Cancer Letters xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Significance of *TP53* mutations determined by next-generation "deep" sequencing in prognosis of estrogen receptor-positive breast cancer

Kumiko Uji, Yasuto Naoi, Naofumi Kagara, Masafumi Shimoda, Atsushi Shimomura, Naomi Maruyama, Kenzo Shimazu, Seung Jin Kim, Shinzaburo Noguchi *

Department of Breast and Endocrine Surgery, Osaka University Graduate School of Medicine, Osaka, Japan

ARTICLE INFO

Article history:
Received 31 May 2013
Received in revised form 31 July 2013
Accepted 15 August 2013
Available online xxxx

Keywords:
Breast cancer
TP53 mutation
Next-generation deep sequencing

ABSTRACT

Next-generation "deep" sequencing (NGS) was used for the mutational analysis of *TP53*, and a DNA microarray was used for the determination of the *TP53* mutation-associated gene expression signature (*TP53* GES) in 115 estrogen receptor (ER)-positive breast cancers. NGS detected 27 *TP53* mutations, of which 20 were also detected by Sanger sequencing (SS) and seven were detected only by NGS. A significantly higher number of mutant alleles (33.9%) was detected in the tumors with *TP53* mutations detected by SS compared with those with *TP53* mutations detected only by NGS (11.1%). The *TP53* mutations detected by NGS were more significantly associated with a large tumor size, a high histological grade, progesterone receptor-negativity, and HER2-positivity compared with those detected by SS. The *TP53* mutations detected by SS, but not those detected by NGS or the p53 immunohistochemistry, exhibited a significant association with poor prognosis. In addition, the *TP53* GES more clearly differentiated low- from high-risk patients for relapse than the *TP53* mutations detected by SS, regardless of the other conventional prognostic factors. Thus, NGS is more sensitive for the detection of *TP53* mutations, but the prognostic significance of these mutations could not be demonstrated. In contrast, the *TP53* GES proved to be a powerful prognostic indicator for ER-positive tumors.

1. Introduction

The tumor suppressor gene *TP53* is a transcription factor that controls the expression of a variety of genes that are implicated in the regulation of the cell cycle, apoptosis, and genomic integrity [1,2]. In addition, this gene has been shown to play an important role in the pathogenesis of various cancers, including breast cancer [3]. It has been reported that 15–71% of breast tumors harbor a *TP53* mutation and that those tumors with a *TP53* mutation have a more aggressive phenotype, such as estrogen receptor (ER)-negativity and high histological grade, compared with those without a *TP53* mutation [4,5]. The correlation between the *TP53* status and prognosis has been studied by many investigators. Most of these studies indicate that a *TP53* mutation is associated with poor prognosis [6], although contradictory results have also been reported, especially for patients treated with chemotherapy [7,8] most likely because the chemo-sensitivity of breast tumors is affected by the presence of a *TP53* mutation [9–11]. In contrast, rather consistent results have been reported for the association between the presence of a *TP53* mutation and poor prognosis in patients treated with hormonal therapy [12,13].

Breast cancer tissue is composed of a variety of constituents, including not only tumor cells but also stromal fibroblasts and inflammatory cells, and the proportion of tumor cells in tumor tissue varies widely from 20% to 95% [14]. The *TP53* status is usually assessed through the Sanger sequencing (SS) method using DNA samples extracted from the whole tumor tissue. Because the detection sensitivity of SS is approximately 20% [15], it is speculated that a significant proportion of *TP53* mutations could be missed due to a low proportion of tumor cells in the tumor tissue.

The advent of next-generation sequencing (NGS) technology has resulted in high-throughput "deep" sequencing with higher sensitivity than that achieved with the conventional SS method. In fact, it has been reported that the use of NGS resulted in the detection of more *EGFR* mutations in lung cancers than those obtained with SS [16,17]. It is therefore tempting to use NGS for the study of *TP53* mutations in breast tumors because a more accurate assessment of *TP53* mutations might result in a further elucidation of the clinicopathological characteristics of breast tumors with a mutation in the *TP53* gene. However, such a study has yet to be reported. Thus, the first aim of the present study was to use NGS for

* Corresponding author. Address: Department of Breast and Endocrine Surgery, Osaka University Graduate School of Medicine, 2-2-E10 Yamadaoka, Suita-shi, Osaka 565-0871, Japan. Tel.: +81 6 6879 3772; fax: +81 6 6879 3779.
E-mail address: noguchi@onsurg.med.osaka-u.ac.jp (S. Noguchi).

0304-3835/$ - see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.canlet.2013.08.028

an analysis of *TP53* mutations to determine whether additional mutations can be identified with NGS and to gain a better understanding of the clinicopathological characteristics of breast tumors with *TP53* mutations.

The second objective was to evaluate the prognostic significance of the *TP53* gene expression signature (GES) that was reported by Miller et al. [18] and Takahashi et al. [19]. The *TP53* GES was developed to distinguish *TP53*-wild-type and -mutant breast cancers using the signature of the genes that are differentially expressed between *TP53*-wild-type and -mutant breast cancers. These researchers found that the prognostic capability of the *TP53* GES is superior to that of the *TP53* mutation status. However, it is speculated that this superiority stems from, at least in part, the fact that the *TP53* mutations are mostly analyzed through SS, which is suspected of overlooking a significant proportion of mutations in tumor tissues with a low tumor cell content. It is thus interesting to compare the prognostic significance of the *TP53* GES with that of *TP53* mutations determined by NGS, which has been shown to be more sensitive than SS. Therefore, these two issues were investigated in the present study using ER-positive breast tumors treated with adjuvant hormonal therapy for two reasons. First, the prognostic significance of the *TP53* mutations detected by SS has been quite consistently demonstrated without the confounding effects of adjuvant chemotherapy. The second reason is that prognostic classification is clinically very important in the decision-making process pertaining to the indication of adjuvant chemotherapy for patients with ER-positive breast tumors.

2. Materials and methods

2.1. Patients and tumor samples

The study cohort consisted of 115 female patients with ER-positive invasive breast cancer who underwent mastectomy or breast-conserving surgery and subsequent radiation therapy and were treated with adjuvant hormonal therapy alone at our hospital between 1998 and 2008. The median follow-up for these patients was 117 months with a range of 21–173 months. Of these 115 patients, 73 were treated postoperatively with tamoxifen (20 mg/day) or toremifene (40 mg/day), 31 were treated with goserelin (3.6 mg/4 weeks) plus tamoxifen (20 mg/day), one was treated with goserelin (3.6 mg/4 weeks) alone, and 10 were treated with an aromatase inhibitor (1 mg/day anastrozole or 25 mg/day exemestane). Tamoxifen, toremifene, and the aromatase inhibitors were administered for approximately 5 years or until recurrence if it occurred earlier, whereas goserelin was administered for approximately 2 years. A total of 32 patients developed recurrences (25 distant and 7 locoregional recurrences). The tumor tissues obtained at surgery were snap frozen in liquid nitrogen and maintained at −80 °C until use for DNA and RNA extraction, and the surgical specimens were fixed in 10% buffered formaldehyde for histological analysis. Informed consent for the study was obtained from each patient before surgery.

2.2. DNA extraction and Sanger sequencing (SS)

DNA was extracted from the tumor tissues using the DNeasy Blood and Tissue Kit® (QIAGEN, Germantown, MD, USA) according to the manufacturer's instructions. The SS analysis of the entire coding region of *TP53* (exon 2–11) was conducted using the Applied Biosystems 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA), as previously described [20]. The Variant Reporter® Software (version 1.0; Applied Biosystems) was used for the *TP53* mutation search.

2.3. Next-generation sequencing (NGS)

The GS Junior system (Roche Diagnostics, Basel, Switzerland) was used for the NGS following the modified protocol described below. (1) *Amplicon preparation*: the purified PCR products used for SS were also used for NGS. The amplicons of each exon derived from an individual's DNA sample were mixed in a length-weighted equal-volume ratio. Then, 500 ng of each mixture was concentrated with Ethachinmate (Nippon Gene, Tokyo, Japan) and eluted in 16 μl of TE buffer. (2) *Amplicon sequencing*: the sequence library was prepared in accordance with the Rapid Library Preparation Method Manual (revised June 2011) with a slight modification [21]. Based on the individual sample concentration, up to 12 libraries were pooled in equimolar amounts and processed according to the emPCR Amplification Method Manual (Lib-L, rev. June 2011). Deep sequencing was performed according to the Sequencing Method Manual (rev. June 2011). The average number of total high-quality reads per run was 108,383, and the average number of coverage reads was 3108 per amplicon. (3) *Analysis of deep sequencing*: the processed and quality-filtered reads were analyzed using the GS Amplicon Variant Analyzer (AVA) software (version 2.7; Roche Diagnostics). The *TP53* reference sequences were extracted from GenBank (Accession number: U94788). The percentage of mutant alleles was calculated by dividing the number of mutant reads by the number of total reads using the AVA software. The percentage of mutant alleles should be more than 1% because the sensitivity of NGS, as reported by Moskalev et al. [17], is 0.5–1%. Those samples with homopolymer stretches and an unequal percentage of variant alleles between forward and reverse alignment were excluded.

2.4. RNA extraction and gene expression profiling

RNA was extracted from the tumor tissues obtained at surgery using the Qiagen RNeasy® mini kit (Qiagen). The extracted RNA (1 μg; RIN value >6) was used for the generation of second-strand cDNA, and cRNA was amplified with the Oligo dT primer, biotinylated, fragmented with the One-Cycle Target Labeling and control reagents (Affymetrix, Santa Clara, CA, USA), and then hybridized overnight (17 h) to a U133 Plus 2.0 array according to the manufacturer's protocol. The hybridized DNA microarray was fluorescence stained with GeneChip® Fluidics Station 450 and scanned with the GeneChip® Scanner 3000 (both from Affymetrix).

2.5. Immunohistochemical (IHC) examination

The expression of ER, the progesterone receptor (PR), Ki67, and p53 in tumor tissues was immunohistochemically examined using a previously described method [22,23]. ER, PR, and p53 were defined as positive if at least 10% of the tumor cells were stained. Ki67 was defined as positive if at least 20% of the tumor cells were stained. The human epidermal growth factor receptor 2 (HER2) was identified immunohistochemically (anti-human c-erbB-2 polyclonal antibody; Nichirei Biosciences, Tokyo, Japan) or through fluorescence *in situ* hybridization (FISH) using PathVysion HER2 DNA probe kits (SRL Inc., Tokyo, Japan) for IHC +2 tumors. A tumor that exhibited +3 immunohistostaining or for which the FISH ratio was $\geqslant$2.0 was considered HER2 positive. The histological grade was determined according to the Scarff–Bloom–Richardson grading system [24].

2.6. Microarray data processing

The RNA extracted from breast tumors was subjected to *TP53* GES analysis using a DNA microarray (Human Genome U133 plus 2.0 Array; Affymetrix) according to a previously described method [19,20], and the breast tumors were classified as *TP53* GES-mutant and *TP53* GES-wild-type tumors. The intrinsic subtypes (Luminal A, Luminal B, HER2-enriched, basal-like, and normal-like) were classified using the PAM50 method reported by Parker et al. [25]. For the PAM50 analysis, 26 ER-negative tumors were added to the 115 ER-positive tumors because the PAM50 method requires a heterogeneous dataset that contains all subtypes.

2.7. Statistics

The association between the various clinicopathological parameters and the *TP53* mutation status determined by SS or NGS were evaluated using the chi-square test or Fisher's exact test. The differences in the frequency of *TP53* mutant alleles between the *TP53* mutations detected by SS and those detected by NGS were assessed with the Mann–Whitney test. The recurrence-free survival rates were calculated with the Kaplan–Meier method and evaluated by the log-rank test (distant recurrences and locoregional recurrences, except ipsilateral in-breast recurrences, were considered recurrence events). The univariate and multivariate analysis of various parameters for the prediction of recurrences was conducted using the Cox proportional hazards model. Regardless of the statistical test performed, differences with $P < 0.05$ were considered statistically significant.

3. Results

3.1. Comparison of SS and NGS for the detection of TP53 mutations

The same DNA samples extracted from 115 breast tumors were subjected to SS and NGS for the detection of *TP53* mutations (Table 1). SS detected 20 *TP53* mutations (SS-*TP53* mutations), and NGS detected 27 *TP53* mutations (NGS-*TP53* mutations). Of the 27 NGS-*TP53* mutations, 20 were also detected by SS, and seven were not. The frequency of mutant alleles was significantly ($P < 0.001$) higher in breast tumors with SS-*TP53* mutations ($n = 20$, average = 33.9%, range: 11.2–63.8%) compared with those with *TP53* mutations detected only by NGS ($n = 7$, average = 11.1%, range: 1.6–24.1%). All of the missense mutations detected by NGS alone

Please cite this article in press as: K. Uji et al., Significance of *TP53* mutations determined by next-generation "deep" sequencing in prognosis of estrogen receptor-positive breast cancer, Cancer Lett. (2013), http://dx.doi.org/10.1016/j.canlet.2013.08.028