# Etiological Observations in 10 Female Breast Cancer Patients, 5 with and 5 without TP53 Mutations in Cell-Free Serum DNA Obtained 30 Years Ago

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**Abstract.**  *Background/Aim:  TP53 is the most frequently altered gene in breast cancer. This is the first report correlating TP53 mutations in cell-free serum DNA with long-term clinical data of women with breast cancer. Patients and Methods:  Sera were obtained from 10 female breast cancer patients between October 1987 and March 1990 in a medical oncology practice in Basel, Switzerland.  Somatic variants of TP53 were identified in the DNA with state of the art high-depth molecularly tagged sequencing and bioinformatic methods.  Results:  TP53 positive patients tend to be older at the time of diagnosis and tend to live longer than TP53 negative patients. TP53 positive patients may also have more evidence for cancer genetic mechanisms. Conclusion: TP53 mutations in cell-free serum DNA may become of prognostic and etiological relevance.*

TP53 mutations are among the earliest events of tumor development across cancer types (1). Early detection may therefore become possible before cancers reach their full malignant potential, e.g. by testing circulating tumor DNA (ctDNA). This approach also has potential for evaluating response and resistance in cancer treatments (2).

TP53 is the most frequently altered gene in breast cancers (3,4). It is mutated in nearly 30% of all cases affecting every molecular subtype of breast cancer (5). TP53 mutations are associated with negative, neutral or positive outcomes (4). DNA sequencing is regarded as the gold standard for identifying TP53 mutations (4).

Cell-free serum is a source for detecting cancer-specific DNA markers (6). Free DNA has been reported in the serum of breast cancer patients for the first time in 1977 (7). The purpose of this article is to present oncogenetic data of 10 female breast cancer patients whose cell-free serum DNA was investigated with modern technology.

**Patients and Methods**

Sera were obtained from 10 female breast cancer patients between October 1987 and March 1990 in a medical oncology practice (Walter Weber) affiliated to the University of Basel, Switzerland. After consenting 10 cc native blood have been drawn into a 10 cc empty vacutainer tube which was then immediately centrifuged for 10 minutes in a large Hettich centrifuge at 5'000 rounds per minute. Within an hour after venipuncture the sera were stored at minus 70 to 80 degrees in 3 cryotubes per patient (2cc, NUNC, Gibko AG Basel, Switzerland). Approval for the use of these samples and correlating data has been granted by the responsible ethics committee (approval number: eknz-2018-00252).

*cfDNA extraction*

Circulating DNA was extracted from 2-4mL of isolated serum with the QIAamp Circulating Nucleic Acid Kit (Qiagen) as previously described (6). DNA was quantified using the Qubit Fluorometer (Invitrogen) and analyzed using the 2200 TapeStation system (Agilent Technologies) with the High Sensitivity DNA Analysis Kit.

*Targeted sequencing and library preparation.* Circulating DNA of the 10 samples was sequenced with the Oncomine Breast cfDNA Assay (A31183, Thermo Fisher Scientific). This panel covers 152 hotspot mutations in 10 genes (*AKT1, EGFR, ERBB2, ERBB3, ESR1, FBXW7, KRAS, PIK3CA, SF3B1*, and *TP53*) across 26 amplicons. This integrates the TagSeq technology (molecular barcode) and allows to detect rare variants present at 0.1% allelic frequency. Library preparation, molecular barcoding, and sequencing were performed according to the instructions and guidelines provided by Thermo Fisher, using 5ng of DNA as input. Briefly, the library preparation protocol was based on a two-step cycle multiplex touch-down polymerase chain reaction (PCR) with a temperature ranging from 64°C to 58°C, which allowed to amplify target regions and to introduce unique molecular identifiers. The obtained tagged amplicons of around 100-140 bp length were then cleaned up using Agencourt AMPure XP (Beckman Coulter), then eluted in 24 µl low TE buffer. A second round of PCR (18 cycles) was performed in a total volume of 50µl to amplify the purified amplicons and to introduce Ion Torrent Tag-Sequencing adapters containing sample-specific barcodes. The resulting library of target DNA fragments was purified by performing a two-step cleanup using Agencourt AMPure XP (Beckman Coulter). The purified libraries were then diluted 1:1000 and quantified by qPCR using the Ion Universal Quantification Kit (Thermo Fisher Scientific). The quantified stock libraries were then diluted to 100pM for downstream template preparation. Subsequentially, sequencing runs were planned on the Torrent Suite Software v5.2, libraries were pooled and loaded on an Ion 540 chip using the Ion Chef Instrument (Thermo Fisher Scientific). The loaded chip was then sequenced using 500 flows on an S5 system (Thermo Fisher Scientific).

*Somatic Variants Identification.* Raw data were processed automatically on the Torrent Server and aligned to the reference hg19 genome. The analysis pipeline included signal processing, base calling, quality score assignment, adapter trimming, PCR duplicate removal, and control of mapping quality. All samples passed the quality check and met the requirements of a minimum average depth of 904X. The round of the sequencing data of the 10 samples was uploaded in BAM format to the Ion Reporter Analysis Server for variant calling and annotation. Variant calling was performed on Ion Reporter (IR) Analysis Software v5.2 using the Oncomine

TagSeq Breast Liquid Biopsy w2.0 workflow. Coverage metrics for each amplicon were obtained by running the Coverage Analysis Plugin software v5.2.1 (Thermo Fisher Scientific). Identified variants were only considered if the variant had a molecular coverage of at least three, indicating that the variant was detected in three independent template molecules. Finally, all candidate mutations were manually reviewed using the Integrative Genomics Viewer37.

*Etiological information* has been obtained from patients with the help of a German translation of the NCI Medical History Questionnaire for Cancer Etiology (8) and through the study of patients' medical charts.

## Results

TP53 is the most studied gene of all time (9). It is a tumor suppressor and is mutated in roughly half of all human cancers. TP53 mutations are thought to inhibit cellular senescense, a critical barrier to carcinogenesis (10).

TP53 mutations were found in sera from 5 of 10 female breast cancer patients (Table I). The median age of diagnosis of TP53 positive patients was 56 years (range: 37 to 67 years). This is higher than in TP53 negative patients: 41 years (range: 37 to 60 years). The median survival of TP53 positive patients was 15 years (range: 5+ to 26 years). This is longer than in TP53 negative patients: 5 years (range: 2 to 19 years). Of special etiological interest is a TP53 positive patient belonging to a BRCA 1 family with following malignancies: breast cancer, ovarian cancer, malignant lymphoma and multiple myeloma (Sample No.051 in Table II).

Table I. *Sequencing with Oncomine TM Breast cfDNA Assay*

| Sample No. | Mapped Reads | On Target (%) | Mean Coverage | KRAS | TP53 | PIK3CA |
|---|---|---|---|---|---|---|
| 012 | 822,290 | 95.84 | 32,400 | p.G12D (0.1%) | | |
| 017 | 1,513,454 | 96.98 | 60,944 | | p.R248W (0.1%) | p.H1047R (2.84%) |
| 026 | 1,430,359 | 95.98 | 56,798 | | | |
| 028 | 1,727,543 | 97.76 | 73,289 | | p.R158L (1.58%) | |
| 031 | 1,719,712 | 96.01 | 69,907 | p.G12C (0.1%) | | |
| 033 | 978,141 | 96.31 | 39,142 | | p.R248W (0.1%) | |
| 035 | 1,084,084 | 95.86 | 43,583 | | | |
| 039 | 135,999 | 97.44 | 5,227 | | p.V173M (0.46%) | |
| 041 | 1,626,548 | 96.08 | 66,433 | p.G154V (0.06%) | | |
| 051 | 2,656,056 | 96.21 | 108,393 | | p.H179R (0.14%) | |

Table II. *10 female breast cancer patients, 5 with and 5 without TP 53 mutations in cell-free serum DNA.*

| Sample No. | TP53 Status | Breast Cancer Histology,Side,Others | Age at Diagnosis (y) | Survival (y) | Findings of Etiological Interest |
|---|---|---|---|---|---|
| 017 | positive | Invasive ductal,left, postmenopausal | 67 | 15 | Son died of dementia at age 59, nv |
| 028 | positive | Invasive ductal,left, premenopausal | 45 | 12 | Sister had lymphocytic NHL in the right breast, v |
| 033 | positive | Invasive ductal,left, postmenopausal | 56 | 5+ | |
| 039 | positive | Mucinous, right,ER+ PR+,postmenopausal | 57 | 16+ | |
| 051 | positive | Invasive ductal,left, triple negative, premenopausal | 37 | 26 | BRCA 1 family ( germline BRCA 1 frameshift mutation 187 del AG ): Relatives with malignancies of breast and ovary; with malignant lymphoma and multiple myeloma, v |
| 012 | negative | Adenocarcinoma,left, postmenopausal | 60 | 5 | Died of ovarian carcinoma at age 65, v |
| 026 | negative | Invasive ductal,ER+ PR+,bilateral, postmenopausal | 37 | 2 | Aunt fs: Breast cancer at age 50, unilateral, nv; Uncle fs: Died of lung cancer at age 40, nv |
| 031 | negative | Invasive ductal, right, ER+PR+,premenopausal | 39 | 3+ | |
| 035 | negative | Invasive ductal,right, premenopausal | 41 | 8 | Aunt ms: Breast cancer at age 67, nv |
| 041 | negative | Invasive ductal,left, ER+PR+,premenopausal | 46 | 19 | |

ER+, estrogen receptor positive; fs, father's side;  NHL, non-Hodgkin lymphoma; ms, mother's side; nv, not verified; PR+, progesterone receptor positive;  v, verified by autopsy/cytology/histology report; y, years;

**Discussion**

This is the first report correlating TP53 mutations in cell-free serum DNA with long-term clinical data of women with breast cancer. The data are only descriptive because of the small sample size.

There were no more tumor samples available. It was impossible to investigate if variants identified were also present in the tumors of the patients.

Comparing 5 patients with TP53 mutations with 5 patients without such mutations it appears that mutation carriers are older and survive longer. In one patient (017) two mutations were found (11). Another mutation carrier (051) belonged to a BRCA1 family. 3 of the women with TP53 mutations were over 55 years of age at the diagnosis of breast cancer and there is a possibility that the variants are a result of clonal expansion of a premalignant leukemia clone (12). The actual coverage has been annotated along with the allelic frequency observed in brackets after the TP53 mutations (Column 6 in Table I).

The detection of tumour mutations using targeted mutation methods can be confounded by natural aging – related clonal haematopoiesis variants that could be misinterpreted as cancer. To avoid these potential false positives and achieve high specificity, mutation-based approaches will require parallel sequencing of white blood cells to filter out these non-cancer variants (13).

Larger studies will have to define the prognostic impact of TP53 mutations in cell free serum DNA of breast cancer patients.

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**Authors' Contributions**

Conception and design of the study, provision of study materials and patients, data analysis and interpretation, as well as final approval of the manuscript were done by Walter Weber and Walter P. Weber. All three authors (W.W., S.S.W. and W.P.W.) contributed to the financial support, collection and assembly of data, and writing of the manuscript.

**Conflicts of Interest**

The authors declare no conflicts of interest