TP53 signature diagnostic system using multiplex reverse transcription–polymerase chain reaction system enables prediction of prognosis of breast cancer patients

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Abstract
Background  TP53 status based on TP53 signature, a gene expression profile to determine the presence or absence of TP53 mutation, is an independent prognostic factor of breast cancer. The purpose of this study was to develop a simple diagnostic system for TP53 signature status.

Methods  We developed a multiplex reverse transcription–polymerase chain reaction system to determine TP53 status. Based on this system, prospectively collected 189 patients with stage I and II breast cancer were determined to have TP53 mutant signature or TP53 wild-type signature. The prognostic significance of the TP53 signature by the diagnostic system was analyzed.

Results  The diagnostic accuracy of TP53 status and reproducibility of this diagnosis system was confirmed. Using the diagnostic system, 89 patients were classified as TP53 mutant signature and the remaining 100 cases were classified as TP53 wild-type signature. Recurrence-free survival (RFS) among patients with TP53 mutant signature was significantly shorter than that among those with TP53 wild-type signature. On univariate and multivariate analyses, the TP53 signature status was an independent predictor of RFS. RFS among patients with TP53 mutant signature was significantly shorter than that among those with TP53 wild-type signature in a cohort of estrogen receptor-positive breast cancer. Although a difference was not significant, no recurrent cases was observed in TP53 wild-type signature group in triple negative breast cancer.

Conclusion  This simple and precise diagnostic system to determine TP53 signature status may help in prognostic assessment, therapeutic decision-making, and treatment optimization in patients with breast cancer.

Keywords  TP53 signature · Breast cancer · Prognostic factor · Diagnostic system

Introduction

The plethora of comprehensive gene expression analyses in the context of breast cancer has gradually helped unravel the molecular biology of breast cancer. In addition, a large number of gene expression profiles that predict prognosis, recurrence, and therapeutic response to anticancer drugs and endocrine therapies has been reported [1]. Representative gene expression profiles, such as Onocotype DX [2–4], Mammaprint [5, 6], and Prosigna [7, 8], have already been approved by US Food and Drug Administration.

Tumor suppressor gene TP53 is the most frequently mutated gene in human cancers, and the patients with TP53...
mutations are known to have poor clinical outcomes [9]. Several large-scale meta-analyses have shown TP53 mutation to be an independent predictor of poor prognosis for breast cancer [10, 11]. Furthermore, TP53 status is a predictive factor for chemotherapy [12, 13].

We had earlier found a gene expression signature (TP53 signature) that correlates with presence or absence of TP53 mutation [14]. The TP53 status determined using the TP53 signature was a prognostic factor independent of other known clinicopathological prognostic factors. Also, the TP53 status determined using gene expression signature was a superior predictor of prognosis compared with that determined using immunohistochemical examination and direct DNA sequencing. Similar results were earlier reported by Miller et al. [15].

The purpose of this study was to develop a simple diagnostic system for TP53 signature using multiplex reverse transcription–polymerase chain reaction (RT–PCR), to test its diagnostic precision and prognostic predictability in a prospective cohort and to examine the clinical significance of TP53 signature among breast cancer subtypes.

Patients and methods

Patients and tumor tissues

This study was approved by the Ethics Committee at the Tohoku University Hospital (TU), Hoshi General Hospital (HG), and Miyagi Cancer Center (MCC). The TU cohort, which was used in our previous study [14], was used for the development of the TP53 signature diagnosis system. Validation cohort is a breast cancer case series from HG and MCC prospectively from September, 2007 to October, 2013 [16]. None of the cases received chemotherapy or endocrine therapy preoperatively. Written informed consent for the study was obtained from all patients. A part of the surgical specimen of breast cancer was stored as fresh frozen (FF) tissue or FFPE tissue by macrodissection technique. Total RNA was extracted from FF tissue or FFPE tissue with use of RNeasy mini kit (Qiagen, Valencia, CA, USA) or RNeasy FFPE kit (Qiagen, Valencia, CA, USA), respectively.

TP53 signature diagnosis system

Genome Lab GeXP Genetic Analysis System (Beckman Coulter, Brea, CA) was used to obtain gene expression profile. To obtain the TP53 signature gene set for GeXP, genes for which the average signal value in the raw data exceeded 1000 in the previous microarray data of the TU cohort [14] and which had less homolog genes were selected. Based on these criteria, 23 genes were chosen among TP53 signature genes. Three genes were added to this gene set as internal control; as a result, a TP53 signature gene set that comprised of 26 genes was established (Supplemental Table 1). Primers for reverse transcription (RT) and for PCR were designed using Genome Lab eXpress Designer GeXP Software (Beckman Coulter, Brea, CA). The multiplex reaction was optimized as per the manual and optimal primer concentrations determined. RT and PCR were performed with Genome Lab GeXP Start Kit (Beckman Coulter, Brea, CA) in accordance to the manual. The quantity of input RNA was 1 μg for FFPE tissues and 50 ng for the FF tissues.

TP53 signature score

TP53 status was determined by TP53 signature score, which is the ratio of the sum of expression levels of 16 genes that were upregulated in tumors with TP53 mutation to the sum of expression values of 7 genes downregulated in tumors with TP53 mutation. The cutoff level for TP53 signature score was determined by Receiver Operating Characteristic curve (ROC) analysis based on the TP53 signature status by microarray of TU cohort [14]. When TP53 signature score of a certain sample was greater than 1.11, the sample was labeled as TP53 mutant signature.

Outcomes

The primary end point of the study was recurrence-free survival (RFS), which was defined as the period from the date of surgery for breast cancer to the date on which tumor
recurrence. Overall survival (OS) was defined as the period from the date of surgery for breast cancer to the date of death. Breast cancer-specific survival (BCSS) was defined as the period from the date of surgery for breast cancer to the date of death by breast cancer.

**Statistical analysis**

All statistical analyses were performed using JMP Pro 14.3.0 (SAS Institute Japan Co., Ltd., Tokyo, Japan). Baseline characteristics of patients (except age) were assessed by chi-squared test. Kruskal–Wallis test was used for statistical analysis of age. Survival curves were made with Kaplan–Meier method, and between-group differences assessed with log-rank test. Univariate and multivariate analyses (Cox proportional hazard model) were conducted to assess the association between clinicopathological factors and the TP53 status for RFS. P value under 0.05 was considered indicative of a statistically significant difference. This study is registered in UMIN-CTR (http://www.umin.ac.jp/ctr/) (000005172).

**Results**

**Patients for analysis**

The TU cohort comprises 40 patients, 34 of whom were included in this analysis. The validation cohort comprised 220 patients who had undergone surgery between October, 2013 and September, 2007. Out of the 220 patients, 31 patients were excluded based on the exclusion criteria (Fig. 1). The remaining 189 patients were included in the analysis. Median duration of observation period was 8.06 years (range 0.91–10.18 years).

**Cutoff value of TP53 signature score**

RNAs extracted from 34 samples of the TU cohort were available for analysis. TP53 signature of these 34 patients was examined with multiplex PCR method. From the result of ROC analysis, the cutoff value for TP53 signature score was set at 1.11 (Area under the curve: AUC = 0.993) (Table 1).

**TP53 signature score for 189 patients in the validation cohort**

TP53 signature scores of 189 cases of the validation cohort were calculated. With use of a cutoff value of 1.11, 89 patients were classified as TP53 mutant signature, and the remaining 100 cases were classified as TP53 wild-type signature. Patient characteristics disaggregated by TP53 status is shown in Table 2 and Fig. 2. A significant difference was observed between the two different TP53 signatures with respect to ER, PgR, HER2, tumor grade, histological type, Ki-67, postoperative adjuvant chemotherapy, and postoperative adjuvant endocrine therapy.

**Recurrence-free survival, overall survival and breast cancer-specific survival by TP53 signature status**

The TP53 mutant signature group showed significantly poorer RFS than that shown by the TP53 wild-type signature group (Fig. 3a). In OS and BCSS, the TP53 mutant signature group showed significantly worse than TP53 wild-type signature group (Fig. 3b, c).

**Univariate and multivariate analyses to identify factors associated with RFS**

On univariate analysis, tumor stage, lymph node and TP53 status by signature were significantly associated with RFS.
(Table 3). On multivariate analysis, only the TP53 status by signature showed a significant association with RFS. Our results indicate that TP53 signature based on multiplex RT-PCR was an independent predictor of RFS.

### RFS by TP53 signature status in subtypes of breast cancer

In ER positive subtype, RFS of TP53 wild-type signature was significantly better than that of TP53 mutant signature ($P = 0.012$) (Fig. 4a). Although a significant difference between TP53 signature status was not shown in ER negative subtype, luminal A like group (ER positive and Ki-67 < 10%) and triple negative breast cancer (TNBC) group, no recurrent cases was observed in TP53 wild-type signature group (Fig. 4b, c, e). On the other hand, a significant difference was observed between TP53 signature status in luminal B like subtype (ER positive and Ki-67 ≥ 10%) (Fig. 4d). A survival analysis in HER2 positive subtype did not be carried out because of lack of patients. In grade 1 and 3, RFS of TP53 mutant signature group was significantly worse than that of wild-type group (Fig. 4f, h). Although the significant difference could not be observed, RFS of TP53 mutant signature group showed worse trend than that of wild-type group in grade 2 (Fig. 4g).

### Discussion

The TP53 mutation has long been known as an independent predictor of poor prognosis among patients with breast cancer [10, 11]. To develop a reliable diagnostic kit, we created the gene expression signature that could diagnose the TP53 gene status using microarray analysis [14]. Uji et al. reported that the TP53 status determined by gene expression signature was a superior predictor of prognosis than TP53 status determined on direct DNA sequencing (including the classical Sanger sequencing and the NGS method) [17]. Today, although the TP53 gene mutation can be analyzed in detail by the cancer genome profiling test, the TP53 signature is considered to have an advantage in terms of prognosis prediction for breast cancer. Lehmann et al. verified the prognostic predictability of 351 reported gene expression profiles on a meta-analysis based on 31 breast cancer cohorts [18]. They found TP53 signature was a robust prognostic factor, and was better than well-known gene expression profiles such as OnctypeDX and Mammaprint. Furthermore, Lehmann et al. verified that TP53 signature was a predictor of therapeutic response in their meta-analysis [18]. Similarly, Oshima et al. reported that signature could predict response to preoperative chemotherapy [19]. As described
## Table 2  Clinicopathological characteristics disaggregated by TP53 status

|                          | Total No. of patients | Mutant signature No. of patients | Wild-type signature No. of patients | P*   |
|--------------------------|----------------------|----------------------------------|------------------------------------|------|
|                          | %                    | %                                | %                                 |      |
| Samples                  | 189                  | 89                               | 47                                 | 53   |
| Age, years (median)      | 29–98 (58.0)         | 29–83 (59.0)                     | 26–98 (56.0)                       | 0.077|
| pStage                   |                      |                                  |                                    | 0.55 |
| I                       | 95                   | 50                               | 41                                 | 54   |
| IIA                     | 65                   | 34                               | 33                                 | 32   |
| IIB                     | 29                   | 15                               | 15                                 | 14   |
| ER                      |                      |                                  |                                    | <0.0001|
| +                       | 138                  | 73                               | 49                                 | 89   |
| −                       | 51                   | 27                               | 40                                 | 11   |
| PgR                     |                      |                                  |                                    | <0.0001|
| +                       | 101                  | 54                               | 34                                 | 67   |
| −                       | 88                   | 46                               | 55                                 | 33   |
| HER2                    |                      |                                  |                                    | 0.025|
| +                       | 18                   | 10                               | 13                                 | 5    |
| −                       | 171                  | 90                               | 76                                 | 95   |
| Pathological tumor size, cm |                      |                                  |                                    | 0.78 |
| ≤ 2                     | 125                  | 66                               | 59                                 | 66   |
| > 2, ≤ 5                | 61                   | 32                               | 28                                 | 33   |
| > 5                     | 3                    | 12                               | 2                                 | 1    |
| pLN                     |                      |                                  |                                    | 0.19 |
| +                       | 57                   | 30                               | 31                                 | 26   |
| −                       | 132                  | 70                               | 58                                 | 74   |
| Grade                   |                      |                                  |                                    | <0.0001|
| 1                       | 46                   | 25                               | 8                                 | 38   |
| 2                       | 82                   | 45                               | 29                                 | 53   |
| 3                       | 55                   | 30                               | 50                                 | 5    |
| NA                      | 6                    | 2                                | 2                                 | 4    |
| Histology               |                      |                                  |                                    | 0.043|
| Invasive ductal carcinoma | 179                 | 95                               | 85                                 | 94   |
| Invasive lobular carcinoma | 3                  | 2                                | 0                                 | 3    |
| Medulary carcinoma       | 3                    | 2                                | 3                                 | 0    |
| Mucinous                | 3                    | 2                                | 0                                 | 3    |
| Undiff. carcinoma        | 1                    | 1                                | 1                                 | 0    |
| Ki-67                   |                      |                                  |                                    | <0.0001|
| < 10                    | 55                   | 32                               | 9                                 | 46   |
| ≥ 10                    | 118                  | 68                               | 37                                 | 45   |
| NA                      | 16                   | 7                                | 9                                 |      |
| Adjuvant chemotherapy    |                      |                                  |                                    | <0.0001|
| +                       | 86                   | 46                               | 54                                 | 32   |
| −                       | 103                  | 54                               | 35                                 | 68   |
| Adjuvant endocrine therapy |                    |                                  |                                    | <0.0001|
| +                       | 137                  | 72                               | 49                                 | 88   |
| −                       | 52                   | 28                               | 40                                 | 12   |

* Chi-square test was used for statistical analysis of patients’ characteristics except for age. Kruskal–Wallis test was used for statistical analysis of patients’ age.

pStage pathological stage, ER estrogen receptor, PgR progesterone receptor, HER2 human epidermal growth factor receptor type 2, pLN pathological lymph node, NA not available.
Fig. 2  *TP53* signature score and clinicopathological characteristics. The upper graph shows the *TP53* signature score of each case in the validation cohort. Red and blue represent *TP53* mutant signature and *TP53* wild-type signature, respectively. The lower figure shows the clinicopathological characteristics of the corresponding cases in the upper graph. Legend of colors is shown in the figure. pStage, pathological stage; pLN, pathological lymph node; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor receptor type 2.

Fig. 3  RFS, OS and BCSS by *TP53* signature status. RFS (a), OS (b), and BCSS (c) by *TP53* status based on the *TP53* signature score in the validation cohort were constructed using the Kaplan–Meier method. The differences were compared using the log-rank test. The short vertical line on the curve represent censored. RFS recurrence-free survival, OS Overall survival, BCSS breast cancer-specific survival.
TP53 signature is confirmed to be both an independent prognostic factor and an independent predictor for response to chemotherapy.

In this report, a simple and easy multiplex RT–PCR diagnostic system for TP53 signature was developed and the rate of agreement of TP53 status by TP53 signature score and the TP53 status by microarray was enough high (97.1%) (Table 1).

In the validation cohort, a significant difference was observed between the two TP53 signatures with respect to ER, PgR, HER2, histological grade, Ki-67 histological type, adjuvant chemotherapy and adjuvant endocrine therapy (Table 2). These results do not contradict those reported from previous studies [14, 15, 20, 21].

The TP53 mutant signature based on the TP53 signature score was associated with significantly poor RFS, OS and BCSS as compared to that associated with the TP53 wild-type signature. On univariate and multivariate analysis, TP53 signature was significantly associated with PFS independent of other clinicopathological factors. These results indicate that the TP53 status diagnosed by this diagnostic system was an independent prognostic factor in patients with breast cancer for whom curative resection (stage I–II) is performed.

In this report, we showed for the first time that there was clinical significance among breast cancer subtypes and grades. In the ER positive, especially in Luminal B like subgroup, Grade1 and 3 subgroup, it was clearly seen that the prognosis was closely associated with the TP53 status. In ER negative group, Luminal A like subtype and TNBC, the significant difference was not observed between TP53 signature status. But, because there was no recurrence in TP53 wild-type signature group, it can be said that TP53 signature had clinical significance in these subtypes.

There are some limitations of the interpretation of this study. First, the sample size was relatively small, and the recurrence events were few so far. We are going to follow up recurrent events sequentially. Second, uniform treatment intervention was not carried out for the study cohort because it is an observational, prospective study. We are currently conducting a large scare retrospective-prospective study to confirm the clinical significance of TP53 signature using several prospective studies conducted in Japan.

In conclusion, we developed a relatively simple multiplex RT–PCR diagnostic system to determine the TP53 signature. Its diagnostic accuracy and prognostic value were verified in a prospective cohort. And we showed the clinical significance of TP53 signature among breast cancer subtypes. This simple and precise diagnostic system may help in prognostic assessment, therapeutic decision-making, and treatment optimization in patients with breast cancer.

**Table 3** Results of uni- and multivariate analysis (Cox proportional hazard model) showing correlation of RFS with clinicopathological factors in patients with breast cancer

| Variable | Univariate | Multivariate |
|----------|------------|--------------|
|          | HR 95% CI  | P            | HR 95% CI  | P            |
| pStage (vs. Stage I) | 2.60 1.01–6.69 0.05 | 1.72 0.48–6.10 0.40 |
| pLN (vs. negative)   | 2.55 1.08–6.01 0.03 | 1.65 0.52–5.17 0.39 |
| Pathological tumor size (vs. T1) | 1.27 0.53–3.07 0.59 | |
| Grade (vs. 1–2)      | 1.14 0.46–2.82 0.78 | |
| ER (vs. positive)    | 1.33 0.54–3.30 0.54 | |
| PR (vs. positive)    | 1.54 0.65–3.67 0.32 | |
| HER2 (vs. negative)  | 0.46 0.06–3.39 0.44 | |
| Ki-67 (vs. <10%)     | 4.15 0.95–18.1 0.06 | |
| Adjuvant chemotherapy (vs. non-therapy) | 1.47 0.62–3.51 0.38 | |
| Adjuvant endocrinetherapy (vs. non-therapy) | 0.97 0.38–2.50 0.95 | |
| TP53 status by signature (vs. wild-type) | 3.96 1.45–10.8 0.01 | 3.73 1.36–10.20 0.01 |

* pStage pathological stage, pLN pathological lymph node, ER estrogen receptor, PgR progesterone receptor, HER2 human epidermal growth factor receptor type 2, HR hazard ratio, CI confidence interval.
Fig. 4 RFS by TP53 signature status in subtypes of breast cancer and in grade. RFS stratified by TP53 status based on the TP53 signature score in patients with ER+ (a), ER− (b), Luminal A like (c), Luminal B Like (d), TNBC (e), Grade 1 (f), Grade 2 (g) and Grade 3 (h) were constructed using the Kaplan–Meier method. The differences were compared using the log-rank test. The short vertical line on the curve represent censored. ER estrogen receptor, TNBC triple negative breast cancer.
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Author contributions Conceptualization: ST, CI; Methodology: ST, CI; Formal analysis and investigation: ST, TF and FF; Writing—original draft preparation: ST; Writing—review and editing: CI, TF, TN, YK, FF, TI, NO; Funding acquisition: ST, CI; Resources: TN, YK, TI, NO.

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Declarations

Conflict of interest Dr. Takahashi reports personal fees from Taiho, Chugai, Asahikasei, Bayer, Japan blood products organization, Medi-con, Terumo, SanoFi, Nippon-kayaku, Takeda, Yakult, grants and personal fees from Merckbiopharma, grants from Ono, outside the submitted work; In addition, Dr. Takahashi has a patent JP4370409B2 issued. Dr. Ishida reports grants from Taiho, Eisai and Kyowa-Kirin, grants and personal fees from Chugai, personal fees from Pfizer and Astra Zeneca, outside the submitted work. Prof. Ishioka reports grants and personal fees from Novartis, Daiichi Sankyo, Bayer, Bristol-Myers Squibb, Nippon-Kayaku, Hitachi, personal fees from Taiho, Ono, Merckbiopharma, AsahiKasei, SanoFi, Takeda, Eisai, Eli Lilly, Mundipharma, Teijin, Chugai, Konica Minolta, Pfizer, Mochida, grants from Riken Genesis, MSD, Linical, outside the submitted work; In addition, Prof. Ishioka has a patent JP4370409B2 issued. All the remaining authors have no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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