Circulating 20S Proteasome in Patients with Non-metastasized Breast Cancer

OLIVER HOFFMANN[1], MARTIN HEUBNER[1], TIMUR ANLASIK[2], MICHAEL WINTERHALTER[2], BURKHARDT DAHLMANN[4], SABINE KASIMIR-BAUER[1], RAINER KIMMIG[1], JEREMIAS WOHLSCHLAEGER[3] and STEPHAN URS SIXT[2]

[1]*Clinic of Obstetrics and Gynaecology, and* [3]*Clinic of Pathology and Neuropathology, Medical Faculty, University of Duisburg-Essen, Germany;*
[2]*Clinic of Anaesthesiology, Medical Faculty, Heinrich Heine University, Duesseldorf, Germany;*
[4]*Institute of Biochemistry/CCM, Charité University Berlin, Berlin, Germany*

Abstract. *Background: Recent data suggest a role of the ubiquitin–proteasome system in various malignancies. In patients with neoplasms, increased extracellular concentrations of circulating 20S proteasome (c-proteasome) have been detected in blood plasma. We tested the hypothesis that the plasma c-proteasome concentration is a biomarker associated with tumor stage and nodal status in patients with the primary diagnosis of non-metastatic breast cancer. Patients and Methods: Venous plasma concentration of 20S proteasome was measured by ELISA technique in 224 non-metastatic breast cancer patients and in 50 healthy volunteers. To assess the relation of proteasome expression to c-proteasome concentration, tumor specimens from 32 patients were immunohistochemically stained for 20S proteasome using an antibody directed against the core subunits of the catalytic domain of the 20S proteasome. Results: The median c-proteasome concentration was higher ($p<0.0001$) in breast cancer patients (397.5 ng/ml, range: 200-50,000 ng/ml) than in healthy controls (305 ng/ml, range: 140-425 ng/ml). There was no significant correlation between c-proteasome concentration and strength of proteasomal staining in tumor specimens. Neither tumor size, nor nodal status, nor any other prognostically important clinical parameter, including the presence of disseminated tumor cells in the bone marrow, correlated with high c-proteasome concentrations. Conclusion: Circulating proteasome concentrations appear to be higher in patients presenting with primary breast cancer than in healthy controls. Thus, the ubiquitin-proteasome system might represent a potential target in breast cancer treatment.*

Correspondence to: Dr. med. Martin Heubner, Clinic of Obstetrics and Gynecology, University of Duisburg-Essen, Hufelandstr. 55, D-45147 Essen, Germany. Tel: +49 2017232441, Fax: +49 2017235962, e-mail: Martin.heubner@uk-essen.de

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Breast cancer is the most frequent malignancy of women and remains a therapeutic challenge. Prognostic and predictive parameters have become of high importance in recent years and impact directly on therapeutic decisions regarding individualized tumor therapy (1). Besides tumor size and nodal status, established parameters are histopathological grading, the hormone receptor status, expression of the Her2neu growth factor receptor, urokinase plasminogen activator and plasminogen activator inhibitor 1. The presence of disseminated tumor cells (DTCs) in the bone marrow (BM), which are observed in up to 40% of breast cancer patients (2-4), is also increasingly being regarded as a clinically relevant prognostic factor for breast cancer (5). However, new prognostic and predictive parameters are warranted for breast cancer patients in order to further optimize and individualize their therapy.

The ubiquitin–proteasome system (UPS) is involved in intracellular protein degradation. Regarding malignancies, the UPS has become increasingly interesting, as it is involved in many regulatory cellular processes, such as gene transcription, apoptosis and signal transduction (6, 7). As these processes are highly relevant to tumor progression and carcinogenesis, impairment of UPS function has been the object of oncologic therapeutic targets (8-12). The 20S proteasome is central to the intracellular UPS. Interestingly, circulating 20S proteasome (c-proteasome) is physiologically present in human blood (13, 14) and increased concentrations have been found in numerous pathological conditions, such as liver and autoimmune disease, trauma, sepsis, acute respiratory distress syndrome, and in patients after abdominal surgery (13, 15-17). There is a growing body of evidence that such c-proteasomes may even have physiological functions (14). With regard to

oncologic disease, higher plasma proteasome concentrations are found in cancer patients compared with healthy controls and might correlate with disease progression in some tumor entities (13, 18-21). Interestingly, there appear to be links between proteasome activity and the expression of estrogen receptor (ER) alpha. The proteasome system is involved in the degradation of ER alpha, which is an important antitumor target of antiestrogen therapies in breast cancer patients (22). Considering these data, we hypothesized that c-proteasome concentrations might be increased in breast cancer patients, which would potentially render the proteasome system a therapeutic target for such patients. Furthermore, we evaluated whether c-proteasome concentrations correlate with established prognostic or predictive markers.

Patients and Methods

Patient characteristics. A total of 224 patients with primary non-metastatic breast cancer who were treated at the Department of Obstetrics and Gynecology at the University Hospital in Essen between 2005 and 2008 were enrolled in this study following Ethics Committee approval and informed consent. The mean age of the patients was 59 years (range: 30-84 years). The control cohort consisted of 50 age-matched healthy volunteer blood donors. Diagnosis was confirmed by histopathology in all cases. The majority of our patients (151/224) had a primary tumor of <2 cm in diameter (stage pT1). A total of 75% of the tumors were histopathologically classified as ductal-invasive, other histologies included lobular, tubular, apocrine, neuroendocrine and papillary differentiation. Well- and moderately differentiated tumors were predominant. No patients with the primary diagnosis of distant metastasis were included in this study. More than two-thirds of the tumors were ER and progesterone receptor (PR) positive. Patients with an Her2 DAKO staining score of 3 (Dako, Glostrup, Denmark) or DAKO 2 with a positive fluorescence *in situ* hybridization analysis were considered Her2-positive (25/224 patients; 11%). Table I summarizes the clinical characteristics of our patient cohort.

Blood samples. To assess proteasomal concentration in blood plasma, venous blood was drawn in EDTA-containing tubes and centrifuged at 500×*g* for 10 minutes at a temperature of 5˚C to separate the plasma from the cell pellet.The samples were drawn on the day of the planned initial surgery and before any invasive interventions were carried out.

Chemicals. Chemicals used were of the highest available or analytical grade. Water was deionized, distilled, and passed through a Milli-Q-System (Millipore, Witten, Germany) before use.

Determination of plasma proteasome concentration. Plasma proteasome concentration was measured by an ELISA assay in patients and controls as previously described (14). Microtitration plates were coated overnight with mouse monoclonal antibody to 20S proteasome subunit α6 (HC2) (Biomol International L.P., Exeter, UK) diluted 1: 4500 in phosphate-buffered saline (PBS) (Invitrogen GmbH, Karlsruhe, Germany), pH 7.4. Plasma samples were diluted 1:1 and 1:5 in PBST-BSA (PBS, 0.1% Tween 20, and 1% bovine serum albumin) and applied to each well for 3 hours at room temperature. All measurements were within the linear portion of the respective ELISA standard curve. Standard curves were established for every microtitration plate using 20S proteasome protein standards (Biomol International L.P.) of concentration ranging from 19.5 ng/ml to 2500 ng/ml (8 linear dilution steps). The 20S proteasome was diluted in PBS-T (PBS and 0.1%Tween 20). The plates were washed once, and a rabbit polyclonal antibody (Biomol International L.P.) to 20S proteasome (dilution 1:4000) was added for 2 hours at room temperature. Following another four washing steps, peroxidase-conjugated mouse anti-rabbit IgG (Sigma-Aldrich, Saint Louis, MO, USA) was used for antigen detection (incubation period: 1 h at room temperature). The bound antibodies were detected using tetramethylbenzidine (Sigma-Aldrich) as substrate. The reaction was stopped with sulphuric acid and OD values were determined at 450 nm. To exclude the possibility of nonspecific binding, wells were filled with bovine serum albumin (Sigma-Aldrich), PBS, or PBS-T instead of blood plasma and incubated with the antibody. No reaction was observed under these control conditions.

Immunohistochemical localization of 20S proteasome in tumor specimens. For localization of 20S proteasome in tumor tissue, formalin-fixed and paraffin-embedded samples from 32 patients with breast cancer in whom c-proteasome concentrations in plasma had been measured were analyzed. Four-μm-thick sections the specimens, collected for routine histopathological examination at the Institute of Pathology and Neuropathology of Essen University Hospital, were deparaffinized and rehydrated according to standard procedures. For antigen retrieval, the slides were heated at 95˚C in citrate buffer at pH 6 for 30 minutes and incubated with antibodies directed against the core subunits of the catalytic domain of the 20S proteasome (Biotrend GmbH, Cologne, Germany; 1:250) in an automatic stainer (Dako). For evaluation of the staining intensity, a three-step quantification score was applied defining low or absent (less than 50%), moderate (50%), and strong staining (50%-100%) of tumor cells. The evaluation of the staining intensity was performed by light microscopy. Per tumor, one representative slide was selected and 10 visual fields were analyzed at 100-fold magnification.

Collection and analysis of BM. At least 10 and 20 ml of BM were aspirated from the anterior iliac crests of 213 primary breast cancer patients before surgery (local anesthesia with mepivacain) and processed within 24 hours. Tumor cell isolation and detection was performed based on the recommendations for standardized tumor cell detection published by the German Consensus group of Senology (23). BM cells were isolated from heparinized BM (5,000 U/ml BM) by Ficoll-Hypaque density gradient centrifugation (density 1.077 g/mol; Pharmacia, Freiburg, Germany) at 400×*g* for 30 min. Interface cells were washed (400×*g* for 15 min) and resuspended in PBS. A total of 1×10^6 mononuclear cells per 240 mm^2 were directly spun onto glass slides (400×*g* for 5 min) coated with poly-L-lysine (Sigma, Deisenhofen, Germany) using a Hettich cytocentrifuge (Tuttlingen, Germany) for the detection of cytokeratin (CK)-positive cells. The slides were air-dried overnight at room temperature.

Immunocytochemistry. Staining for CK cells was performed using the murine monoclonal antibody Mab A45-B/B3 (Micromet, Munich, Germany), directed against a common epitope of CK polypeptides, including the CK heterodimers 8/18 and 8/19. The protocol has been described in detail elsewhere (23, 24). Briefly, the method includes a)

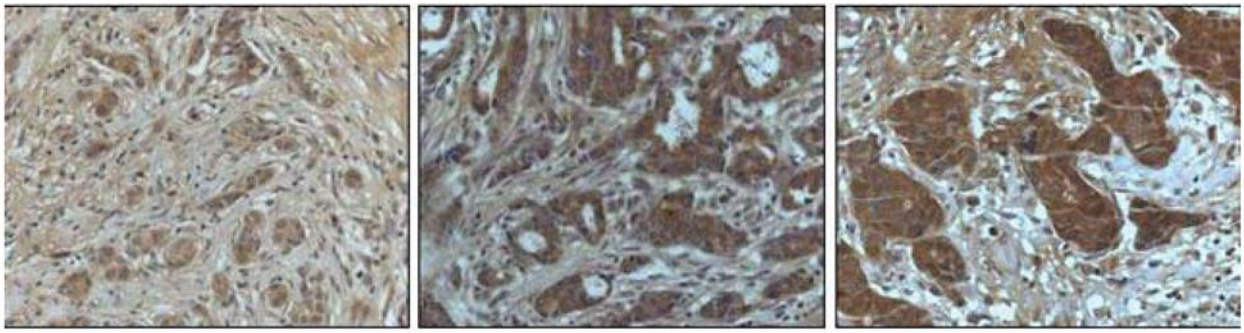

Figure 1. *Immunohistochemical investigation of 20S proteasome in invasive breast carcinoma. The 20S proteasome protein expression was predominantly noted in the cytoplasm of the tumor cells, and to a lesser extent also in the nuclei. The reaction pattern was quite uniform, but showed variable staining intensity (score 1: low staining intensity, 2: moderate staining intensity, and 3: strong staining intensity).*

permeabilization of the cells with a detergent (5 min), b) fixation with a formaldehyde-based solution (10 min), c) binding of the conjugate Mab A45-B/B3-alkaline phosphatase to cytoskeletal CKs (45 min) and d) formation of an insoluble red reaction product at the site of binding of the specific conjugate (15 min) using the DAKO-APAAP detection kit (DakoCytomation, Glostrup, Denmark) according to the manufacturer's instructions. Subsequently, the cells were mounted with Kaiser's glycerole/ gelatine (Merck, Darmstadt, Germany) in Tris EDTA buffer (Sigma, Deisenhofen, Germany). A control antibody (conjugate of Fab-fragment; Micromet) served as negative control. For each test, a positive control slide with the breast carcinoma cell line MCF-7 (ATCC, Rockville, MD, USA) was treated under the same conditions.

Evaluation of CK cells. Microscopic evaluation of the slides was carried out using the ARIOL SL-50 system (Applied Imaging International, Newcastle upon Tyne, UK) according to the International Society for Hematotherapy and Graft Engineering (ISHAGE) evaluation criteria and the DTC consensus (23, 24). These automated scanning microscopes and image analysis systems consist of a slide loader, camera, computer and software for the detection and classification of cells of interest based on particular colour, intensity, size, pattern, and shape.

Statistical analysis. Analyses were performed with SPSS version 17.0© (SPSS, Inc., Chicago, IL, USA). Data are presented as medians and range. The statistical analysis for correlations of c-proteasome concentrations with prognostically relevant variables including the patients' age, histopathologic grading, tumor stage, nodal status, hormone receptor status, Her2 status and presence of DTC in the BM, was conducted using the Mann-Whitney-*U*-test and the Kruskal-Wallis test for continuous variables. To test patients and controls for normal distribution of c-proteasome concentration, the Kolmogorov-Smirnov statistic was used. An *a priori* α-error (p) of less than 0.05 was considered statistically significant.

Results

Measured concentrations of c-proteasome were significantly higher in patients with breast cancer (median: 397.5 ng/ml, range: 200-50,000; $p<0.0001$) than in controls (median: 305 ng/ml, range: 140-425 ng/ml) and showed greater variance. While the serum concentrations in the control cohort showed a normal Gaussian distribution according to the Kolmogorov-Smirnov test, the c-proteasome concentrations of the patient samples did not. Prognostic and predictive clinical variables of breast cancer patients were analyzed for possible associations with c-proteasome concentrations. We found no significant associations of c-proteasome concentration with variables such as tumor size, nodal status, histologic grading, FIGO stage, hormone receptor status, Her2 status, histopathologic tumor type, or age at first diagnosis. Table I summarizes the correlation of clinical variables with c-proteasome concentrations. The presence of DTCs in the BM was assessed in 213/224 patients before surgery. In 34% (72/213) of these patients, DTCs in the BM were detected. We found no significant association of their presence with c-proteasome plasma concentration ($p=0.194$). Immunohistochemistry of 32 tumor specimens revealed 4 patients with low, 16 patients with moderate, and 16 patients with strong 20S proteasome staining. Comparisons between these groups showed no significant differences in median c-proteasome concentrations (1,970 ng/ml, 706 ng/ml, and 690 ng/ml, respectively, $p=0.930$, Kruskal-Wallis test).

Discussion

We were able to show that patients with non metastasized breast cancer, analogous to patients with other malignancies (13,18-20), have greater serum concentrations of 20S c-proteasome than healthy volunteers. As c-proteasomes may be considered intact and enzymatically active (25), it can be hypothesized that they exert functions in terms of tumor activity or tumor-associated effects. However, relevant predictive or prognostic markers did not correlate with c-proteasome concentrations. Previous data suggested that c-proteasome concentrations correlate with the tumor burden

Table I. *Clinical data in correlation with c-proteasome concentrations.*

	Patients (%) n=224	Mean (range) c-proteasome concentration (ng/ml)	*P*-Value*
Tumor stage			
pT1	158 (71%)	990 (200-50000)	0.489
pT2	59 (26%)	2138 (200-50000)	
pT3	7 (3%)	486 (250-970)	
Grading			
G1	43 (19%)	1778 (200-50000)	0.546
G2	114 (51%)	1486 (200-50000)	
G3	67 (30%)	598 (200-4810)	
Nodal status			
pN0	155 (70%)	1136 (200-50000)	0.209
pN1-3	68 (30%)	1610 (200-50000)	
Histopathology			
Ductal-invasive carcinoma	169 (75%)	957 (200-20000)	0.742
Other	55 (25%)	1381 (200-50000)	
Estrogen receptor status			
Positive	187 (83%)	1402 (200-50000)	0.874
Negative	37 (17%)	643 (200-3370)	
Progesterone receptor status			
Positive	179 (80%)	1448 (200-50000)	0.742
Negative	45 (20%)	594 (200-3370)	
Her2/Neu			
Positive	25 (11%)	688 (215-4500)	0.586
Negative	199 (89%)	1351 (200-50000)	

*According to Mann-Whitney *U*-test/Kruskal-Wallis test.

of patients (18-21). Our findings do not necessarily contradict this thesis. Accounting for the fact that patients with non-metastatic breast cancer do not suffer from large amounts of tumor mass, these results would indeed fit findings for other neoplasms. In particular, in our patient cohort with patients with mainly small primary tumors of less than 2 cm (pT1) in diameter, it can be hypothesized that the tumor load is small. Likely, the median c-proteasome concentration was notably lower in our patient collective (397.5 ng/ml) compared with ovarian cancer patients, who mainly suffer from advanced tumor stages (457.5 ng/ml) (21). It is striking that the variance of c-proteasome concentrations in the patient cohort is considerable. We can only hypothesize about the cause for this phenomenon. Apart from tumor mass, the histopathological expression of proteasomes in the primary tumor might affect the concentration of c-proteasome, as patients with strong tissue staining showed higher concentrations of 20S c-proteasome. However, the observed differences are not statistically significant. Previously, we were able to show that the concentration of c-proteasomes does not correlate with lactate dehydrogenase concentration and thus does not solely derive from cell lysis (21). Without doubt, one should keep in mind that c-proteasome might derive from neoplastic diseases, but has also been described to correlate with multiple other benign conditions such as vascular, pulmonary, or autoimmune diseases (14, 15, 26). Accordingly, differences in c-proteasome concentrations might also originate from co-morbidities and states other than tumor alone. There have been several attempts at targeting proteasomes in cancer with small molecules. In the treatment of multiple myeloma patients, the proteasome inhibitor bortezomib showed promising results (27-29); efforts for its implementation in breast cancer therapy have been made (30-32). However, the role of the proteasome system in tumor progression, particularly of breast cancer, is not fully clear. The inhibition of proteasomal degradation with small molecules appears an attractive therapeutic approach for ER-expressing tumors, especially (33). It is possible that patients with a considerable expression of proteasome in tumor tissue or with high concentrations of c-proteasome might indeed benefit from these targeted therapies.

Conflict of Interest Statement

This manuscript has been approved by all the Authors. There are no relationships that may present conflict of interest.

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