Abstract
Triple negative (TN) and younger cases in breast cancer have poorer clinical outcome data. We evaluated the relationship between outcome of triple negative and younger cases and the proliferation potency of breast cancer cells. 94 cases of operated breast cancer were classified by subtype according to 2011 St. Gallen consensus meeting. Each subtype group and younger patients (=or<45) were investigated Ki-67 proliferation index indicated proliferation potency of cancer, nuclear grade (NG) and histological grade(HG). Out of 94 cases 48 were classified as Luminal A, 30 as Luminal B, 8 as HER2 and 8 as TN. The average proliferation index of Ki-67 was 6.4 ± 3.7 in Luminal A, 24.6 ± 11.2 in Luminal B, 20.2 ± 6.6 in HER2, and 62.6 ± 15.7 in TN. There was a significant higher proliferation index of Ki-67 in the TN group. There were significantly higher NG and HG in the TN group compare with Luminal A and B. Mean Ki-67 proliferation index, NG and HG in cases which were 45 years old and less was significantly higher than those in cases which were more than 46 years old. The poor prognosis in the TN group and younger patients is supposedly caused by the proliferation potency, NG and HG in breast cancer.

Key word: Ki-67, triple negative, young patient, breast cancer

Introduction
Triple negative (TN) status was one of the major contributors for the increase in recurrence and the worse in survival. One of the reason why TN has poor prognosis is supposed to unresponsiveness to hormonal therapy and molecular target drug to HER2 (human epidermal growth factor receptor type 2). Patients with TN phenotype showed higher response rate and pathologic complete response rate to chemotherapy. However, relapse free survival and overall survival were significantly worse in TN breast cancer patients. Chacon et al reported that TN patients had a higher histological grade which induced a worse prognosis.

Another question is that younger patients supposedly have poor outcomes. Higher percentage of young patients at presentation developed metastasis and had a worse survival. Maggard et al reported that young patients have more advanced stage in breast cancer and have more aggressive tumor characteristics and higher grade tumor. Ki-67 is one of the markers for proliferating cells and is over-expressed in many breast cancers. If the reason why TN and young patients have poor prognosis is high proliferation of cancer cells, they might have a high Ki-67 proliferation index. Therefore, we evaluated the relationship using Ki-67 proliferation index, nuclear grade (NG), and histological grade(HG) between poor outcome of TN cases or younger patients and the proliferation potency of breast cancer.

Patients and Methods
94 cases of breast cancer which was operated at
Iwai hospital between June 2009 and May 2011 were classified by subtype according to 2011 St. Gallen consensus meeting, and investigated Ki-67 proliferation index indicated proliferation potency of cancer, NG and HG in each group by immunohistochemical staining to the specimens obtained from breast cancer surgery.

**Immunohistochemistry**

Formalin-fixed and paraffin-embedded tissue sections from breast cancers were derived from the archive of the Department of Pathology, Iwai Hospital. Immunohistochemical stains of ER, PR, HER2 and Ki-67 tissue sections followed the recommended staining protocols.

Immunohistochemical staining for anti-estrogen receptor (ER) rabbit monoclonal antibody (SP1) (NICHIREI BIOSCIENCES INC. Tokyo, Japan), anti-progesterone receptor (PgR) mouse monoclonal antibody (A9621A) (NICHIREI BIOSCIENCES INC. Tokyo, Japan) and anti-Ki-67 antibody (Invitrogen, Camarillo, CA) were performed with standard protocol and Histofine Simple Stain MAX-PO (MULTI) (NICHIREI BIOSCIENCES INC. Tokyo, Japan). Immunoreactions were developed using diaminobenzidine as the chromogen. Immunostain for HER2 was used Histofine HER2 KIT (MONO) and Universal KIT (NICHIREI BIOSCIENCES INC. Tokyo, Japan). Patients in the 2+ range for HER2 were examined by the fluorescence in situ hybridization (FISH) procedure, and were judged whether HER2 is positive or not.

NG (nuclear atypia score + mitotic counts score) was counted according to the General Rules for Clinical and Pathological Recording of Breast Cancer (The 17th Edition)\(^{12}\). HG (tubular formation score + nuclear atypia score + mitotic counts score) was counted by Nottingham classification\(^{13}\).

**Statistics**

Analyses were performed using Tukey-Kramer test.

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<th>Subtype</th>
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<td>Luminal A</td>
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<td>Luminal B</td>
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<td>HER2</td>
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<td>Triple negative (basal-like)</td>
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2011 St. Gallen consensus meeting

**Fig. 1** There was significantly higher proliferation index of Ki-67 in TN group ($p<0.01$).

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All results were considered significant if $p \leq 0.05$.

**Result**

94 cases in breast cancer were classified the subtype according to 2011 St. Gallen consensus meeting. The Luminal A subtype was the most common subtype in breast cancer (48 cases), which was followed by the Luminal B subtype (30 cases), HER2 (8 cases) and TN (8 cases), respectively (Table 1).

The average ($\pm$ standard deviation) proliferation index of Ki-67 in each subtype group is 6.4 $\pm$ 3.7 in Luminal A, 24.6 $\pm$ 11.2 in Luminal B, 20.2 $\pm$ 6.6 in HER2 and 62.6 $\pm$ 15.7 in TN. There was significantly higher proliferation index of Ki-67 in TN group with Tukey-Kramer method ($p<0.01$) (Fig. 1).

NG and HG in invasive ductal carcinoma cases (=82 cases) except for DCIS (ductal carcinoma in situ) are 1.1 $\pm$ 0.4 and 1.3 $\pm$ 0.5 in Luminal A, 1.8 $\pm$ 0.7 and 1.9 $\pm$ 0.6 in Luminal B, 2.5 $\pm$ 0.5 and 2.3 $\pm$ 0.5 in HER2, and 3 $\pm$ 0 and 3 $\pm$ 0 in TN (Fig. 2, 3). There were significantly higher NG and HG in TN group when compared with Luminal A and B ($p<0.01$). TN subtype has high NG, HG and more frequent expression of Ki-67.

Mean Ki-67 in cases (n=15) which were 45 years old and less was 28.56 $\pm$ 25.0, and was significantly higher than that in cases which were more than 46 years old ($p=0.041$) (Fig. 4A).

Mean NG and HG in cases which were 45 years old and less were 1.92 $\pm$ 0.86 and 2 $\pm$ 0.82, respectively, and were significantly higher than those in cases which were more than 46 years old ($p=0.045$ and 0.047, respectively) (Fig. 4B).
**Discussion**

At 2011 St. Gallen consensus meeting, the subtype classification in breast cancer was changed as Luminal A was defined with HER2(-), ER(+) or PgR(+) and less than 14% of Ki-67 proliferation index; HER2 was defined with HER2(+), ER(-), and PgR(-); TN was defined with HER2(-), ER(-), and PgR(-); Luminal B was defined without Luminal A, HER2 and TN. Moreover the treatment is different from each subtype classification. Therefore, Ki-67 proliferation index have to be measured to determine the sub-classification and the treatment.

Ki-67 protein is present during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent from resting cells (G0). It is an ideal biomarker therefore to assess the growth fraction of a given cell population\(^{16}\). Although the functional significance of Ki-67 remains to be fully elucidated, it has valuable prognostic utility in assessing solid tumors and hematological malignancies\(^{15, 16}\).

Ki-67 is a marker for proliferating cells and is over-expressed in many breast cancers\(^{11}\). Low levels of Ki-67 were associated with prolonged relapse free survival\(^{17}\). Railo et al reported that the dis-
ease-free survival in Ki-67 positive patients who have more than 10% of Ki-67 proliferation index was significantly shorter than in Ki-67 negative patients\(^7\). Multiple-analysis showed that Ki-67 proliferation index was an independent prognostic factor\(^17,18\).

Using Ki-67 proliferation index, the reason why TN patient has poor prognosis in breast cancer is suspected to be from the high proliferation of cancer cell because of higher titter of Ki-67, NG and HG as we indicated in our study.

Many reports indicated that TN breast cancer cases had poorer clinical outcome data\(^1-4\). TN was associated with shorter relapse free survival and overall survival, even though it was associated with a higher response rate to neoadjuvant chemotherapy\(^6\). The reason why TN cases have poor outcome is that hormonal therapy and molecular targeted therapy are not effective, and TN cases have few other therapy options for breast cancer\(^9\).

On the other hand, Chacon et al reported that characteristics of TN are high proliferation index, and high NG and HG\(^7\). Their characteristics easily induce a higher rate of early recurrence\(^7,19\), low disease free survival and low overall survival\(^7,20\). Therapeutically, despite being highly chemo-sensitive, their progression free time is generally short\(^7\).

It is also supposed that young patients with breast cancer induce poor outcomes because of high estrogen levels which stimulate breast cancer cell growth in young patients. TN is also more likely to be more prevalent in young women\(^7,12,21\).

The other reason why young, breast cancer patients have poor prognoses is due to the high proliferation of cancer cells apparently caused by higher titter of Ki-67, NG, and HG as indicated in our study. Anders et al reported that if 45 years old and less patients in breast cancer defined as young group, young group had larger tumor and high grade tumor, and trended toward inferior disease survival\(^22\). Therefore, we also defined young group as 45 years old and less, we investigated Ki-67 proliferation index, NG and HG in cases 45 years old and less compared with more than 46 years old.

Characteristics of young breast cancer are; poor prognosis despite of early stage\(^14,16\), more advanced stage, more aggressive tumor, high grade tumor, hormonal receptor(-)\(^16\). The grade of tumor in young breast cancer was higher as patient’s age at on set was lower\(^14\). A multivariate regression showed that young age was an independent risk factor for death\(^8\). Maggard et al reported that young patients had worse 5-year survival when compared with older patient groups\(^8\).

Therefore, if the stage of young patients with TN subtype of breast cancer is not too advanced, we have to treat with the best chemotherapy available, and then, follow up very carefully.

References

Triple negative in breast cancer showed higher Ki-67


