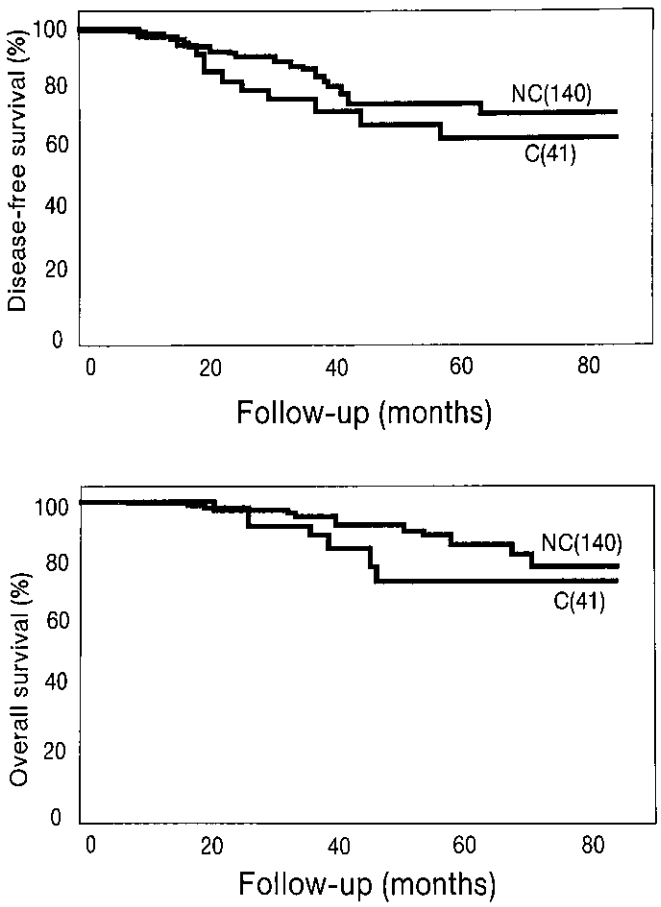

Fig. 1. Disease-free and overall survival of all patients categorized according to intraductal component morphological type ($P=0.2477$, $P=0.2069$). Numbers in parentheses, the total number of patients per group. NC, non-comedo group; C, comedo group.

sion ($P=0.2091$), ER status ($P=0.1684$), type of surgery ($P=0.3556$), or adjuvant therapy ($P=0.4799$). The mean MIB1 labeling index of the comedo group was significantly higher than that of the non-comedo group (230 vs. 101) ($P<0.0001$).

Associations of MIB1 labeling index with various clinicopathological variables: The MIB1 labeling index correlated with two prognostically relevant factors: ER status ($P=0.0003$) and tumor size ($P=0.0033$). The MIB1 labeling index did not correlate with age ($P=0.4333$), menopausal status ($P=0.8198$), axillary lymph node status ($P=0.2495$), peritumoral lymphatic vascular invasion ($P=0.3621$), type of surgery ($P=0.3556$), or adjuvant therapy ($P=0.2388$).

Association of intraductal component morphology with survival: Fig. 1 shows disease-free and overall survival curves stratified by intraductal component morphology. These curves illustrate the increased hazard rates for patients with breast carcinoma associated with comedo type intraductal components, but the differences did not reach statistical significance ($P=0.2477$, $P=0.2069$).

Table II. Multivariate Cox Analysis of Disease-free and Overall Survival

	Disease-free survival		Overall survival	
	relative risk	P	relative risk	P
Age		0.9446		0.3771
Menopausal status		0.7420		0.7335
Tumor size		0.0820	**2.107**	**0.0273**
Lymphnode status	**2.760**	**0.0058**		0.0625
Peritumoral lymphatic vascular invasion		0.5842		0.7315
ER status		0.6335		0.5596
Intraductal component morphology		0.9927		0.9121
MIB1 labeling index	**2.408**	**0.0160**	**2.923**	**0.0035**
Type of surgery		0.8165		0.4551
Adjuvant therapy		0.7497		0.5634

Univariate and multivariate analysis of survival: Univariate analysis focusing on disease-free survival revealed tumor size ($P=0.0012$), axillary lymphnode status ($P=0.0002$), peritumoral lymphatic vascular invasion ($P=0.0218$), intraductal component morphology ($P=0.0366$) and MIB1 labeling index ($P<0.0001$) to be significant prognostic factors in 181 breast cancer patients. On univariate analysis concerning overall survival, tumor size ($P=0.0123$), axillary lymphnode status ($P=0.0122$) and MIB1 labeling index ($P<0.0001$) were significant prognostic factors. Following the univariate study, multivariate analysis was conducted to test the independent prognostic value of these variables. When all variables were taken into account, axillary lymphnode status ($P=0.0058$) and the MIB1 labeling index ($P=0.0160$) were significant prognostic factors predicting disease-free survival, whereas tumor size ($P=0.0273$) and MIB1 labeling index ($P=0.0035$) were significant prognostic factors predicting overall survival (Table II).

To assess the importance of risk factors in intraductal component morphology, multivariate analysis was conducted separately for the 140 non-comedo and the 41 comedo cases. In the non-comedo group, the MIB1 labeling index remained the most important variable predicting disease-free and overall survival ($P=0.0122$, relative risk $=2.507$; and $P=0.0040$, relative risk $=2.875$; respectively). Nodal status came next, but was minimally significant ($P=0.0133$, relative risk $=2.475$; and $P=0.0251$, relative risk $=2.241$; respectively), while tumor size had no prognostic value. In the comedo group, no significant variables were recognized.

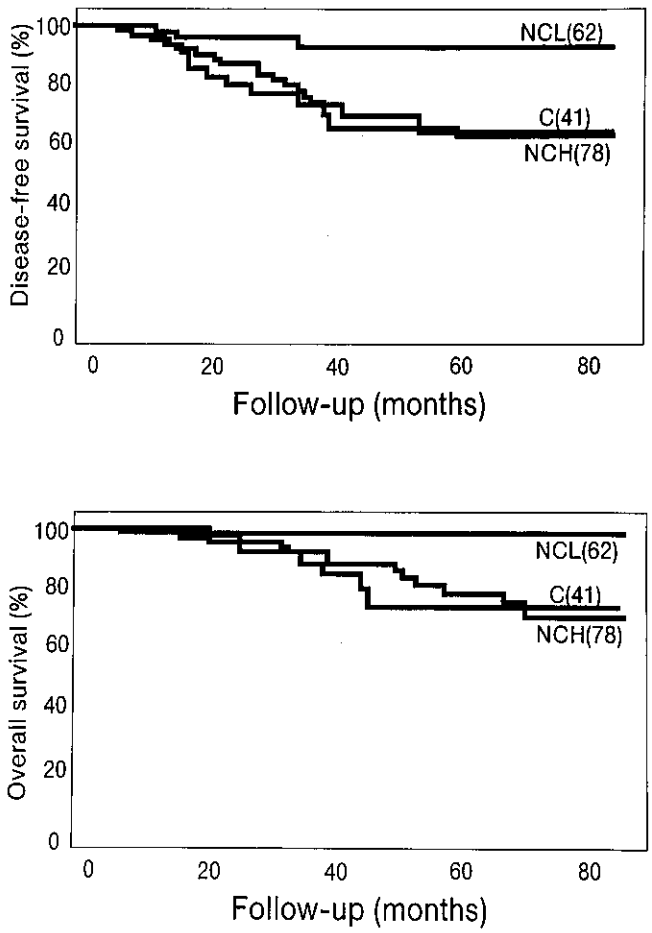

Fig. 2. Disease-free and overall survival of all patients categorized according to intraductal component morphological type and MIB1 labeling index. The cut-off point for the MIB1 labeling index was set at 50, and 140 non-comedo cases were accordingly divided into two groups ($P=0.0040$, $P=0.0402$). NCL, non-comedo with low MIB1 labeling index group; NCH, non-comedo with high MIB1 labeling index group; C, comedo group.

Prognostic significance of MIB1 labeling index in the non-comedo group: To clarify the prognostic value of the MIB1 labeling index in the non-comedo group, the cut-off point was set at 50 and the 140 non-comedo cases were subdivided into two groups: high MIB1 labeling index (>50, 78 cases) and low MIB1 labeling index (≤ 50, 62 cases). Then, disease-free and overall survival rates were compared among the three groups. As shown in Fig. 2, the non-comedo group with a low MIB1 labeling index had significantly better disease-free and overall survival rates than the non-comedo group with a high MIB1 labeling index and the comedo group ($P=0.0040$, $P=0.0402$).

DISCUSSION

Invasive ductal breast carcinoma accounts for approximately 85% of all breast carcinomas in Japanese women, and structurally these cancers are usually made up of invasive foci and intraductal components. In the past, attention was generally focused on the invasive foci when discussing the prognosis of invasive ductal breast carcinoma, and most histological classifications have been based on the morphology of the invasive foci. For example, the Japanese Breast Cancer Society has classified invasive ductal carcinoma into three types using primarily the morphological features of invasive foci as criteria. As a result of the widespread adoption of breast-conserving therapy, a great deal of significance is now attached to the intraductal components, because extensive intraductal components have been reported to be significantly associated with local recurrence.[1–7] Moreover, early breast cancer in which intraductal components predominate is now being encountered more often in clinical settings. Nevertheless, it is not known how these intraductal components are related to the prognosis of patients receiving breast-conserving therapy, or to the prognosis of breast cancer patients as a whole, including those undergoing mastectomy. Accordingly, in the present study we investigated how the morphological and biological behavior of the intraductal components affects postoperative survival in breast cancer. In order to assess the effect of morphological behavior on postoperative survival, we first divided invasive ductal breast carcinoma into a comedo group and a non-comedo group based on intraductal component morphology and determined how their postoperative outcomes differed. The differences in disease-free survival and overall survival between these two groups were not significant, apparently due to the non-comedo group having a mixture of intraductal components displaying a variety of biological behaviors. When proliferative activities based on MIB1 were compared, the non-comedo group was found to have significantly lower proliferative activity than the comedo group, but the distribution covered a broad range, from 4 to 540, suggesting a need for further subclassification of the non-comedo group aimed at refining the prediction of outcome.

The results of multivariate analysis for the entire subject group showed the MIB1 labeling index to be an independent predictor of both disease-free and overall survival. When multivariate analysis was then performed for the non-comedo group only, the MIB1 labeling index was found to be a better predictor than nodal status of both disease-free and overall survival. It was also found that the non-comedo patients with a high MIB1 labeling index had a significantly worse prognosis than the non-comedo patients with a low MIB1 labeling index. The

former had the same prognosis as the comedo group. These findings mean that it is possible to predict outcome according to the non-comedo group subcategory using the MIB1 labeling index as a criterion. Classifying intraductal components into comedo and non-comedo types on the basis of architecture and the absence or presence of necrosis is convenient, but overly simple. In recent years, with the increase in ductal carcinoma *in situ*, methods of classifying intraductal components have been proposed that would allow outcome prediction on the basis not only of morphological behavior, but also biological behavior.[9–13] Lampejo *et al.*[9] subclassified invasive ductal carcinoma using the differentiation of cancer cells in intraductal components as the major criterion, and Holland *et al.*[10] proposed subclassification based on nuclear grade. However, both systems were hampered by the problem that subjective elements could not be eliminated. By contrast, the MIB1 method is apparently superior in terms of being more objective. The MIB1 antibody raised by Cattoretti *et al.*[14] reacts with the Ki-67 nuclear antigen (345 and 395 kD double band on western blot analysis of proliferating cells), which is associated with cell proliferation and is found throughout the cell cycle (G1, S, G2, M-phase). It is not present in resting (G0) cells. This antibody recognizes native Ki-67 antigen and recombinant fragments of the Ki-67 molecule. Cattoretti *et al.* demonstrated that the nuclear staining with MIB1 seen in paraffin sections after microwave pretreatment coincides well with that of Ki-67 seen in frozen sections, and studies have been done using this antibody.[15–18] Thus, we employed the MIB1 antibody to detect proliferative activity in the present study.

Multivariate analysis in the comedo group showed that the MIB1 labeling index, tumor size, and nodal status were not significant predictors of survival. It is possible that the large number of biologically aggressive cancers in the comedo group may have masked significant differences.

It was concluded that the MIB1 labeling index is an independent predictor of both disease-free and overall survival. In particular, the MIB1 labeling index was demonstrated to be a better predictor than nodal status in the non-comedo type. It was also shown that it is possible to predict outcome more accurately by subclassifying the non-comedo type using the MIB1 labeling index. Long-term follow-up, based on the accumulation of more cases, is needed to confirm the usefulness of this method.

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