Plasma DNA integrity indicates response to neoadjuvant chemotherapy in patients with locally confined breast cancer

Julia Lehner¹, Oliver J. Stötzer², Debora M.I. Fersching¹, Dorothea Nagel¹ and Stefan Holdenrieder¹,³

¹Institute of Clinical Chemistry, University Hospital Munich-Grosshadern, ²Hematology/Oncology Outpatient Center Munich, Munich and ³Institute of Clinical Chemistry and Clinical Pharmacology, University Hospital Bonn, Bonn, Germany

Introduction

Since the establishment of the mammography screening program, breast cancer is often diagnosed at a very early stage [1]. The conventional tumor markers CEA and CA 15-3 are usually slightly elevated in early stage breast cancer, but they are not accurate enough for diagnosis support nor for the exact response monitoring during neoadjuvant chemotherapy [2]. New biomarkers for this setting are therefore urgently needed.

The absolute level of cell-free DNA (cfDNA) is known to be a promising new biomarker for cancer diagnosis [3] although it is also elevated in various other diseases where there is a high turnover tissue such as sepsis, stroke and autoimmune diseases [4]. When measuring cell-free DNA, one can use several DNA fragments in serum or plasma. Two such DNA fragments are ALU 115 and ALU 247, with 115 and 247 base pair lengths respectively. These fragments are known to be distributed at several different points in the genome [3]. A new approach for using these DNA fragments as a biomarker is based on the theory that cancer cells often undergo necrotic cell death, whereas apoptotic cell death occurs more frequently in normal tissue. Umetani et al. [5, 6] and Wang et al. [7] used this theory to calculate two different DNA integrity indices. These indices calculate the ratio of a longer DNA fragment, e.g. ALU 247, to a shorter DNA fragment, e.g. ALU 115. As necrosis is an unorganized cell death, DNA fragments that are longer than 180 bp are supposed to occur more often in plasma than under apoptotic conditions [8]. Consequently, the calculated DNA integrity ratio should be higher in cancer patients than in healthy individuals. We measured both ALU 115 and ALU 247 in breast cancer patients during neoadjuvant chemotherapy and calculated both DNA integrity indices in order to investigate their relevance as predictive markers in a clinical setting.

Patients and methods

Between 2007 and 2011, plasma samples of 49 breast cancer patients undergoing neoadjuvant chemotherapy were collected. All patients had locally confined breast cancer (46 patients with invasive ductal carcinoma and 3 patients with invasive lobular carcinoma) and underwent pretherapeutic staging by mammography, mamma-sonography, chest X-ray, abdomen sonography and bone szintigraphy. This pretherapeutic staging was also used to determine the tumor size at therapy start as well as the TNM stage (75% of all patients with T1/2, 25% with T3/4, 80% with N1-3, 20% N0). Neoadjuvant chemotherapy consisted of four cycles of epirubicine (90 mg/msq) and cyclophosphamide (600 mg/msq), followed by 4 cycles of docetaxel (75 mg/msq) or paclitaxel (175 mg/msq). Herceptin was added to docetaxel in Her2/neu overexpressing cases. Surgical tumor resection was performed after 6 – 8 chemotherapy cycles and outcome of the neoadjuvant chemotherapy was determined by histopathology according to RECIST criteria: No change (NC, means less than 30% tumor regression, 14 patients), partial remission (PR, 30 to 99% tumor regression, 25 patients) and complete remission (CR, 10 patients).
During the chemotherapy cycles, plasma samples were taken before the start of chemotherapy cycle two (C2) and before cycle 5 or 6 (C6). In order to create a comparable study design, the plasma sample C6 was taken approximately at Day 60 to 70 after therapy start.

**Plasma preparation and qPCR**

For the plasma preparation for qPCR 4.4 ml of plasma samples were collected in an EDTA collection tube (Sarstedt, Germany), centrifuged, separated, aliquoted and cryopreserved at –80°C. DNA isolation was done with a DNA isolation kit (QiAamp DNA Mini Kit; Qiagen, Duesseldorf, Germany). Lysis of the plasma was followed by two washing steps and an elution step. 5 µl of this eluate were used as a template for the qPCR. For the qPCR of the ALU repeats ALU 115 and ALU 247, the same primers as described in Umetani et al. 2006 [6] were used. The reaction mixture for the qPCR contained 5 µl of template, 0.25 µl of Uracil DNA Glycosylase (UNG, Roche Diagnostics, Mannheim, Germany), 2 µl of each primer (forward and reverse), 6.75 µl of PCR grade H2O and 4 µl of Mastermix SYBR Green (Roche), resulting in 20 µl of reaction volume. The Real-Time PCR amplification was performed with the LightCycler® 480 Instrument II by Roche Diagnostics. Ten minutes of incubation time were followed by 10 minutes UNG inactivation time at 95 °C. 45 PCR cycles were performed with denaturation at 95 °C for 10 seconds, annealing at 62 °C for 15 seconds and extension at 72 °C for 15 seconds, respectively. A standard curve was performed for each DNA fragment with serial dilutions of a provided DNA (efficiency for ALU 115 1.95, ALU 247 1.84). All measurements were done in duplicates and controls as well as plasma pools were included into every plate.

**Calculation of the DNA-integrity index**

Two different algorithms were used for calculation of the DNA Integrity Index: DNA Int 1 according to Umetani et al. [5, 6] and DNA Int 2 according to Wang et al. [7]. For the DNA Int 1, the ratio of the concentrations of ALU 247 to ALU 115 sequences was calculated. As the ALU 115 sequences are represented within the annealing sites of ALU 247 [6], DNA Int 1 can theoretically vary between 0 and 1. A high index would indicate a considerable contribution of non-apoptotic cell death such as necrosis.

For DNA Int 2, the difference between the Cp value of a standard pool of human genomic DNA (measured with every PCR plate) and the Cp value of each sample for Alu 115 and Alu 247 to obtain ΔCp 115 and ΔCp 247 was calculated. Those two ΔCp values where subtracted (ΔCp115 – ΔCp247) to obtain ΔΔCp. The formula for DNA Int 2 was calculated as: DNA Int 2 = e^{(–ΔΔCp × ln(2))}.

**Results**

According to histopathologic staging after surgery, 14 patients had no change of disease (NC), 25 partial remission (PR) and 10 complete remission (CR).

Concerning the comparison of patients with no change (NC) with remissive patients (PR+CR), neither the values before Cycle 2 nor the data before Cycle 6 showed any statistically significant data. At Cycle 2, the remissive patients showed the following median values (ALU 115: 21 ng/ml, ALU 247: 25 ng/ml, DNA Int 1: 1.3, DNA Int 2: 0.3) compared to the NC patients (ALU 115: 29 ng/ml, ALU 247: 31 ng/ml, DNA Int 1: 1.2, DNA Int 2: 0.4). For C6, the PR/CR group showed slightly higher levels in ALUs compared to C2 (ALU 115: 27 ng/ml, ALU 247: 34 ng/ml, DNA Int 1: 1.0, DNA Int 2: 0.3) whereas lower levels were measured for the NC group (ALU 115: 21 ng/ml, ALU 247: 27 ng/ml, DNA Int 1: 1.1, DNA Int 2: 0.3).

Concerning the comparison of patients with complete remission (CR) with no change or partial remission (NC+PR), DNA Int 2 values measured before cycle 2 showed a borderline significant difference between the response groups (medians CR: 0.6; NC+PR: 0.4; p = 0.05) while for both ALU values and DNA Int 1 no difference was detected (DNA Int 1: CR 1.3, NC+PR 1.3). At C6, ~ 60 – 70 days after the start of the neoadjuvant chemotherapy, a differentiation between the CR group and the PR+NC group
was achieved by DNA Int 2 (medians CR: 0.7; NC+PR: 0.3; p = 0.004) as well as by DNA Int 1 (medians CR: 1.3; NC+PR: 1.0; p = 0.05) but not for the ALU values (ALU 115: CR 12 ng/ml, NC+PR 27 ng/ml, ALU 247: CR 16 ng/ml, NC+PR 35 ng/ml).

**Discussion**

Neoadjuvant chemotherapy is applied in parts of patients suffering from locally confined breast cancer in order to reduce the tumor load before surgical resection. While in many patients a reduction of tumor volume is observed, some patients do not respond sufficiently to this treatment. Therefore it would be desirable to identify those non-responding patients in order to early adapt or change the therapy regimen. However, the established tumor markers CEA and CA 15-3 were found not to be able to accurately indicate the response to neoadjuvant chemotherapy [2]. In the present study, we focused on a new biomarker, the DNA integrity in plasma of breast cancer patients. Earlier reports described a role of DNA integrity in the diagnosis of cancer patients as a higher ratio of longer to shorter circulating DNA fragments was found to be present in cancer patients [5, 6, 7]. Those longer fragments (> 180 bp) are supposed to result from the relatively unorganized cell death mode necrosis while shorter fragments are assumed to originate from apoptotic cell death [8].

Because DNA integrity was calculated by two different methods in the past [5, 6, 7], we compared both algorithms in a sample of breast cancer patients questioning whether they correlate with the response to neoadjuvant chemotherapy. Irrespective of the formula, DNA integrity was not able to distinguish between patients with remission and those without remission, neither at Cycle 2 nor at Cycle 6 of the treatment. This might be due to the fact that the remission group is quite heterogenic as the partial remission group contains patients with a very good and with moderate response to therapy (30 – 99% tumor reduction). Nevertheless this differentiation would have been valuable to offer patients with insufficient therapy response additional treatment options in time. However, DNA integrity indicated complete response (CR) to therapy using DNA Int 2 already before Cycle 2.

This data is clinically useful, as it allows an early estimation of very good response to chemotherapy. In further trials, it should be checked whether these differences are present already during the first week after treatment as they were found for circulating nucleosomes in other settings when the tumor destruction gains its highest levels [4]. In addition, DNA integrity discriminated response groups before Cycle 6 by use of both formulas. As Cycle 6 is applied ~ 60 days after therapy start, the objective response to chemotherapy is already more visible. Most of the patients that finally achieved a complete remission during neoadjuvant chemotherapy had no tumor residuals in sonography at that time. Interestingly, the values of both DNA Integrity Indices were higher in the CR group, potentially mirroring a higher tumor destruction level in the complete remission group.

**Conclusion**

DNA Integrity Indices have shown to be useful to indicate a complete response to neoadjuvant chemotherapy in patients suffering from locally confined breast cancer at Cycle 2 and ~ 60 – 70 days after therapy start. Further investigations will show their clinical usefulness in comparison with other relevant biomarkers.

**Acknowledgments**

This work is part of Julia Lehner’s doctoral thesis.

**References**


