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Irradiated Breast Cancer Patients Demonstrate Subgroup-specific Regularities in Protein Expression Patterns of Circulating Leukocytes

KRISTINA YEGHIAZARYAN[1], SOULAFA MAMLOUK[1], DANIELA TROG[1], HEIKE MOENKEMANN[1], MICHAEL BRAUN[2], WALTER KUHN[2], HANS H. SCHILD[1,3] and OLGA GOLUBNITSCHAJA[1,3]

Departments of [1]Radiology and [2]Gynecology, and [3]Center of Excellence for Breast Cancer Research, Friedrich Wilhelms University of Bonn, D-53105 Germany

Abstract. *Background: Breast cancer is one of the most frequent types of cancer with fatal outcome worldwide. The use of breast conserving lumpectomy followed by radiation therapy is common and has been shown to be a strategy competitive to mastectomy in preventing mortality caused by breast cancer. However, breast irradiation, particularly applied after pre-irradiation chemotherapy, frequently leads to serious short- and long-term side-effects, the prediction of which is highly desirable in terms of individual therapy planning. For these purposes, minimal-invasive molecular blood analysis is considered as a powerful diagnostic tool: molecular interplay in blood is highly informative and may predict individual side-effects of therapy. Materials and Methods: Ex vivo comparative protein expression profiling was performed in circulating leukocytes isolated from fresh blood samples of seven breast cancer patients before lumpectomy and consequently at several checkpoints under radiation treatment (0-60 Gy). Protein expression patterns were investigated by two-dimensional polyacrylamide gel electrophoresis followed by protein spot identification using matrix assisted laser desorption/ ionisation – time of flight. Specific expression levels of highly affected differentially expressed proteins were quantified by Western blotting. Results: The radiation treatment caused individual extensive alterations in expression patterns of leukocytes in the patients tested. In particular, a key regulator of redox status, thioredoxin, and the free-radical detoxification cascade members, SOD-2 and catalase, were highly affected. In spite of the high diversity of individual expression levels, characteristic protein expression patterns were recognized and patients were grouped according to the similarities found. Conclusion: Characteristic expression patterns in circulating leukocytes might provide novel molecular targets for prediction of therapy side-effects and improve individual therapy planning for breast cancer patients, thus avoiding unnecessary and excessive treatment-related toxicity. Molecular candidates and specific patterns are demonstrated in this work.*

Correspondence to: Professor Dr. Olga Golubnitschaja, Department of Radiology, Division of Molecular/Experimental Radiology, University of Bonn, Sigmund-Freud-Str. 25, D-53105 Bonn, Germany. Fax: +49-228 287 15983, e-mail: Olga.Golubnitschaja@ukb.uni-bonn.de

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Breast cancer is one of the most frequent tumour disease particularly affecting the female sub-population with an average incidence rate of 10-12 per 100 women. Early metastatic activity of the tumour hinders the full recovery of breast cancer patients. The most common approach currently applied in the treatment of this patient cohort is breast conserving surgery followed by radiation therapy. Besides some annoying short-term effects such as blood flow disturbances, painful erythema and irritation in skin (1), breast irradiation causes severe medium- and long-term side-effects such as leukopenia, anemia, breast edema, fibrosis, and increased risk for angiosarcoma, leukaemia and myelodysplastic syndromes (2-7). With regard to leukopenia and anemia, grade 1 toxicity has been demonstrated in 26% and 21% of the cases, respectively, and most of the patients who experienced grade 1 or grade 2 of toxicity had received pre-irradiation chemotherapy (8). Radiotherapy for left-sided breast cancer is also considered as an independent risk factor in the long-term development of ischemic heart disease (4, 9, 10).

The side-effects dramatically decrease the overall quality of irradiated breast cancer patients' lines (11). Therefore, there is an acute need for the identification of individual susceptibility to radiation reactions, in order to avoid unnecessary and excessive treatment-related toxicity as well as to substantially improve patient-specific radiotherapy

planning in terms of doses applied. Several cellular- and molecular-based approaches have been undertaken in order to develop some reliable tools with predictive power. Some are focused on minimally invasive blood analysis as a valuable source of information about individual genetic characteristics, stress factors and actual molecular interplay. Thus, an important prognostic role of leukocyte activity in breast cancer patients is longstanding. Fluctuating considerably among both node- negative and -positive patients, this activity has been demonstrated to be significantly lower in patients with a greater tumour burden (12). Although no prognostic power has been demonstrated by *in vitro* Comet assay quantification of individual DNA-damage and -repair capacity in phytohemagglutinin-stimulated lymphocytes of breast cancer patients, the authors stressed the necessity for more comprehensive analysis which would consider late effects of radiotherapy, stress-response and repair kinetics in terms of diagnostic and prognostic purposes (13).

Further blood analysis demonstrated a dramatically reduced GSH:GSSH (reduced *versus* oxidized form of glutathione) ratio in a dose-response fashion after fraction radiotherapy as applied in animal models as well as breast and lung cancer patients (14). Although the general tendency was clear, high fluctuations in individual oxidative stress responses have been found. The authors speculated about individually scaled organ-dependent (*e.g.* from the liver) release of GSSH in the blood resulting in generally dysregulated production of harmful free-radicals, which is very well documented in the literature (15).

Our current study was designed making use of detection advantages provided by clinical proteomics in favor of potential diagnostic tools. The approach was based on *ex vivo* monitoring of the individual molecular interplay in peripheral leukocytes isolated from fresh blood samples collected step-by-step at consequent checkpoints from breast cancer patients who underwent diagnosis then breast-conserving surgery followed by fractionated radiotherapy (2 Gy each fraction), with a final application of 60 Gy. Individual stress responses were monitored by comparative protein profiling. Potential molecular targets with prognostic power are discussed.

Materials and Methods

Recruitment of patients and check-points for blood sample collection. From the current pool of breast cancer patients treated at the Centre of Excellence for Brest Cancer Research, Friedrich Wilhelms University of Bonn, seven patients were recruited for the study at diagnosis of the disease, and would undergo complete treatment starting with breast conserving surgery till the end of the radiation therapy at the same centre. Patient data are given in Table I. No preferences for any demographic parameters were made on recruitment. All investigations conformed to the principles outlined in the Declaration of Helsinki and were performed with permission from the responsible Ethics Committee of the Medical Faculty, University of Bonn.

Table I. *Patient data and acquisition blood samples for Western blot analysis*

		Check-point (date)			
Patient	Age	A	B	E	F
1	42	11.04.06	14.09.06	05.10.06	27.10.06
2	52	15.05.06	12.10.06	02.11.06	no data
3	58	25.07.06	23.08.06	12.09.06	04.10.06
4	42	25.07.06	04.10.06	18.10.06	07.11.06
5	64	15.08.06	11.10.06	no data	27.11.06
6	53	24.10.06	18.12.06	10.01.07	30.01.07
7	48	23.11.06	03.01.07	22.01.07	09.02.07

A: at initial diagnosis, B: after lumpectomy and before radiation therapy , E: middle of radiation therapy, after 30 Gy were applied, F: end of last radiation session after 60 Gy were applied.

The blood samples were collected at the following check-points: i) At the first investigation, before biopsy, ii) after performance of lumpectomy, prior to radiation therapy, iii) after the first irradiation with 2 Gy, iv) after the application of 14 Gy (7th irradiation), v) after the application of 30 Gy (15th irradiation), vi) at the end of the radiation therapy (60 Gy, 30th irradiation). Each blood sample underwent an immediate isolation of circulating leukocytes.

Isolation of leukocytes. Blood samples (20 ml) anti-coagulated with lithium-heparin were collected from recruited patients. Leukocytes were separated using Ficoll-Histopaque gradients (Histopaque 1077, Sigma-Aldrich, USA) as described elsewhere (16), frozen as dry pellets and processed for complete molecular biological analysis.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Altogether six 2D-PAGE-images were performed for expression patterns in circulating leukocytes of breast cancer patient numbered 6 as shown in Table I under at all checkpoints used as given above. A 200 μg aliquot of each protein sample was used for 2D-PAGE analysis, which was performed twice for each sample. First-dimensional separation was performed in immobilised pH gradient (IPG) strips (Bio-Rad, USA) in the range of IP 4-7 as described by the supplier. Protein samples of 125 μl containing re-hydration buffer (8 M urea, 10 mM DTT, 1% CHAPS, 0.25% Bio-Lyte, pH 4-7) were loaded onto the IPG-strips and subjected to 14 kVh overnight at 20˚C in a PROTEAN IEF Cell (Bio-Rad, USA). Following first-dimensional separation, the extruded IPG-strips were equilibrated in gel equilibration buffer I (50 mM Tris-HCl, 6 M urea, 30% glycerol, 2% SDS, 1% DTT), followed by equilibration in buffer II (50 mM Tris-HCl, 6 M urea, 30% glycerol, 2% SDS and 260 mM iodacetamide) for 10 min before loading them onto polyacrylamide gels (12% SDS-PAGE) for the second-dimensional resolution in Mini-PROTEAN 3 (Bio-Rad). After electrophoresis, the separated proteins were visualised using silver staining (Silver Stain Plus™; Bio-Rad). Differential gene expression was analysed using specific software (Bio-Rad).

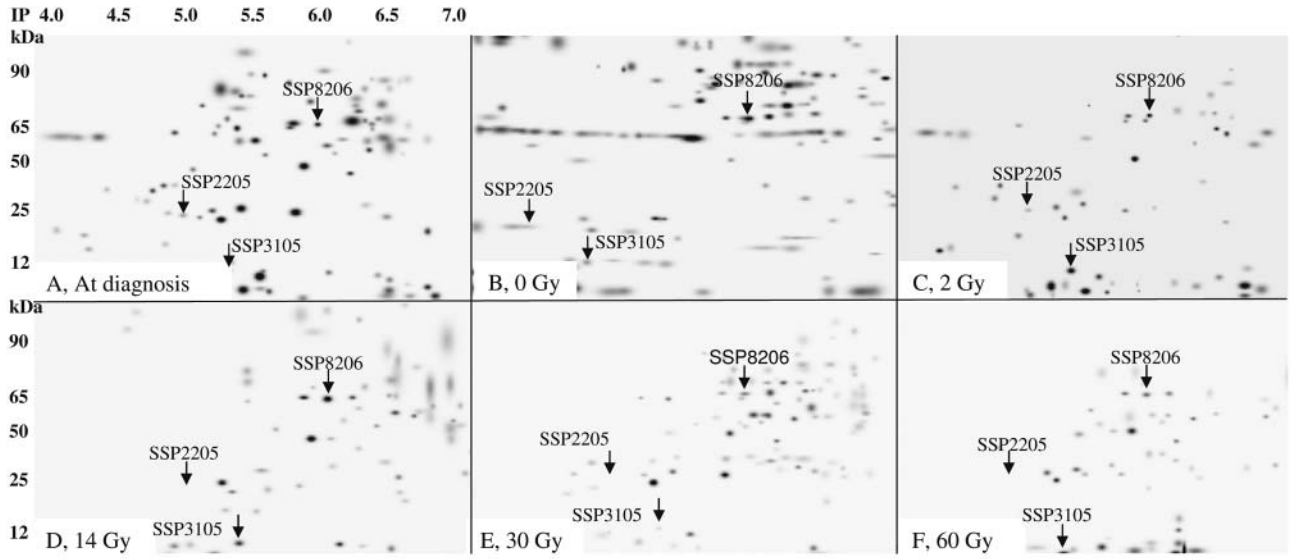

Figure 1. *2D-PAGE images of differential gene expression patterns in circulating leukocytes of a breast cancer patient (53 years old, patient 6 in Table I) at the following checkpoints: A, at initial diagnosis (A diagnosing); B, before radiotherapy, after a lumpectomy (0 Gy), during radiotherapy application after: C, 2 Gy; D, 14 Gy; E, 30 Gy, and after F, radiotherapy (60 Gy). For these checkpoints, the respective treatment conditions A-F are described in Materials and methods. The spots SSP3105, SSP2205 and SSP8206 were identified as thioredoxin, SOD-2 and catalase, respectively.*

Matrix assisted laser desorption/ionisation – time of flight (MALDI-TOF). Selected spots were cut out from gels. Sample preparation was performed as described elswhere (17). Proteins were in-gel digested by incubating the samples with porcine trypsin (Promega, USA) at 37˚C overnight. Peptide mixtures were then purified by ZipTip C18 according to the manufacturer's instructions (Millipore, USA). Elution was performed using 50% ACN/water solution saturated with CHCA. A total of 1 μl of each sample was spotted onto sample anchor and allowed to dry at room temperature. After drying, 0.7 μl of re-crystallization solution (ethanol:acetone:1% TFA in the ratio 60:30:10) was added. Peptide mass fingerprint (PMF) were performed using a MALDI-TOF mass spectrometer (Bruker Daltonics, USA) operated in positive ion reflector mode with an acceleration voltage of 25 kV. For internal calibration the trypsin autolysis peptides were applied. Mass spectra were analyzed automatically using Bruker software. The following search parameters were set: a) monoisotopic masses, b) mass tolerance of 50 ppm, c) one missing cleavage per peptide, and d) possible oxidation of methionine-residues. No restrictions on M_r or pI were made. A minimum of four matching peptides covering at least 15% of the overall sequence was required for protein identification. The sequence similarity search was carried out using BLASTP software.

Western blot analysis. The acquisition of blood samples is given in Table I. All analyses were performed twice for each sample as described previously. Primary anti-body incubation was performed at room temperature using a 1:200 dilution of the specific goat polyclonal IgG anti-bodies: thioredoxin (*sc-18215*; Santa Cruz, USA), superoxide dismutase-2 (*sc-18503*; Santa Cruz), catalase (*sc-34283*; Santa Cruz), and actin (*sc-1616*; Santa Cruz). The protein-specific signals were measured densitometrically using the Quantity One® imaging system (Bio-Rad). The expression rates of the target genes were normalized through the corresponding expression rates of actin (the house-keeping gene).

Results

Differential protein expression analysis shows a significant shift in protein expression patterns of circulating leukocytes under all steps of breast cancer treatment. Differential protein expression patterns are demonstrated in Figure 1. Altogether, six 2D-PAGE images were evaluated during the treatment of the breast cancer patients as shown in Table I. At all five checkpoints, significant alterations in expression patterns were demonstrated, when compared to the baseline image of expression before any treatment was applied ("At diagnosing" in Figure 1) as well as when expression patterns of single time points were compared to each other. No one conservative protein spot with constant expression level in all six images was registered.

Highly affected differentially expressed protein spots were identified as redox status regulator and free-radical detoxification cascade members. Among differentially expressed protein spots, SSP2205, SSP3105 and SSP8206 were chosen as highly affected and identified by MALDI-TOF as mitochondrial superoxide dismutase (SOD-2), thioredoxin, and catalase with 69%, 46% and 35% overall sequence coverage, respectively (Table II).

Table II. *MALDI-TOF analysis of the selected differentially expressed protein spots. 2D-PAGE-images are given in Figure 1. Expression levels at the baseline checkpoint A (initial diagnosing) are taken as reference, i.e. = 1.*

SSP number	Ratio of expression						NCBI/Acc. No	Protein identified	Peptide number measured/ matched	Sequence coverage (%)	Potential function
	A	B	C	D	E	F					
2205	1	1	0.5	0	0	0	P04179	Human superoxide dismutase, mitochondrial precursor	31/21	69	Mitochondrial manganese superoxide dismutase
3105	1	1	3	3	1.5	3	CAA38410	Human thioredoxin	11/8	46	Multifunctional redox control
8205	1	1	1	1	1	2	CAA27721	Human catalase	47/18	35	Peroxidase

Western blot quantification of the target protein expression levels is shown in Figure 2 which demonstrates a high diversity of expression patterns among patients. However, some similarities among individual patterns were recognized. The patients were grouped according to those similarities as shown in Figure 2. Patients 4, 5, and 7 demonstrated no SOD-2 expression at either the initial checkpoint (A) or at the end of the therapy (F) (see subgroup II, Figure 2a). There are two opposite therapy effects recorded in subgroups I and III: whereas in subgroup I the expression of SOD-2 was fully suppressed by the therapy, in subgroup III this expression was strongly induced as shown at the last checkpoint (F).

A very important result was achieved considering simultaneously the expression patterns of both catalase (Figure 2b) and thioredoxin (Figure 2c): namely, with the exception of patient 7, the identical grouping of patients was reached – patients 1, 5 and 6 (subgroup I), patients 2, 3 and 4 (subgroup II). In the case of catalase, the therapy resulted either in high expression induction (subgroup I) or had no visible effect on final expression rates (subgroup II). Noteworthy, the initial expression of catalase (checkpoint A) was similarly low in subgroup II, whereas subgroup I demonstrated extremely varied values (note the corresponding standard deviation in Figure 2b). Similarly, highly similar values for catalase at the final checkpoint were found in subgroup II.

A high diversity in thioredoxin expression levels, either very high or very low with the difference up to 25-fold, and even higher at both initial and end checkpoints (A and F, respectively) were found in the patients tested. The therapy resulted in substantial induction of thioredoxin activity in subgroup I, whereas subgroup II demonstrated no expression differences between checkpoints A and F.

Discussion

An acute need in identification of individual susceptibility to radiation reactions is currently being discussed by the broad scientific forum focused on breast cancer treatment, in order to avoid unnecessary and excessive treatment-related toxicity as well as to substantially improve patient-specific radiotherapy planning in terms of doses applied. Although currently not available, minimally invasive blood analysis is considered as a potential tool with high predictive power (18) and can contribute to both improved early diagnosis and optimized patient-specific therapy treatment. This work deals with the latter issue and was focused on *ex vivo* individual profiling of differential gene expression, identification of selected highly affected proteins, and recognition of possible similarities in individual expression patterns in circulating leukocytes isolated from breast cancer patients who underwent diagnosis and breast-conserving surgery followed by fractionated radiotherapy by application of a total of 60 Gy.

Protein expression profiling demonstrated a significant shift in individual protein expression patterns of all recruited patients under the treatment conditions tested in the study. Moreover, not a single conservative protein spot was found to have a constant expression under the treatment conditions used. Further high diversity of expression patterns within the patient group was demonstrated. This makes a diagnostic application of blood proteome seem an extremely complicated task and highlights the importance of recognizing similarities in expression in such cases.

Among the highly affected differentially expressed proteins, the key regulator of redox status, thioredoxin, was identified. Thioredoxin is a member of the minimal stress proteome of cellular organisms, *i.e.* it belongs to the

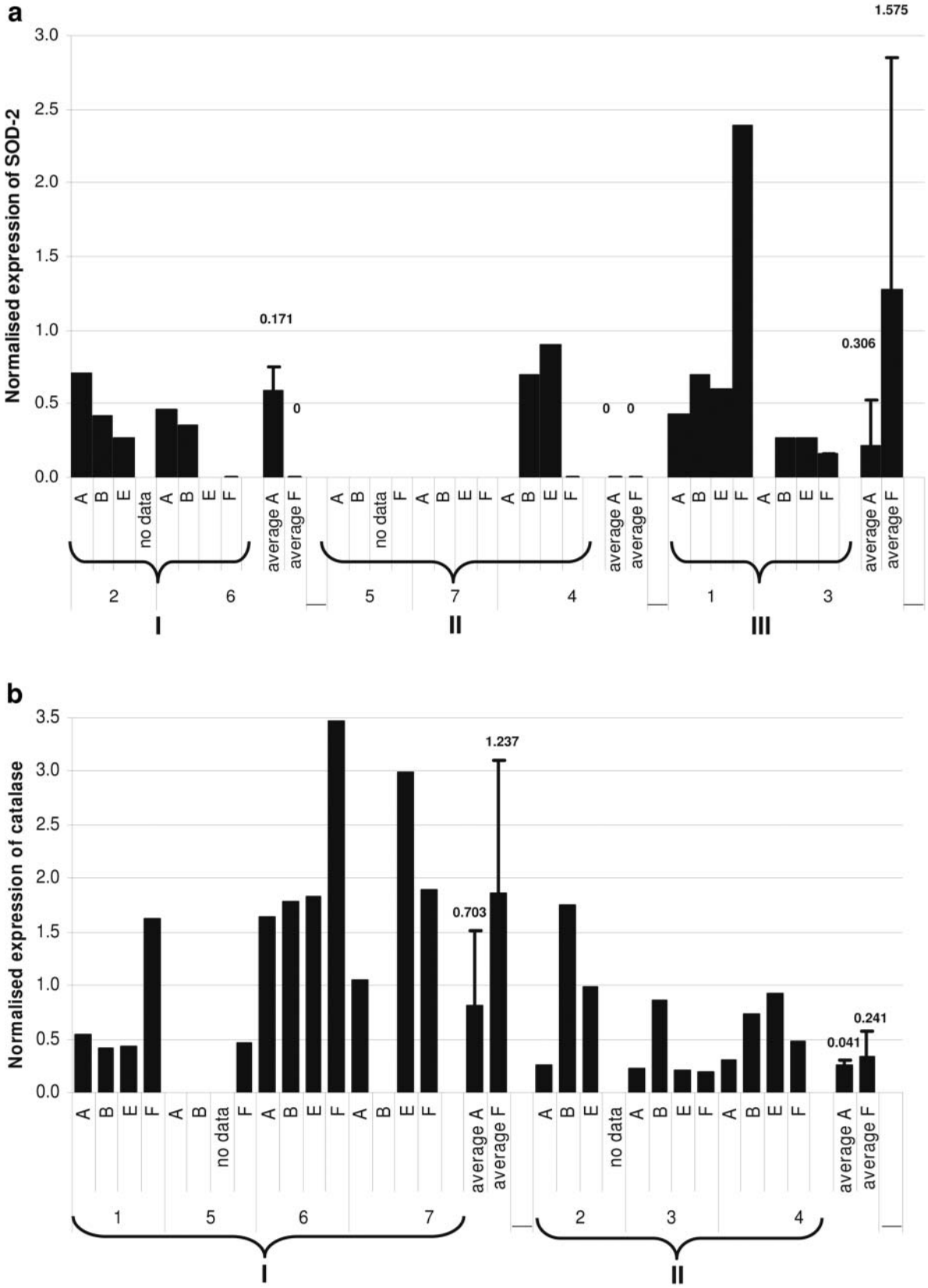

Figure 2. *continued*

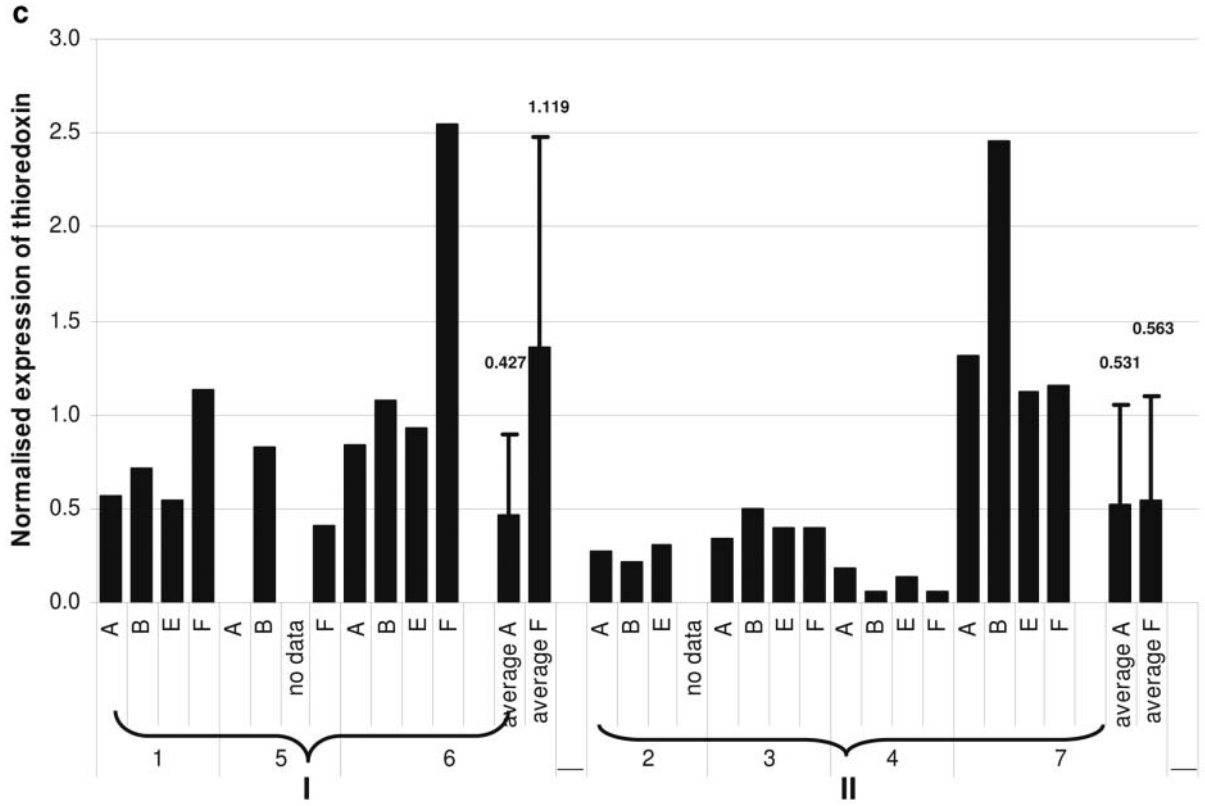

Figure 2. *Expression levels in circulating leukocytes of seven breast cancer patients, as determined by Western blotting. Expression of a) SOD-2, b) catalase and c) thioredoxin at A, initial diagnosis; B, before radiotherapy; E, after 30 Gy radiotherapy; and F, after 60 Gy radiotherapy. Patients were grouped according to their similarity of protein expression, with average standard deviation for each group shown.*

essential pool of subset molecules, the interplay of which form a basis for mechanisms of adaptation to stressful, toxic and extreme environments (19). The thioredoxin system is the key regulator of intra-cellular redox status, and in this way it directs many central cellular events such as protein disulfide reduction, protein- and DNA-repair, protein-folding, balance between apoptosis and proliferation, transcription regulation, and protection against oxidative stress (20). Redox activity of thioredoxin further strongly modulates cellular resistance towards aging and even global longevity of Man as, reviewed by Golubnitschaja (21). Thioredoxin has been shown to be an attractive molecular target for anti-tumour therapy and reliable inhibitors of thioredoxin are currently under intensive consideration (22). An imbalanced redox status has been demonstrated in patients with breast cancer (23).

Two further proteins which were identified among those highly affected and differentially expressed are the members of the free-radical detoxification cascade, SOD-2 and catalase. Like thioredoxin, SOD-2 (MnSOD) is also a member of the minimal stress proteome of cellular organisms (19). MnSOD is an essential mitochondrial free-radical scavenger, the function of which is to balance the production of free-radicals such as reactive oxygen species (ROS), in the electron transport chain in order to avoid harmful side-effects such as oxidative damage to DNA by endogenous ROS. Increased production of endogenous ROS and consequent oxidative damage in mitochondria has been associated with the etiology of various pathological states such as accelerated aging, and neurodegenerative and cancer diseases, as reviewed by Alexeyev *et al.* (24). It is very well documented that MnSOD is poorly expressed in cancer cells. The epigenetic origin of silencing MnSOD expression has been reported (25). In our study, 4 patients out of 7 demonstrated a lack of MnSOD expression as early as the initial checkpoint (A), and 5 patients demonstrated a considerable decrease or even complete gene silencing during and/or after therapy application. If confirmed by large-scale studies, this finding might be important for some prognostic evaluations in early/preventive breast cancer diagnosis and individual therapy design. Catalase is the downstream enzyme in the free-radical detoxification cascade which has high substrate

specificity to hydrogen peroxide – the product of SOD detoxification activity. Blood analysis demonstrated a simultaneous imbalance in redox status and depletion of both antioxidants – SOD and catalase - in breast cancer patients compared to negative controls (23).

Our investigations demonstrated a high diversity in levels of the target proteins in untreated patients as well as large differences in individual expression patterns under the treatment conditions used. In spite of the small size of the patient group and the high diversity in individual expression levels, some subgroup-specific patterns can be clearly recognized: i) In patients with initially low or even no target expression compared to those with a relatively high one, the levels differed by 5-to 10-fold or more; ii) patients who demonstrated no change in target protein expression levels at initial and end checkpoints (A and F, respectively) *versus* those who demonstrated dramatic expressional alterations after therapy. Very important observation was made considering expression patterns of catalase *versus* those of thioredoxin: sub-groups selected for catalase were almost identical with those selected according to thioredoxin patterns. This fact supports the assumption about the functional connection between redox imbalance and antioxidant dysfunction in breast cancer pathology. Therefore, the combination of thioredoxin and catalase blood analysis at the initial checkpoint might provide useful information for preventive molecular diagnosis of breast cancer and better therapy planning for individual patients.

Currently we can only speculate about the meaning of differences recognized between the subgroups selected according to their expression patterns. Thus, a dramatic increase in thioredoxin levels under therapy might support the invasive potential of metastases, which can better survive due to the high induction of thioredoxin in the whole organism. In contrast, reduced levels of both SOD-2 and catalase lead to increased production of harmful free-radicals, causing damage to DNA and increasing the risk of secondary cancer and other diseases. Further large-scale studies are necessary, in order to estimate the long-term effects and potential prognostic power of the expressional patterns demonstrated in this work.

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