Original Article



# Five-year survival in luminal breast cancer patients: relation with intratumoral activity of proteasomes

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**Background:** The purpose of the study was to analyze the relationship between the caspase-like (CL) and chymotrypsin-like (ChTL) activities of proteasomes and the 5-year overall and metastasis-free survival rates in patients with luminal breast cancer.

**Methods:** The study included 117 patients with primary operable invasive breast cancer ($T_{1-2}N_{0-1}M_0$). Tissue samples from breast cancer patients were obtained as a result of the radical mastectomy or breast conserving surgery, which was a first line of therapy. The ChTL and CL proteasomes activities in the tumor tissue and in the surrounding adjacent breast tissues were assessed using the fluorometric method. The coefficients of ChTL (cChTL) and CL (cCL) proteasomes activities were also determined. The coefficients were calculated as the ratio of the corresponding proteasomes activity in the tumor tissue to the surrounding adjacent breast tissues. Within 5 years of follow-up, hematogenous metastases occurred in 14% of patients with luminal A breast cancer, in 31% of patients with luminal B human epidermal growth factor receptor-2 (HER-2) negative and in 23% of patients with luminal B HER-2 positive breast cancers. The study protocol was approved by the Local Ethics Committee of the Cancer Research Institute of Tomsk National Research Medical Center. Written informed consent was obtained from all patients.

**Results:** An increase in the ChTL and CL proteasomes activities was shown in all studied molecular subtypes of breast cancer compared to adjacent tissues. It was found that the cChTL of >35.9 U/mg protein and the cCL of >2.21 in breast cancer patients were associated with the development of distant metastases. In patients with luminal A breast cancer, the 5-year metastasis-free survival rates were associated only with the value of cCL of proteasomes (log-rank test: P=0.008). In patients with luminal B HER-2 negative breast cancer, the 5-year metastasis-free survival rates were associated with the levels of ChTL and cCL proteasomes activities (log-rank test: P=0.02 and P=0.04, respectively).

**Conclusions:** The data obtained on the correlation of 5-year metastasis-free survival rates with the level of

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proteasomes activities indicate the possibility of their use as additional prognostic criteria for breast cancer.

**Keywords:** Luminal breast cancer; hematogenous metastases; proteasomes activity; long-term outcome; prognosis

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## Introduction

Breast cancer is the most common malignancy in women worldwide (1-4). Breast tumor heterogeneity is a major challenge in the clinical management of breast cancer patients (5-9). Therefore, the search for markers that could predict the disease prognosis is of great importance. Luminal hormone receptor-positive ($HR^+$) breast cancer is the most common molecular subtype, accounting for 60–75% of breast cancers worldwide. According to the 15th St. Gallen Consensus, $HR^+$ tumors are classified into luminal A and luminal B molecular subtypes (10). Estrogen receptors (ERs) are key points of ERα signaling in luminal cancer. The activation of the signaling cascade mediated by ERs is often observed in the response to therapy for luminal A breast cancer, which determines the sensitivity of these tumors to endocrine therapy (11,12). In luminal A cancer, the ERα signaling pathway was shown to maintain the epithelial phenotype of the tumor and inhibit the epithelial-mesenchymal transition (EMT) of breast cancer. Moreover, a positive relationship between the Notch3 transcription factor and ERα was demonstrated in breast cancer cell lines and in human breast cancer tissues. Notch3 specifically binds to the CSL binding element of the ERα promoter and activates ERα expression. Notch3 depletion in luminal breast cancer promotes metastasis (13). The luminal B subtype of breast cancer is characterized by high proliferative activity and positive expression of ER/progesterone receptor (PR). Human epidermal growth factor receptor-2 (HER-2) receptor can be either over expressed or not over expressed. The prognosis for luminal B breast cancer is less favorable compared to that for luminal A cancer (14,15). Luminal B tumors are characterized by activation of either ER- or HER-2-mediated signaling cascades. Taking into account the molecular features of luminal B breast cancer, a patient with this molecular subtype of the tumor is recommended to undergo both hormone therapy and chemotherapy. However, not all luminal breast cancer patients respond to treatment and which leads to the development of distant metastases (16). Currently, there is active research into predictive biomarkers for selection of personalized drug treatment. In particular, the effectiveness of using various gene signatures to select the most adequate therapy is being evaluated (17). However, the problem of breast cancer progression remains relevant. Each molecular subtype of breast cancer has its own characteristics that have not been sufficiently studied. Therefore, further studies on breast cancer molecular subtypes are needed.

The ubiquitin-proteasome system plays an important role in the regulation of the expression of biologically active molecules. The ubiquitin-proteasome system is a universal intracellular system of protein degradation and quality control by turning on/off the cascade of enzymatic reactions, as well as a complex regulation of the composition and proteolytic activities of the proteasomes. Proteolysis occurs in proteasomes that degrade cytosolic and nuclear proteins, growth factors, as well as turn inactive proteins into active ones and participate in the formation of regulatory peptides, presentation of type I histocompatibility complex, and regulation of gene transcription (18). Proteins are broken down in the inner chamber of the proteasomes, which is formed by two β-rings, consisting of β subunits that exhibit proteolytic activity: chymotrypsin-like (ChTL) activity, trypsin-like activity, and caspase-like (CL) activity. The relative content of proteasomes, their composition and localization in the cell change dynamically and adjust in accordance with the cell requirements and particular stress conditions (19). In addition, a change in the qualitative composition of proteasomes affects the efficiency of proteolysis (20,21).

Also, proteasomes are involved in the regulation of key processes associated with tumor progression—proliferative activity, apoptosis, neoangiogenesis, and changes in cell motility (18,22,23). In addition, the effective regulation of the number and functions of many proteins, including receptors, growth and transcription factors, is provided by adequate proteolysis processes (24-26). In particular, the issue of regulation of the content of ERs by proteasomes is

 

discussed in detail. The ubiquitin-proteasome system can regulate the ER in two ways: by modifying receptors by attaching 1–2 ubiquitin molecules and thereby altering their functioning (27) and by regulating the content by cleavage in proteasomes (28-30). In addition, the ubiquitination of ERα is also associated with its phosphorylation state, which can lead to the modification of ERα (31-33). Thus, the phosphorylation and ubiquitination of ERα are interrelated to control both the quantity and the functions of this receptor. In addition, the ChTL proteasomes activity is required to regulate the expression of the gene encoding ERα (*ESR1*) (34).

Proteasomes are an interesting object for studying different breast cancer molecular subtypes in order to assess their contribution to the pathogenesis of the disease. They can be candidates for prognostic markers, since, despite their versatility, these systems exhibit a certain specificity in various pathological processes, including cancers of the lung, prostate, stomach, breast, pancreas, and head and neck (35-39).

Using various databases and platforms (Oncomine, cBioPortal and Cytoscaped and METABRIC, TCGA, CCLE Project and STRING), numerous published studies demonstrated the prognostic value of the gene expressions of various proteasome subunits both in breast cancer and in other cancers in terms of long-term overall and long-term progression-free survivals. In particular, the authors reported on the prognostic significance of the expression of the *PSMC* family genes encoding subunits of the 19S regulatory complex of the proteasomes, the expression of the *PSMA* family genes encoding the α subunits of the proteasome nucleus, and the expression of the *PSMB8* gene responsible for the formation of the immune *LMP*7 subunit (40-42). Almost all databases concern the expression of genes encoding various subunits of the proteasome complex. Nevertheless, in the available literature, we did not find publications on the assessment of the prognostic significance of proteasomes activity. Our previous studies have shown the relationship between the activity of proteasomes system and the stage of the disease and lymphogenous metastasis in breast cancer (43,44). We previously published results on the study of the subunit composition of proteasomes using Western blot analysis and immunohistochemistry (IHC) (20,45,46). We have shown an increase in the level of the total pool of proteasomes in terms of the content of α1α2α3α5α6α7 subunits and the level of the activator subunit Rpt6 in breast cancer tissue compared to unchanged tissue (45). Also, it was found by immunofluorescence method that breast tumor cells contained immune proteasomes, as well as PA700 and PA28αβ activators. In addition, in breast cancer, a significant number of immune proteasomes were present in tumor cells, and not in normal ones. The PA28αβ activator and immune proteasomes were characterized only by cytoplasmic localization, while the α1α2α3α5α6α7 subunits of the total pool of proteasomes and the PA700 activator associated with the degradation of large molecules and/or ubiquitinated substrates were present both in the cytoplasm and in the nuclei of tumor cells. The association of the subunit composition of the proteasomes with the status of ERs as well as with lymph node metastasis was also demonstrated (20,45,46). Due to the multifaceted effects of proteasomes on both key processes for the development and progression of tumors, as well as on the main therapeutic targets, it is necessary to study the prognostic role of proteolytic activities of proteasomes in breast cancer.

The purpose of the study was to analyze the relationship between the ChTL and CL activities of proteasomes and the 5-yaer overall/metastasis-free survival rates in patients with luminal breast cancer. We present the following article in accordance with the MDAR reporting checklist (available at https://tbcr.amegroups.com/article/view/10.21037/tbcr-22-22/rc).

## Methods

### *Human samples*

A total of 117 patients with stage $T_{1-2}N_{0-1}M_0$ breast cancer were included into the study. The luminal A breast cancer molecular subtype was diagnosed in 65 (56%) of patients, luminal B breast cancer without over expression of the HER-2 receptor was found in 40 (34%) of patients and luminal B HER-2-positive breast cancer was diagnosed in 12 (10%) of patients (*Table 1*).

The first stage in the treatment of patients was radical mastectomy or breast conserving surgery. In the adjuvant regimen, patients were treated according to the molecular subtype of the tumor. Patients with luminal A breast cancer received adjuvant chemotherapy with combination of adriamycine and cyclophosphamide (AC) and Taxotere as monotherapy. Patients with luminal B HER-2 negative breast cancer received 4–6 cycles of chemotherapy with AC or fluorouracil, doxorubicin, cyclophosphamide (FAC) regimen. Chemotherapy and anti-HER-2 therapy with Herceptin was given to patients with luminal B HER-2 positive breast cancer. All patients with luminal A and

 

**Table 1** Clinical characteristics of breast cancer patients

| Clinical parameters | Luminal A (n=65) | Luminal B | | Total patients (n=117) |
|---|---|---|---|---|
| | | HER-2 negative (n=40) | HER-2 positive (n=12) | |
| Tumor stage, n [%] | | | | |
| T1 | 22 [34] | 15 [37.5] | 4 [33] | 41 [35] |
| T2 | 43 [66] | 25 [62.5] | 8 [64] | 76 [65] |
| Nodal status, n [%] | | | | |
| N0 | 38 [58] | 25 [62.5] | 5 [42] | 68 [58] |
| N1 | 27 [42] | 15 [37.5] | 7 [58] | 49 [42] |
| 5-year overall survival, n [%] | 62 [95] | 35 [88] | 12 [100] | 109 [93.2] |
| 5-year metastasis-free survival, n [%] | 56 [86] | 28 [69] | 9 [77] | 93 [79.5] |

HER-2, human epidermal growth factor receptor-2.

luminal B breast cancer received hormone therapy with Tamoxifen or Aromatase inhibitor for 5 years. Radiation therapy was performed as indicated. The criteria for exclusion of patients from the study were as follows: patients over 75 years; patients with hematogenous metastases ($M_1$); patients who received neoadjuvant chemotherapy.

When analyzing the survival rate, the calculation was carried out in the fifth year after surgery, taking into account the patients who were lost to follow-up and who died from concomitant non-cancer diseases. The overall and metastasis-free survival rates were calculated in months from the onset of radical mastectomy or sector resection of the breast until the time of the patient's last visit to the oncologist or the identification of the outcome (died or cancer progression). The follow-up period ranged from 3 to 60 months. The 5-year overall survival rate in breast cancer patients was 93.2%, with the median survival rate of 54 months. The 5-year metastasis-free survival rate in breast cancer patients was 79.5%, with the median rate of 46 months. Disease progression (metastases in the pelvic bones, spine bones, skull bones, liver, lungs, brain, and multiple distant metastases) within 5-year follow-up was observed in 14% of patients with luminal A breast cancer, in 31% of patients with luminal B HER-2-negative breast cancer and in 23% of patients with luminal B HER-2-positive breast cancer.

Ethics approval and consent to participate: the study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study protocol was approved by the Local Ethics Committee of the Cancer Research Institute of Tomsk National Research Medical Center (protocol No. 2 from 4 April 2017) and informed consent was taken from all the patients.

Tissue samples from all enrolled patients were collected before therapy. Tumor and noncancerous adjacent tissue samples were studied. These tissues were subjected to morphological verification. Unchanged tissues were represented by visually unchanged breast tissues taken at a distance of 1–3 cm from the tumor margin. Tissue samples were frozen and stored at –80 ℃.

### *Antibodies and reagents*

Proteasomes substrate N-succinyl-leu-leu-val-tyr7-amido-4-methyl coumarin (Suc-LLVY-AMC; Product Number: S6510), Substrate Z-Leu-Leu-Glu-7-amido-4-methylcoumarin is acts as a substrate for CL activity (Cbz-LLG-AMC; Product Number: C0483) and proteasomes inhibitor Z-Leu-Leu-Leu-al (MG132; Product Number: C2211) were purchased from Sigma-Aldrich (USA). Peroxidase blocking reagent, LSAB2 System-HRP (Code K0672), mouse mAbs to ERa (clone 1D5; Code M7047), mouse mAbs to PR (clone PgR 636; Code M3569) and biotinilated anti-mouse IgG antibodies were purchased from Dako (Denmark).

### *IHC*

Molecular subtype of breast cancer was determined after assessing the receptor status [expression of ER and PR], HER-2 status and proliferative activity (Ki67 expression) in biopsy and surgical specimens using the

 

immunohistochemical method. Staining was performed on 5–6-µm-thick formalin-fixed paraffin-embedded sections according to routine laboratory protocols. The sections were deparaffinized in xylene, rehydrated through a graded ethanol series and pretreated with 0.01 M sodium citrate buffer. Endogenous peroxidase activity was blocked with a peroxidase blocking reagent (Dako). The sections were then incubated for 60 min with primary antibodies against ER (clone 1D5, Dako), PR (clone PgR636, Dako) HER-2 (polyclonal, Dako, Denmark) and Ki-67 (clone MIB-1, Dako, Denmark). After a second incubation with biotinylated antibodies (Dako, Denmark) for 10 min, the sections were labeled with streptavidin-biotin complex. The immunoreactions were visualized with 3,3'-diaminobenzidine (Dako) and counterstained with hematoxylin (47).

According to the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines, ER and PR expression was considered positive when nuclear immunoreactivity was ≥1% of tumor cells (42). HER-2-positive protein status was defined as a score of 3+ or 2+ by IHC along with confirmed amplification of the *c-erbB2* gene using fluorescence in situ hybridization (FISH).

According to the recommendations of the St. Gallen Consensus (10), luminal A molecular subtype of breast cancer was determined as being ER and/or PR positive and HER-2 negative and low Ki-67 (≤20%); and luminal B subtype as ER and/or PR positive and HER-2 negative and high Ki-67 (>20%) or ER and/or PR positive and HER-2 positive.

### *Clarified tissue homogenates*

To obtain clarified homogenates, frozen tissue samples (100 mg) were homogenized in liquid nitrogen and resuspended then in 300 µL of 50 mM Tris-HCl buffer (pH 7.5) containing 2 mM ATP, 5 mM magnesium chloride, 1 mM dithiothreitol, 1 mM EDTA and 100 mM sodium chloride. The homogenate was centrifuged for 60 minutes at 10,000 g and 4 ℃.

### *Determination of proteasomes activity*

ChTL and CL activities of proteasomes were determined in clarified tissue homogenates of breast cancer patients by hydrolysis of the substrates: fluorogenic oligopeptide Suc-LLVY-AMC or Cbz-LLG-AMC, respectively (43). The reaction mixture for the determination of ChTL and CL activity of proteasomes contained 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 30 µM corresponding substrate, 5 mM $MgCl_2$, and 1 mM ATP. The reaction was carried out at 37 ℃ for 20 min. The resulting product was recorded on a Hitachi-850 fluorometer (Japan) at an excitation wavelength of 380 nm and emission of 440 nm. The unit of proteasomes activity was defined as the amount of enzyme at which 1 nmol of Suc-LLVY-AMC or Cbz-LLG-AMC was hydrolyzed for 1 min. To assess the activity of impurity proteases, a specific proteasomes inhibitor MG132 (Sigma) was used. The specific activity of proteasomes was expressed in units of activity per 1 mg of protein. Protein content was determined by the Lowry method.

### *Determination of the coefficients of ChTL (cChTL) and CL (cCL) activities of proteasomes*

The cChTL and cCL activities of proteasomes were calculated as the ratio of the proteasomes activity in the tumor tissue to the activity in the unchanged tissue. The need to determine the coefficients of proteasomes activity is explained by the fact that in a personalized analysis of changes in proteasomes activity in tissues, a strong variability was recorded in the ratio of the activity of the studied proteases in the tumor and in the unchanged tissue, which is probably due to the intensity of proteolysis processes both in the tumor and outside the tumor. The use of such coefficients made it possible to more objectively assess the changes occurring in the tumor, taking into account the proteasomes activities of the unchanged tissues.

### *Statistical analysis*

The data were processed by Statistica 10.0 software and presented as median and interquartile range (25%, 75%). To evaluate the difference, either Mann-Whitney or Kruskal-Wallis test was used. When assessing the relationship between the parameters of intracellular proteolytic systems and the 5-year overall and metastasis-free survival rates, the generalized log-rank test and the $\chi^2$ test were used to compare multiple groups. Survival curves were constructed using the Kaplan-Meier method. When analyzing the survival rate, the calculation was carried out in the fifth year after surgery, taking into account the patients who were lost to follow-up and patients who died from concomitant non-cancer diseases. The overall and metastasis-free survival rates were calculated in months from the onset of radical mastectomy or sector resection of the breast until

 

**Table 2** ChTL and CL activities of proteasomes in luminal breast cancer tissues

| Indicators | Luminal A (n=65) | Luminal B | | Total patients (n=117) |
|---|---|---|---|---|
| | | HER-2 negative (n=40) | HER-2 positive (n=12) | |
| ChTL proteasomes activity ($10^3$ U/mg protein) | | | | |
| Adjacent tissues | 13.06 (6.4, 33.3) | 22.48 (8.9, 61.7) | 14.07 (11.3, 21.2) | 17.0 (7.52, 40.81) |
| Cancer tissues | 31.57* (16.4, 64.3) | 45.71* (22.6, 148.9) | 52.01* (19.8, 69.5) | 35.9* (17.8, 81.94) |
| CL proteasomes activity ($10^3$ U/mg protein) | | | | |
| Adjacent tissues | 14.25 (8.3,32.2) | 20.83 (10.9, 71.6) | 16.14 (9.9, 36.9) | 18.2 (9.6, 46.8) |
| Cancer tissues | 31.81* (16.7,116.1) | 106.81* (28.2, 298.4) | 59.33* (16.7, 121.2) | 46.4* (18.7, 163.8) |

Data were present as median and range value (25%, 75%). *, significance of differences compared to adjacent tissues group, P<0.01. ChTL, chymotrypsin-like; CL, caspase-like; HER-2, human epidermal growth factor receptor-2.

**Table 3** cChTL and cCL activities of proteasomes in tissues of luminal breast cancer

| Indicators | Luminal A (n=65) | Luminal B | | Total patients (n=117) |
|---|---|---|---|---|
| | | HER-2 negative (n=40) | HER-2 positive (n=12) | |
| cChTL proteasomes activity | 2.11 (1.1, 3.8) | 1.90 (1.1, 3.3) | 3.31 (1.4, 4.4) | 2.16 (1.10, 4.02) |
| cCL proteasomes activity | 2.14 (1.2, 4.7) | 2.94 (1.4, 6.2) | 2.89 (1.3, 8.1) | 2.21 (1.3, 5.2) |

Data were present as median and range value (25%, 75%). cChTL, coefficient of chymotrypsin-like; cCL, coefficient of caspase-like; HER-2, human epidermal growth factor receptor-2.

the time of the patient's last visit to the oncologist or the identification of the outcome. The follow-up time ranged from 3 to 60 months. P values <0.05 were considered statistically significant.

## Results

### *Study of proteasomes activities in the tumor and adjacent tissues of breast cancer patients*

An increase in proteasomes ChTL and CL activities in tumors compared to adjacent tissues were observed in all molecular subtypes of breast cancer (*Table 2*). The median values of ChTL and CL proteasomes activities were respectively 2.4 and 2.7 times higher in the tumor tissues than in the unchanged tissues (P=0.000, P=0.000, respectively).

The data analysis showed a strong variability in the ratio of the activity of the studied proteases in the tumor and in the unchanged tissue, which is likely due to the intensity of proteolysis processes both in the tumor and outside of it. Therefore, a personalized assessment of changes in proteasomes activity in tissues was carried out. We proposed to determine the cChTL and cCL activities of proteasomes for each patient included in the study. The introduction of these coefficients made it possible to more objectively assess the changes of proteasomes activities occurring in the tumor, taking into account the of normal and tumor tissues. The data presented in *Table 3*, which reflect the median values of the proteasomes activity coefficients (*Table 3*).

### *Survival of breast cancer patients depending on the activity of proteasomes in tumor tissues*

The 5-year overall and metastasis-free survival rates were compared with proteolytic activity of proteasomes in breast tissues (*Table 4*). The median values of the corresponding parameters presented in *Tables 2,3* were chosen as reference levels dividing the proteolytic activities into high and low in the total group of breast cancer patients. The analysis of survival revealed the difference in the 5-year metastasis-free survival rates with regard to the ChTL and cCL activities of proteasomes in the tumor (log-rank test: P=0.02 and P=0.002, respectively). However, the values of cChTL and CL were not associated with survival rates. There were no differences in overall survival rates depending on proteasomes activity (*Table 4*).

**Table 4** The 5-year overall and metastasis-free survival rates depending on proteasomes activity in breast cancer tissues

| Factors | Number of patients | Cumulative proportion of survivors (%) | |
|---|---|---|---|
| | | 5-year overall survival | 5-year metastasis-free survival |
| ChTL proteasomes activity in cancer | | P>0.05 | P=0.02 |
| >35.9 U/mg protein | 58 | 94.83 | 89.83 |
| ≤35.9 U/mg protein | 59 | 91.23 | 72.43 |
| cChTL proteasomes activity | | P>0.05 | P>0.05 |
| >2.16 | 58 | 92.16 | 86.27 |
| ≤2.16 | 59 | 94.12 | 76.47 |
| CL proteasomes activity in cancer | | P>0.05 | P>0.05 |
| >46.4 U/mg protein | 61 | 93.88 | 85.71 |
| ≤46.4 U/mg protein | 56 | 93.48 | 78.26 |
| cCL proteasomes activity | | P>0.05 | P=0.002 |
| >2.21 | 56 | 93.02 | 93.02 |
| ≤2.21 | 61 | 93.62 | 70.21 |

ChTL, chymotrypsin-like; cChTL, coefficient of chymotrypsin-like; CL, caspase-like; cCL, coefficient of caspase-like.

The prognosis of the disease in relation to the development of distant metastases was significantly worse with high values of ChTL and cCL activities. With the ChTL proteasomes activity of more than 35.9 U/mg protein, the 5-year metastasis-free survival rate was 68.31%±6.27%, while with the ChTL activity of less than 35.9 U/mg protein, the 5-year metastasis-free survival rate was 85.82%±5.56% (P=0.02) (*Figure 1A*). With the cCL value of more than 2.21, the cumulative 5-year metastasis-free survival rate was 59.56%±7.49%, while with a low value of this coefficient, the metastasis-free survival rate was 90.17%±6.28% (P=0.002) (*Figure 1B*).

Thus, the ChTL proteasomes activity of more than 35.9 U/mg protein and the cCL value of more than 2.21 were associated with unfavorable prognosis (development of distant metastases). The data obtained are fundamentally new and make it possible to consider the activity of proteasomes in breast tissues as important additional prognostic criteria in assessing the disease outcome.

### *The 5-year overall and metastasis-free survival rates in patients with different molecular breast cancer subtypes*

A significant difference in the 5-year overall and metastasis-free survival rates between patients with luminal A, luminal B HER-2 negative and luminal B HER-2 positive breast cancer was not found. The 5-year overall survival rate was 95% in patients with luminal A breast cancer. The 5-year overall survival rates were 88% and 100% in patients with luminal B HER-2 negative and luminal B HER-2 positive breast cancers, respectively (P=0.21; $\chi^2$=3.09) (*Figure 2A*); for metastasis-free survival (P=0.26; $\chi^2$=2.67) (*Figure 2B*).

### *Relationship between the 5-year overall/metastasis-free survival and proteasomes activity in patients with different molecular subtypes of breast cancer*

The relationship between the 5-year overall and metastasis-free survival rates and proteasomes activity in breast cancer tissues with various molecular subtypes was studied. In patients with luminal A breast cancer, the 5-year metastasis-free survival rates were associated with cCL values of proteasomes (log-rank test: P=0.008). In patients with cCL of more than 2.21, the 5-year metastasis-free survival rate was 68.4%. In patients with cCL of less than 2.21, the 5-year survival metastasis-free survival rate was 96.4% (P=0.008) (*Figure 3*).

In patients with luminal B HER-2 negative breast cancer, the 5-year metastasis-free survival rates were associated with the levels of ChTL activity and cCL activity of proteasomes (log-rank test: P=0.02 and P=0.04, respectively) (*Figure 4A,4B*).

 

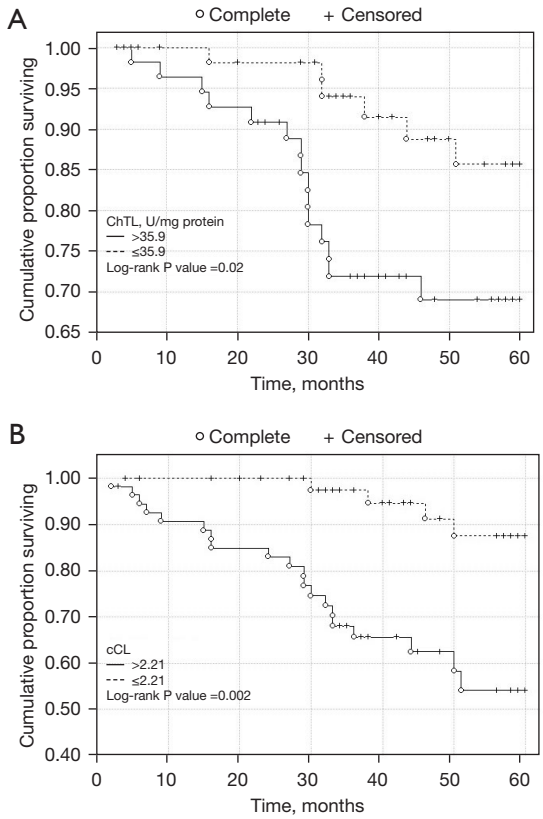


**Figure 1** Kaplan-Meier curves of metastasis-free survival depending on (A) the value of ChTL proteasomes activity in the tumor and (B) the cCL proteasomes activity in luminal breast cancer patients. ChTL, chymotrypsin-like; cCL, coefficient of caspase-like.

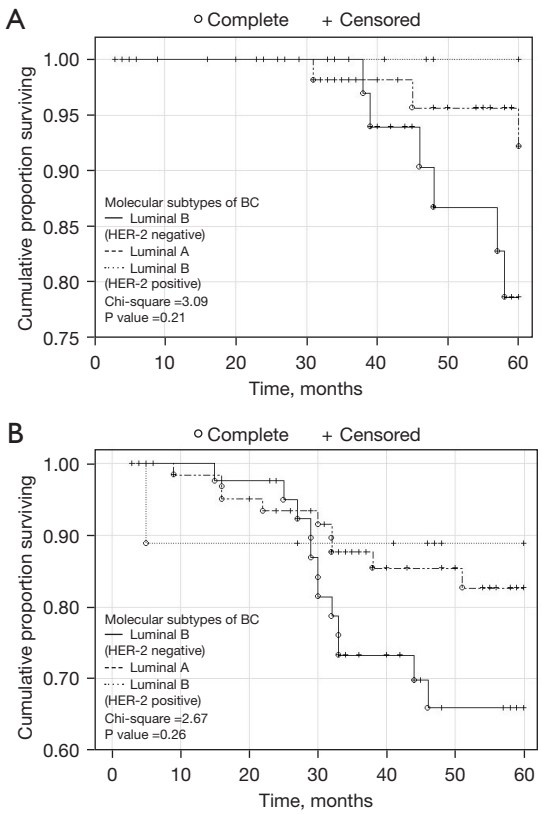


**Figure 2** Kaplan-Meier curves of overall survival (A) and metastasis-free survival (B) of breast cancer patients in different molecular subtypes. Disease progression (development of hematogenous metastases) at 5 years of follow-up occurred in 31% patients with luminal B HER-2 negative breast cancer; in 23% of patients with luminal B HER-2 positive breast cancer and in 16% of patients with luminal A breast cancer. BC, breast cancer; HER-2, human epidermal growth factor receptor-2.

The threshold value of the ChTL proteasomes activity was 35.9 U/mg protein. In the case of the increased threshold value of the ChTL activity, the 5-year metastasis-free survival rate was 60.87%. In patients with ChTL proteasomes activity of less than 35.9 U/mg protein, the 5-year metastasis-free survival rates were significantly higher and amounted to 88.24% (P=0.02) (*Figure 4A*). The cCL proteasomes activity above 2.21 was associated with distant metastases and poor outcome, with the survival rate of 65%. In patients with the cCL of less than 2.21, the survival rate was 85.7% (P=0.04) (*Figure 4B*).

In cases with luminal B HER-2 positive breast cancer, the activity of intracellular proteolytic systems was not associated with the 5-year metastasis-free survival, which may be due to the small number of patients included in this study group.

Thus, in luminal A breast cancer, it is advisable to use the definition of cCL, while in luminal B HER-2 negative breast cancer, it is recommended to use the definition of ChTL and cCL as additional prognostic criteria associated with the development of distant metastases.

## Discussion

The present study shows an increase in the processes of intracellular proteolysis in the tumor compared to the unchanged tissues. This may be associated with intensive metabolic processes that characterize the malignant tumors. The rapid intracellular exchange of proteins is accompanied by their increased degradation by proteolytic systems, which ensure the safety of the cellular proteome (23). In addition,

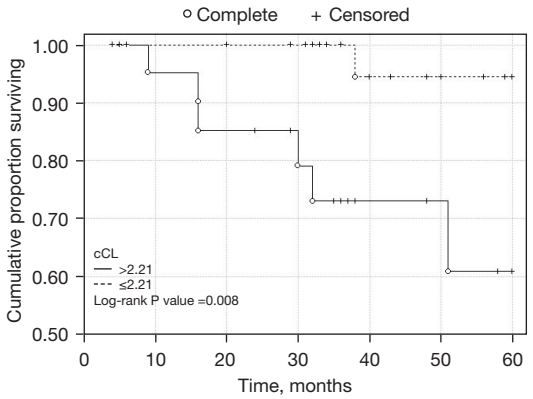


**Figure 3** Kaplan-Meier curves of metastasis-free survival depending on the cCL proteasomes activity in patients with luminal A breast cancer. cCL, coefficient of caspase-like.

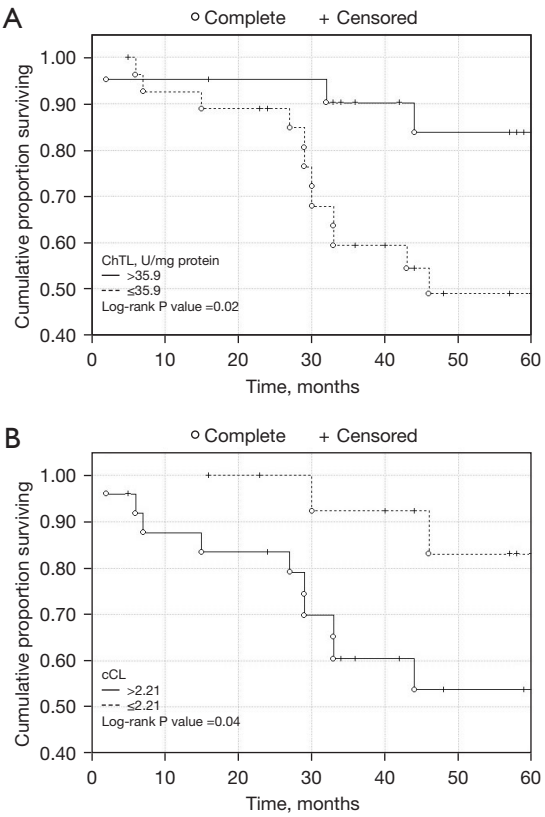


**Figure 4** Kaplan-Meier curves of metastasis-free survival depending on (A) the values of ChTL proteasomes activity and (B) depending on the level of the cCL proteasomes activity in patients with luminal B HER-2 negative breast cancer. ChTL, chymotrypsin-like; cCL, coefficient of caspase-like; HER-2, human epidermal growth factor receptor-2.

the results obtained are consistent with other recent studies. Chen and Madura [2005] in their studies showed that the activity of proteasomes was significantly higher in tumors compared to adjacent tissues taken from the same breast cancer patients (48,49).

It should be noted that the results on the feasibility of using proteasomes activity as additional prognostic markers of distant metastasis in different molecular subtypes of breast cancer are new.

We have shown for the first time that in breast cancer it is advisable to use the ratio of the activity values in the tumor and the unaltered tissues surrounding the tumor (proteasomes activity coefficients). It has been also shown that the determination of the value of the cCL proteasomes activity above 2.21 is associated with a poor prognosis in patients with luminal A and luminal B HER-2 negative breast cancers. In addition, the ChTL value in the tumor above 35.9 U/mg protein is an unfavorable prognostic sign only for luminal B HER-2 negative breast cancer. Our data on the feasibility of using proteasome activity to predict long-term outcomes are consistent with other recent studies. Kao *et al.* (40), studied the prognostic significance of the expression of *PSMC* family genes encoding the subunits of the 19S regulatory complex of proteasomes responsible for the hydrolysis of substrates conjugated with polyubiquitin. The study included data on 2,898 breast cancer cases. There were used Oncomine platform, GEPIA2 platform, the Human Protein Atlas, the Kaplan-Meier plotter database, METABRIC database and the Metacore platform. The authors showed overexpression of the *PSMC* 6 gene in luminal breast cancer. In addition, it was shown that high expression of *PSMC1*, *PSMC3*, and *PSMC6* and low expression of *PSMC2* were poor predictors of distant metastasis-free survival in breast cancer (40). Using the cBioPortal, Oncomine, and the Kaplan-Meier plotter databases, another group of authors studied the relationship between the expression of *PSMA* family genes encoding α subunits of the proteasome core and recurrence-free survival in breast cancer. Breast cancer tissues were shown to have higher levels of *PSMA* gene expression compared to normal breast tissues. In addition, high expression levels of *PSMA2*, *PSMA3*, *PSMA4*, *PSMA6*, and *PSMA7* correlated with poor recurrence-free survival in breast cancer patients, while high expression levels of PSMA5 and *PSMA8* were associated with a good prognosis (41). Chen *et al.* (42) evaluated pan-cancer expression profile analysis PSMB8 expression using datasets from TCGA and the GTEx databases. The authors found a statistically significant increase in PSMB8 expression

 

in luminal breast cancer tissue compared to that in normal breast tissue, as well as in tumors of other localizations ($P<0.05$), with the exception of lung squamous cell carcinoma and adrenocortical carcinoma. In addition, using datasets from the Te Cancer Genome Atlas (TCGA) database, the relationship of PSMB8 expression and prognostic value in 33 types of cancer, including breast cancer, was evaluated. In breast cancer, a low level of PSMB8 expression was shown to be a poor prognostic factor for overall and progression-free survivals (42). Enhanced protein metabolism inherent in tumors is accompanied by increased sensitivity to proteasome inhibitors. This sensitivity, along with the potential to stabilize pro-apoptotic signaling pathways, makes the proteasome an attractive clinical target for the development of new therapies. At present, the development of targeted drugs directed to the 19S regulatory subunit, immune subunits, and inhibitors of deubiquitinating and ubiquitin ligase enzymes, is being actively carried out (50). *In vitro* studies using a triple-negative breast cancer model showed that bortezomib and carfilzomib, proteasomes inhibitors, induced proteotoxic stress and apoptosis through an expanded protein response, and the sensitivity correlated with the expression of proteasomes immune subunits (*PSMB8/9/10*). Moreover, the expression of inducible immune proteasomes subunit β5i in brain metastases was lower than in primary breast tumors (38).

The probable involvement of proteasomes in the mechanisms of tumor progression in luminal breast cancer can be related to the fact that a number of signaling molecules and targets of therapeutic action act as substrates for proteasome degradation which are actively involved in these processes. First, proteasomes can cleave cytoskeletal proteins, actin-binding proteins, and transcription factors that regulate cell motility and thus participate in the process of metastasis (26,51). Several studies have confirmed that β-catenin plays a significant role in tumor progression. In a cell, β-catenin can be a component of the catenin-cadherin complex, providing adhesion and reducing cell migration activity, as well as having a significant impact on the reorganization of actin filaments. On the other hand, β-catenin is involved in the activation of gene transcription of the Wnt/β-catenin signaling pathway, leading to increased migration, decreased adhesion, and increased cell proliferation (52). The stability of β-catenin is controlled through ubiquitin-proteasome degradation. In the absence of Wnt, free β-catenin is delivered to proteasome for degradation by the cytoplasmic destruction complex (53).

The proteasome system is also involved in the regulation of the level and activity of molecular markers currently used for molecular subtyping of breast cancer. These markers also play an important role in tumor progression. In particular, ERs, which play an important role in the transmission of the estrogen signal and in the realization of the effects of hormones in luminal cancers, are partially regulated by the ubiquitin-proteasome system, influencing the degradation of the receptors and regulators of their transcription, as well as the expression of receptor genes (2,54,*55*). A study of the effect of proteasome inhibitors on the content of ER-α in $ER^+$ breast cancer cell cultures showed that bortezomib, a proteasome inhibitor, inhibited receptor proteolysis for several hours (56). Some ER ligands may also promote ubiquitination of the receptor, provoking its rapid degradation through the proteasome pathway (57). Degradation of PRs is also partially mediated by proteasomes. Ligand-dependent decrease in the expression of PRs occurs through a mechanism that includes receptor phosphorylation, ubiquitination, and destruction by 26S proteasomes (*55*). It was shown that activated phospho-PR receptors are susceptible to more rapid suppression of ligand-dependent receptors by the ubiquitin-proteasome system as compared to dephosphorylated PR species (2,58). The proteasome system plays an important role in the activation/inactivation of the HER-2 receptor. In particular, proteasome inhibition has been shown to effectively prevent galdamycin-dependent cleavage of the *ERBB2* gene and induce the accumulation of polyubiquitinated *ERBB2*. Galdamycin is an inhibitor of Hsp90, a protein currently considered as a promising therapeutic target. The function of Hsp90 is to ensure the stability of the HER-2 receptor on the membrane (59). Inhibition of Hsp90 facilitates the intracellular delivery of tracizumab-conjugated nanoparticles capable of transporting doxorubicin to $ERBB2^+$ breast cancer cells, thus leading to the enhanced antitumor activity (60). In an experiment on HER-2 positive cell cultures, HER-2/neu overexpression was shown to activate the Akt signaling pathway by the NF-κB through the action of calpains, while in the absence of expression of this molecule, the Akt signaling pathway is classically activated, through the IκB kinase complex and involvement of the ubiquitin-proteasome system (61).

Thus, the proteasome system is actively involved in cancer progression.

## Conclusions

The present study shows an increase in the processes

 

of intracellular proteolysis in the tumor compared to the unchanged tissues. It has been also shown that the determination of the value of the cCL activity of proteasomes above 2.21 is associated with a poor prognosis in patients with luminal A and luminal B HER-2 negative breast cancers. In addition, the ChTL value in the tumor above 35.9 U/mg protein is an unfavorable prognostic sign only for luminal B HER-2 negative breast cancer. Thus, in the case of luminal breast cancer, it is advisable to use the definition of the cCL activity, and also in the case of luminal B HER-2 negative breast cancer, it is advisable to use the definition of ChTL and cCL activities as an additional prognostic factor associated with the development of distant metastases.

The data obtained on the relationship between the 5-year metastasis-free survival and the levels of proteasome activities indicate the feasibility of their use as additional prognostic criteria for breast cancer. In addition, the results can be used as a theoretical basis for the development of new anticancer drugs aimed at eliminating the imbalance in the functioning of the proteasomes system.

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## Footnote

*Reporting Checklist:* The authors have completed the MDAR reporting checklist. Available at https://tbcr.amegroups.com/article/view/10.21037/tbcr-22-22/rc

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*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at https://tbcr.amegroups.com/article/view/10.21037/tbcr-22-22/coif). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study protocol was approved by the Local Ethics Committee of the Cancer Research Institute of Tomsk National Research Medical Center (protocol No. 2 from 4 April 2017) and informed consent was taken from all the patients. Written informed consent was obtained from all patients.



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