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Original

Analysis of clinicopathological features and proliferation-related protein expressions in breast cancer based on tumor intrinsic phenotype

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Abstract

We analyzed the clinicopathological features of breast cancer in relation to proliferation-related protein expressions based on intrinsic phenotype. The study included 175 patients with primary invasive breast cancer. The intrinsic phenotype is classified into the luminal A, luminal B, HER2, and triple-negative (TN) subtypes. Labeling indices (LIs) of Ki-67, cyclin A, cyclin D1 and phosphorylated retinoblastoma protein (p-Rb) were identified in tissues by immunohistochemistry. In addition, p53 overexpression was examined. The numbers of positive cells were determined using automated analysis software. We found a high incidence of papillotubular carcinoma in the luminal A and B types, while solid tubular carcinoma was common in the TN type. Furthermore, HER2 types exhibited high Ki-67, cyclin A and p-Rb LIs, and p53 overexpression, whereas the luminal A type showed low LIs of those markers. On the other hand, luminal A and B types expressed high levels of cyclin D1, whereas HER2 and TN types expressed low levels. These results revealed a relationship between the Ki-67 LI and tumor intrinsic phenotype, and this relationship may be reflected on the basis of cyclin A and p-Rb LIs or p53 overexpression. The results also indicate that cyclin D1 LI is inversely correlated to these intrinsic phenotypes.

Key words : *breast cancer, Ki-67, cyclin D1, cyclin A, p-Rb*

I. Introduction

Breast cancer is a serious malignancy with highest rate of morbidity among malignant neoplasms and is the leading cause of death among women in Japan [1]. Most tumor diagnoses depend on pathological examination, which is essential for making definitive diagnoses of malignant tumors. Similarly, pathological analysis plays a key role in the diagnosis of breast cancer. Recent studies have shown that both clinicopathological findings and therapeutic outcomes of breast cancer are closely related to breast cancer cell phenotypes, which have become indispensable factors in the pathological diagnosis of breast cancer [2-4].

To date, breast cancer cell intrinsic phenotypes have been broadly classified

into luminal A, luminal B, human epidermal growth factor receptor 2 (HER2), and triple-negative (TN) groups [4, 5], according to the presence or absence of estrogen receptor (ER), progesterone receptor (PgR), and HER2 protein expression [5, 6]. The luminal A type is only positive for ER and PgR expression, whereas the luminal B type is additionally positive for HER2 expression. On the other hand, the HER2 type is only positive for HER2 expression, while the TN type is negative for ER, PgR, and HER2 expression. It has been revealed that the prognosis of the luminal types is good, whereas that of the TN type is poor [7]. Although the prognosis of the HER2 type has been dramatically improved with the advent of trastuzumab, a molecular targeting agent, it was previously considered to be associated with a poor prognosis [7].

Recently, it has been found that proliferative potential is correlated with breast cancer intrinsic phenotype and that the Ki-67-positive cell fraction or labeling index (LI) may determine the phenotype [8]. For instance, in the existing classification, when the Ki-67 LI exceeds 14%, the luminal type is changed from A to B. Thus, evaluation of proliferative potential (Ki-67 LI) is indispensable for the appropriate treatment of breast cancer [9].

The cell cycle mechanisms through which proliferation is controlled have been elucidated. Cell cycle progression is controlled by a balance between factors that positively and negatively regulate proliferation [10]. Retinoblastoma (Rb) protein phosphorylation is considered the most essential factor for accelerating the synthesis (S) phase of the cell cycle, and related factors, including cyclin D1 and cyclin A, have been shown to be important indicators of proliferative potential [11-13]. Alternatively, p53 protein negatively regulates the cell cycle via p21 inhibition, and p53 mutation results in the loss of p21 function and may result in failure to regulate the cell cycle [14]. In breast cancer cells, although cell proliferation is inhibited through a similar mechanism, the relationship between increased Ki-67 expression (increased proliferative potential) and the aforementioned cell cycle-related expression of protein in each phenotype remains unclear. In the present study, we classified breast cancer into 4 intrinsic phenotypes and, for each type, analyzed the relationship between clinicopathological features, proliferative potential (Ki-67 LI), and expression of cell cycle-related proteins.

II. Materials and methods

The study included 175 women with primary breast cancer who underwent surgical resection at the Department of Surgery at Iwate Medical University between April 2009 and April 2013 but did not undergo pre-operative chemotherapy. The diagnosis of histological type and cancer staging were conducted on the basis of the clinical practice guidelines for breast cancer [15]. The clinical grade of breast cancer was determined in accordance with the Nottingham classification [16].

The present study was conducted with the approval of the ethics committee of Iwate Medical University School of Medicine (approval number H25-122).

Resected specimens obtained in accordance with the clinical practice guidelines for breast cancer were fixed in 10% buffered formalin for 1–2 days after resection [15]. All resected

Table 1. Experimental conditions of immunohistochemistry assays

Antibody	Clone	Dilution	Source
ER	SP1	Ready-to-use	Dako
PgR	PgR636	Ready-to-use	Dako
HER2	Polyclonal	Ready-to-use	Dako
Ki-67	MIB-1	Ready-to-use	Dako
Cyclin A	6E6	× 100	Leica Biosystems
Cyclin D1	SP4	× 100	Nichirei Biosciences
p-Rb	Polyclonal	× 300	Leica Biosystems
p53	DO-7	× 100	Cell Signaling Technology

ER : estrogen receptor, PgR : progesterone receptor, HER2 : human epidermal growth factor receptor type 2, p-Rb : phosphorylated-Retinoblastoma
Dako : Glostrup, Denmark. Leica Biosystems : Wetzlar, Germany. Nichirei biosciences : Toyko, Japan.

Table 2. Determination of breast cancer phenotype based on expression of ER, PgR, HER2 and Ki-67 LI.

Phenotype	ER	PgR	HER2	Ki-67 LI
Luminal A	(+) and of	(+)	(−)	< 14%
Luminal B	(+)	(+)	(−) (+)	≧ 14% any
HER2	(−)	(−)	(+)	any
TN	(−)	(−)	(−)	any

ER : estrogen receptor, PgR : progesterone receptor, HER2 : human epidermal growth factor receptor type 2, TN : Triple negative breast cancer, LI: labeling index

specimens were embedded in paraffin and stained with hematoxylin and eosin (HE).

1. Immunohistochemical classification of intrinsic phenotypes

Representative sections from the resected specimens were selected, and 3-μm-thick serial sections were then divided into those for HE staining and those for immunostaining. Immunostaining was performed using the Dako EnVision+ system with an autostaining device (Autostainer, Dako, Denmark) [17]. The antibodies utilized in the immunostaining are listed in Table 1.

2. Immunohistochemical staining criteria

1) Intrinsic phenotype determination

Phenotypes were determined according to the criteria listed in Table 2 [5, 6]. Each antibody was considered positive on the basis of the following criteria: ER and PgR were considered positive if ≥1% of the tumor cells showed positive staining [15]. For HER2, the staining characteristics of positive cells ranging from 0 to 3+ were assessed in accordance with the HER2 test guidelines, with 3+ considered to be positive [18]. Moreover, cases with negative staining for HER2 but positive staining for ER and PgR and a Ki-67 LI of ≥14% were classified as the luminal B type [8].

2) Calculation of the positive-cell fraction according to Ki-67, cyclin A, cyclin D1, and phosphorylated (p)-Rb immunostaining

Each section subjected to immunostaining was digitized as a virtual slide using ScanScope AT Turbo (Aperio, Leica Biosystems Imaging, Germany), and the LI was calculated using automated measuring software (Aperio Pathology System, Germany). The LI was calculated from hot spots on the slide (i.e., the area most densely stained within the specimen). The degree of staining was

Table 3. Clinicopathological features breast cancer examined based on intrinsic phenotype.

	Molecular subtype				P
	Luminal A	Luminal B	HER2	TN	
Total	40	40	35	60	
Age	62	59.3	62.5	61.2	
Size	16mm (8-40mm)	14mm (7-21mm)	19mm (5-45mm)	18mm (4-66mm)	
Histological subtype					<0.01
Papillotubular carcinoma	19 (47%)	2 (6%)	10 (29%)	5 (8%)	
Solid-tubular carcinoma	10 (25%)	24 (59%)	15 (42%)	35 (58%)	
Scirrhous carcinoma	11 (28%)	14 (35%)	10 (29%)	20 (34%)	
pTNM stage					0.13
I	30 (75%)	25 (63%)	18 (51%)	35 (58%)	
II	2 (5%)	10 (25%)	7 (20%)	18 (30%)	
III	8 (20%)	5 (12%)	10 (29%)	7 (12%)	
IV	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
Histological Grade					<0.01
1	21 (53%)	5 (12%)	1 (4%)	0 (0%)	
2	19 (47%)	24 (59%)	5 (13%)	11 (18%)	
3	0 (0%)	11 (29%)	29 (83%)	49 (82%)	

HER2 : human epidermal growth factor receptor type 2, TN : Triple negative breast cancer.

classified into 0, 1+, 2+, and 3+ for analysis, and staining of 2+ (i.e., cells with unevenly stained nuclei) was considered positive. LI was calculated as the number of positive cells per 1000 cells.

3. Statistical analysis

The data were statistically analyzed using the statistical software Stat Mate for Windows Version 3.07 (Atom, Tokyo, Japan). The relationships among the LIs for Ki-67, cyclin A, cyclin D1, p-Rb, and p53 were examined using the Mann–Whitney U test, and multiple comparisons of the clinicopathological findings were performed using Fischer's exact test. A p value of <0.05 was considered significant.

III. Results

1. Clinicopathological examination of breast cancer intrinsic phenotype

The clinicopathological features of each intrinsic phenotype are shown in Table 3. No difference was found in age or tumor diameter between each intrinsic phenotype. Among the histological types, the frequencies of papillotubular carcinoma and solid-tubular carcinoma were significantly higher in the luminal A and B types and the TN type ($p<0.01$). However, no differences were observed in the incidences of scirrhous carcinoma among phenotypes. Moreover, no significant correlation was observed between any phenotype and pathological stage (i.e., pathological tumor–node–metastasis stage). The histological grade was mostly grade 1 for the luminal A type and grade 2 for the luminal B type. A higher incidence of grade 3 was observed in HER2 and TN type cases ($p<0.01$).

2. Immunohistochemical analysis of breast cancer intrinsic phenotype

Representative stains with each antibody are shown in Figure 1.

1) A comparison of the Ki-67 LI across intrinsic phenotypes

Figure 2 depicts the distribution of the Ki-67 LI for each intrinsic phenotype. The median Ki-67 LI for the luminal A, luminal B,

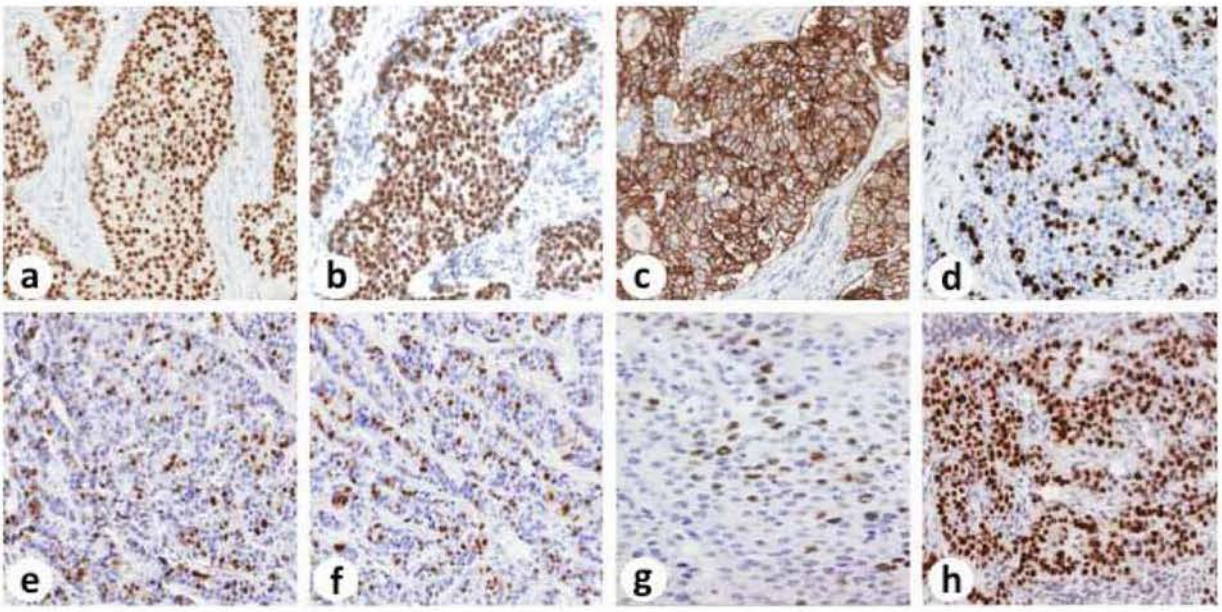

Fig. 1. Representative immunohistochemical findings. (a) Positive nuclear ER staining. This tumor was classified as ER-positive. (b) PgR staining in the nucleus. This tumor was scored as PgR-positive. (c) Positive HER2 membrane staining. The HER2 expression of this tumor was scored as 3+. (d) Positive nuclear Ki-67 staining. The Ki-67 LI of this specimen was 29%. (e) Positive nuclear cyclin A staining. The cyclin A LI of this tumor was 22%. (f) Cyclin D1 staining in the nucleus. The cyclin D1 LI of this tumor was 24%. (g) Positive nuclear p-Rb staining. The p-Rb LI of this tumor was 20%. (h) Positive nuclear p53 staining. The p53 LI of this tumor was 96%. The magnification of all figures is × 200.

HER2, and TN types was 8%, 36.5%, 45%, and 54%, respectively. The TN and HER2 types had significantly higher Ki-67 LIs than did the luminal A and B types ($p<0.01$). Furthermore, although the luminal B type had a significantly higher Ki-67 LI than did the luminal A type ($p<0.01$), no significant difference was observed between the TN and HER2 types.

2) A comparison of the cyclin A LI across intrinsic phenotypes

Figure 3-1 depicts the distribution of the cyclin A LI for each type. The median cyclin A LI for the luminal A, luminal B, HER2, and TN types was 2%, 6%, 10.5%, and 16.5%, respectively. The TN type had a significantly higher cyclin A LI than did the luminal A, luminal B, and HER2 types ($p<0.01$), and the cyclin A LI for the HER2 type was significantly different from that for the luminal A and B types ($p<0.01$, $p<0.05$, respectively). On the other hand, the cyclin A LI for the luminal B type was significantly higher than

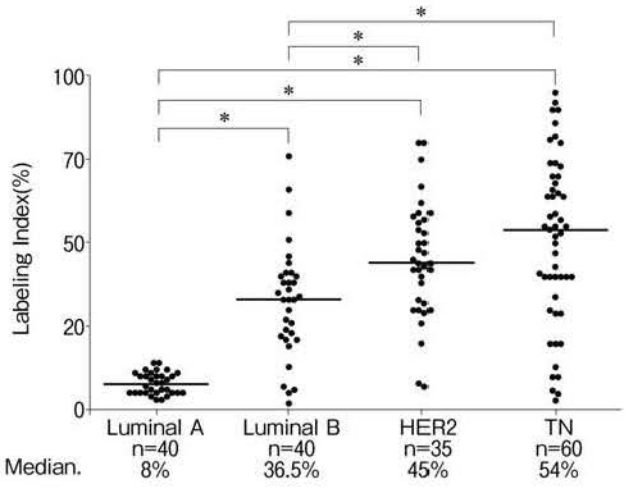

Fig. 2. Ki-67 LI based on intrinsic phenotype.
*$p < 0.01$

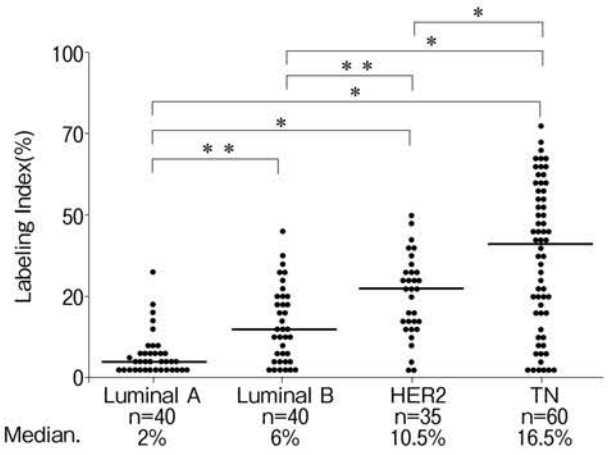

Fig. 3-1. Cyclin A LI based on intrinsic phenotype.
*p < 0.01 **p < 0.05

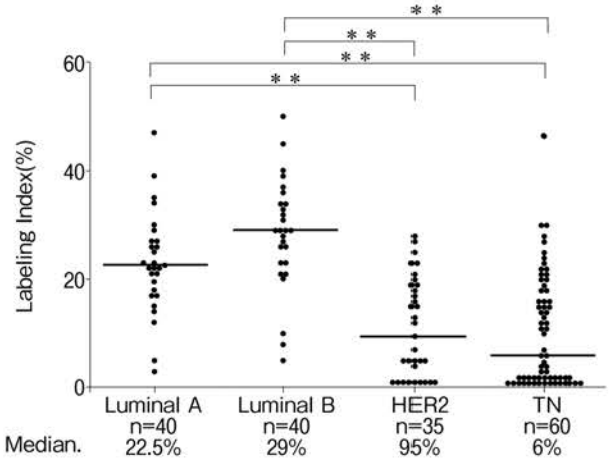

Fig. 3-2. Cyclin D1 LI based on intrinsic phenotype.
**p < 0.05

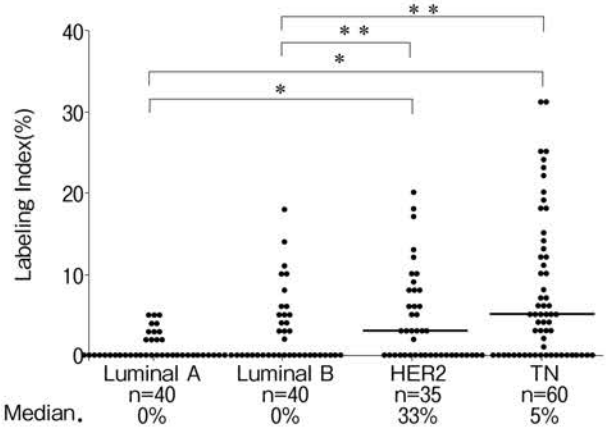

Fig. 4-1. p-Rb LI based on intrinsic phenotype.
*p < 0.01 **p < 0.05

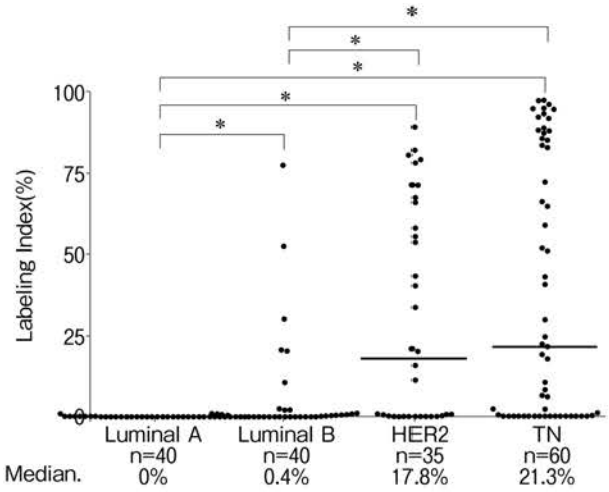

Fig. 4-2. p53 LI based on intrinsic phenotype.
*p < 0.01

that for the luminal A type ($p<0.05$).

3) A comparison of the cyclin D1 LI across intrinsic phenotypes

The distribution of the cyclin D1 LI for each intrinsic phenotype is shown in Figure 3-2. The median cyclin D1 LI for the luminal A, luminal B, HER2, and TN types was 22.5%, 29%, 9.5%, and 6%, respectively. The cyclin D1 LI for the luminal A type was significantly higher than that for the HER2 and TN types ($p<0.05$), and the cyclin D1 LI for the luminal B type was significantly higher than that for the HER2 and TN types ($p<0.05$). No significant difference was observed for the cyclin D1 LI between the luminal A and B types.

4) A comparison of the p-Rb LI across phenotypes

The distribution of the p-Rb LI for each phenotype is shown in Figure 4-1. The median p-Rb LI for the luminal A, luminal B, HER2, and TN types was 0%, 0%, 3%, and 5%, respectively. The p-Rb LI for the TN type was significantly higher than that for the luminal A and B types ($p<0.01$). The p-Rb LI for the

HER2 type was significantly different from that for the luminal A and B types ($p<0.05$). No significant difference was observed in the p-Rb LI between the TN and HER2 types.

5) A comparison of the p53 LI across phenotypes

Figure 4-2 shows the distribution of the p53 LI for each phenotype. The median p53 LI for the luminal A, luminal B, HER2, and TN types was 0%, 0.4%, 17.8%, and 21.3%, respectively. The p53 LI for the TN and HER2 types was significantly different from that for the luminal A and B types ($p<0.01$). The p53 LI was significantly higher for the luminal B type than for the luminal A type ($p<0.01$); however, no significant difference was found between the TN and HER2 types.

IV. Discussion

Intrinsic phenotypic classification provides information that is indispensable for the diagnosis and treatment of breast cancer [19]. It is particularly useful in the selection of various chemotherapies and molecular targeting agents and is highly relied upon by breast cancer specialists. Clinicopathologically, each intrinsic phenotype has been found to exhibit distinctive features, although the details have not been fully examined. Assessment of the proliferative potential of breast cancer cells is becoming necessary to stratify breast cancer patients [8, 9], and is closely linked to the classification of breast cancer phenotypes. Cell proliferative potential is strictly regulated by the cell cycle through various proteins and is determined by a balance between factors that positively and negatively regulate proliferation [10]. In addition, it is assumed that the Ki-67 LI ultimately indicates cell proliferative potential via the balance of expression of these proteins. Nevertheless, Ki-67-positive cells express Ki-67 throughout the entire cell cycle, excluding part of the G1 phase [20], and care must be taken to ensure that the Ki-67 LI is similar to the growth fraction. Although the former finding indicates that the Ki-67 LI is not related to DNA synthesis, which is a direct indicator of the cell proliferative potential, in general, there is a correlation [21, 22]. However, the relationship between Ki-67 expression and proliferation-related factors has not been fully examined in breast cancer.

In Japan, invasive ductal carcinoma in breast cancer is divided into the following main histological types: papillotubular carcinoma, solid tubular carcinoma, and scirrhous carcinoma [15]. A unique classification of intrinsic phenotype proposed in Japan has been used for some time to classify these subtypes and has been shown to be clinicopathologically useful [23]. This classification is actively utilized in the actual treatment of breast cancer and, in Japan, it is considered an essential diagnostic factor. In the pathology of breast cancer, if intrinsic phenotypic classification is to be established as necessary data, then its relationship with the histological classification of breast cancer currently in use needs to be examined. The present study revealed a significantly high incidence of papillotubular carcinoma in the luminal A tumor type and a significantly high incidence of solid tubular carcinoma in the luminal B and TN types. However, scirrhous carcinoma was common in each phenotype. These results indicate that the Japanese criteria of histological types correlate with

the international phenotypic classification, and these results suggest that the histological classification in the Japanese clinical practice guidelines is useful. Although the above classification is uniquely used in Japan and has not gained popularity throughout the world, it provides valuable evidence for improving international standards.

It has been pointed out that histological grade is a useful indicator for predicting the prognosis of breast cancer [24]. While several classifications have been proposed for the histological grade of breast cancer [16, 25], the histological grade classification system used throughout the world was applied in the present study. In this grade classification, the degree of tubular formation, nuclear atypia, and mitotic figures are used as indicators and scored, and correlate well with prognosis [16]. The present study shows that luminal A type cases included many cancers with a low histological grade, whereas HER2 and TN type cases showed a high histological grade. However, the histological grade of luminal B type cases was intermediate. The results of the present study are comparable with the those of previous study [26], and confirm the relationship between histological grade and breast cancer phenotype.

The Ki-67 LI has been shown to be a useful indicator for predicting the prognosis of breast cancer, and many reports indicate that cancers with a high Ki-67 LI have a poor prognosis [21, 22]. In the actual treatment of breast cancer, the Ki-67 LI is considered an indispensable factor, and it would be safe to state that Ki-67 LI measurement should be included in routine medical practice. In the present study, no statistically significant difference was identified between the HER2 and TN types; however, the Ki-67 LI increased in all types in the following order, from luminal A, luminal B, HER2, and TN. This suggests a positive correlation between proliferative potential and phenotypic classification. Nishimura et al. also disclosed that proliferative potential is closely related to phenotypic classification [27].

Cyclin D1 overexpression is associated with cancer cell aggressiveness in esophageal and pancreatic cancers 28). However, the inverse relationship has been reported in breast cancer [29]. According to Bostrom et al., cyclin D1 overexpression is seen in ER-positive breast cancers [29]. These results suggest that prognosis is good in breast cancers with cyclin D1 overexpression. In breast cancer, cyclin D1 overexpression is a biological marker that may indicate good prognosis. Conversely, it has been reported that cyclin D1 overexpression indicates poor prognosis in the ER-positive group [30]. Therefore, the clinicopathological significance of cyclin D1 overexpression in ER-positive tumors is controversial. Cyclin D1 is overexpressed in ER-positive breast cancers; however, the exact mechanism for this overexpression has yet to be clarified. Analtonen et al. demonstrated that the role of cyclin D1 may differ in the cell cycles of ER-positive and -negative cells 30). Although the mechanism of cyclin D1 expression in breast cancer is not fully understood, the role of cyclin D1 overexpression of the ER-positive tumor cells may be different from that of ER-negative tumor cells. This finding supports the notion that can be classified breast cancer based on ER expression.

High levels of cyclin A overexpression were

shown, in descending order, in the luminal A, luminal B, HER2, and TN types, whereas the inverse was the case with cyclin D1. Cyclin A is also referred to as the S-phase cyclin and positively regulates DNA synthesis [10]. Cyclin A overexpression by itself is an indicator for poor prognosis in breast cancer [31], and the results of the present study support this finding. It is important to analyze the significance of cyclin A overexpression in terms of phenotype.

Rb phosphorylation is responsible for the transition of cells from the G1 phase to the S phase [11]. Thus, anti-p-Rb antibodies can identify cells in the S phase by immunostaining [32]. In the present study, we examined the distribution of cells positive for p-Rb in normal mucosa of the large intestines and found that staining was limited to the crypt proliferation zone (data not shown). To date, the identification of cells in the S phase has required the injection of 3H-thymidine into the body or incorporation of 5-bromo-2′ -deoxyuridine into cellular DNA 33); however, immunostaining using p-Rb antibodies is easily applicable for identification of cells in the S phase [33]. The present study showed high levels of p-Rb in all four tumor intrinsic phenotypes, listed here in descending order: luminal A, luminal B, HER2, and TN. These results are similar to those for the Ki-67 LI. As mentioned above, the Ki-67 LI theoretically represents the growth fraction; however, proliferative potential may be overestimated. Therefore, p-Rb expression and cyclin A overexpression are more appropriate for evaluating proliferative potential. To date, no reports have investigated the relationship with phenotype using p-Rb antibodies, and the present study disclosed the usefulness of p-Rb expression in evaluation of proliferative potential of tumors. Further studies should be conducted using p-Rb antibodies to examine the proliferative potential more accurately.

p53 overexpression is generally considered to reflect p53 mutations and is utilized to identify these mutations, which are often detected in malignant tumors [34, 35] and have been found to contribute to cancer progression. Although the incidence of p53 overexpression in invasive breast cancer varies among the different reports, p53 overexpression has a close association with carcinogenesis in a subset of breast cancer (approximately 40%) [36], which suggests that p53 abnormalities (i.e., p53 overexpression or p53 mutation) play a key role in determining the invasive potential of breast cancer cells. Examination of the relationship between p53 abnormalities and phenotypic classification may provide a valuable perspective on the treatment of breast cancer. In the present study, there was a high incidence of cells positive for p53 overexpression: in descending order, in luminal A, luminal B, HER2, and TN types; however, no significant difference was observed between HER2 and TN types. Calza et al. reported results different from ours; in their report, the relative order of p53 overexpression was, from highest to lowest, luminal A, luminal B, HER2, and TN [37], with no difference observed between HER2 and TN types. Calza' s group determined the presence or absence of p53 mutations on a genetic level, whereas our results were based on immunostaining. Thus, the difference in detection method may have influenced the results. In general, p53 abnormalities are

detected with higher sensitivity by analysis of abnormal expression than by analysis of mutations; however, there is no clear reason that explains the difference between these results [38]. There was a high incidence of p53 abnormalities in HER2 and TN types in the present study. p53 overexpression may play a key role in the development of both phenotypes.

In conclusion, we examined differencies of clinicopathological findings and expression of proliferation-related markers between each intrinsic phenotype. The results of the present study reveal that the intrinsic phenotype of breast cancer is characterized by the clinicopathological findings and the molecular alterations. There was a close association of Ki-67 LI with breast cancer phenotype in the present study. This finding is supported by the notion that Ki-67 LI is defined by cyclin A overexpression and/or p-Rb expression level. Cyclin D1 overexpression of the intrinsic phenotype was inversely correlated with Ki-67 LI, cyclin A, and p-Rb of the intrinsic phenotype. We suggest that phenotypic classification is an indispensable element in the routine treatment of breast cancer.

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Conflict of interest: We declare that we have no conflict of interest.

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乳癌における形質分類に基づいた臨床病理学的及び増殖関連タンパク発現の検討

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要旨

我々は乳癌における各形質に基づいて乳癌の臨床病理学的特徴と増殖能および細胞周期関連タンパクとの関連性について検討した．乳癌の形質は Luminal A 型，Luminal B 型，HER2 型および TN 型に分類される．各形質ごとに Ki-67, サイクリン A, サイクリン D1,p-Rb,p53 の発現について免疫組織化学を用いて陽性細胞率を解析した．Ki-67、サイクリン A，p-Rb および p53 は TN 型，HER2 型の順で高値を示し，Luminal A 型で低値を示した．一方サイクリン D1 では Luminal A 型および Luminal B 型が高値であった．上記の結果から Ki-67 陽性細胞率と形質分類間には関連性がみられ，この関係はサイクリン A および p-Rb の発現もしくは p53 の発現によって規定されている可能性が示唆された．一方サイクリン D1 についてはそれらとは逆の関係にあることが示された．