

Serum cathepsin K and cystatin C concentration in patients with advanced non-small-cell lung cancer during chemotherapy

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Abstract: A pathogenic implication of cathepsin K (Cath K) and its inhibitor – cystatin C (Cyst C) occur to be of growing importance in the mechanisms of tumor invasiveness in lung cancer. This study was conducted to investigate the prognostic role and the effects of chemotherapy on serum Cath K and Cyst C (ELISA) in patients with advanced stage non-small cell lung cancer (NSCLC). The study entered 40 patients (32 men) and 15 healthy volunteers (control group). Peripheral blood samples were taken before and after four cycles of chemotherapy. The mean serum Cyst C levels were significantly higher in patients with advanced NSCLC than in controls ($p=0.003$). The levels of Cath K in serum of NSCLC are comparable to those in controls. No correlation was found between Cath K and Cyst C concentrations and the histological type and staging of lung cancer. Patients with T4-stage had a lower level of Cyst C, than those with T2 ($p=0.033$). No correlation was found between the concentrations of Cath K, Cyst C and the effect of chemotherapy. However, Cyst C level positively correlated with serum creatinine concentration ($R=0.535$; $p=0.005$) in patients who responded to chemotherapy and with patient's age ($R=0.456$; $p=0.018$) in whole group. When the cut-off values of serum Cath K and Cyst C (23.35 pmol/l, 1.29 mg/l, respectively) were used, the prognoses of high and low groups were not different. Concluding, patients with lung cancer have a higher serum concentration of Cyst C compared to healthy people. In our opinion, determination of Cath K and Cyst C concentrations has no clinical significance in the prognosis of the survival time in lung cancer.

Key words: lung cancer, NSCLC, non-small cell lung cancer, chemotherapy, cathepsin K, cystatin C

Introduction

Cathepsin K is an elastase that belongs to the papain-cysteine proteinase family and possesses an unique collagenolytic activity [1]. It plays a well – known role in bone turnover, but also in nonosseous lesions, possibly through remodeling of the extracellular matrix [1]. Tissue remodeling is crucial in different lung diseases, in the embryonal development as well as in bronchial carcinoma [2]. Cathepsin K is expressed by osteoclasts and macrophages and in the lung by bronchial epithelial and alveolar cells [3,4]. The role of cathepsin K in neoplastic conditions is not fully understood. The reports on prostate and breast carcinomas indicate that cathepsin K may be produced by epithelial neoplastic

cells and that its expression is associated with the increased invasive potential [5,6]. Rapa *et al.* [7] demonstrate that Cath K is newly produced in the stroma of invasive tumors of the lung and can favor or modulate the invasive growth of tumor cells. The intracellular activity of Cath K is regulated by specific endogenous cysteine proteinase inhibitors such as cystatin C [8].

Cystatin C is a non-glycosylated low molecular weight (13kD) basic protein that is produced by all nucleated cells [9]. This protein is a member of the cystatin superfamily of cysteine protease inhibitors; the production rate of cystatin C is stable and does not change in inflammatory conditions [9]. Due to its nature, the serum level of cystatin C is known to be a better marker for GFR (glomerular filtration rate) than serum creatinine [9]. Cystatin C is the most important extracellular inhibitor of several cysteine proteinases, and it has been postulated that the imbalance between cysteine proteinases and cystatins can contribute to

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connective tissue remodeling [10]. Moreover, in patients with colorectal cancer and malignant melanoma, a significant correlation has been revealed between the increased serum cystatin C level and malignant progression [11]. High cystatin C levels were associated with a tumor's size, postmenopausal status and a patient's age [12]. There are no findings about concentrations of Cath K and Cyst C in the serum of lung cancer patients. In our study, we measured the serum levels of Cath K and Cyst C in lung cancer patients to assess their correlations and their clinical significance.

Materials and methods

Patients. The study involved 40 patients diagnosed and treated in the Department of Lung Diseases and Tuberculosis in Białystok (32 men and 8 women) with the histological diagnosis of NSCLC.

The mean age of patients was 61.7 ± 6 years (Patients' characteristics is shown in Table 1). The study patients had neither been treated with any anticancer medication nor undergone radiotherapy. Squamous cell carcinoma (SCC) comprised 42.5% (17 individuals) of patients with NSCLC, adenocarcinoma was revealed in 27.5% (11 patients), whereas NSCLC was diagnosed in 30% (12 patients). Serum samples, obtained from the whole blood of patients with lung cancer before cytoreduction treatment and after four cycles of chemotherapy, were used as the study material. To exclude the possible interference of chemotherapy, subsequent blood samples were obtained at least 28 days after the last administration of cytotoxic drugs. Blood serum was stored at -80°C immediately after separation by centrifugation (3000 rpm) until the assay was performed. At the first stage, blood samples were taken to assess Cath K and Cyst C after complete diagnostics of lung cancer had been made, including X-ray and CT of the chest, bronchofiberscopy with H+P examination lung transbronchial biopsy (TBB), or transbronchial needle aspiration biopsy (TBNA). The clinical analysis comprised the evaluation of clinical staging of NSCLC (TNM, AJCC), and the performance stage according to Zubrod. The response to therapy was estimated according to the WHO criteria. All patients underwent basic laboratory tests and accessory investigations (ultrasonography of the abdominal cavity, if necessary, of the chest, EEG, and CT of the central nervous system). At the next stage, after termination of chemotherapy, during the evaluation of therapy outcome, blood samples were collected to determine the concentrations of Cath K and Cyst C.

Controls. The control group consisted of 15 healthy volunteers (12 men and 3 women) without any acute or chronic inflammatory conditions, without history of any kidney diseases, hypertension, diabetes mellitus. The mean age of controls at the time of sampling was 62.1 ± 4 year. There were no significant differences in age and sex between patients and controls.

Therapy. Chemotherapy was carried out in a 21-day cycle using cisplatin at a dose of 30 mg/m^2 on days 1, 2, and 3 and gemcytabine at a dose of 1000 mg/m^2 on days 1 and 8 of the cycle. All patients received four cycles of chemotherapy. Some of the patients underwent later radiotherapy or next cycles of chemotherapy.

Serum Cath K and Cyst C analysis. Cathepsin K (Cathepsin K Elisa Kit, Biomedica Austria), cystatin C (Human Cystatin C Quantikine ELISA Kit, R&D System, USA) concentrations were determined by means of an enzyme-linked immunosorbent assay (ELISA) method according to the manufacturer's instructions. All specimens were assayed in duplicates. The detection limit of Cath

K was 1.1 pmol/l ($0 \text{ pmol/l} + 3 \text{ SD}$). The median range was 8.7 pmol/l . The standard range was from 0 pmol/l to 300 pmol/l . The minimum detectable dose (MDD) of cystatin C ranged from $0.030 - 0.227 \text{ ng/ml}$. The mean MDD was 0.102 ng/ml .

Ethical issues. In accordance with the declaration of Helsinki, the study protocol was approved by the local ethics committee and a written informed consent was obtained from all participants.

Statistical analysis. Statistical analysis was performed using Statistica 8.0 software (Stat Soft Inc., Tulsa, USA). The compatibility test of Shapiro-Wilk was used for measurable features consistent with normal distribution. The Student *t* test was applied to compare the respective groups and for pairs to compare features in two time intervals. Correlations between the parameters were calculated by the Pearson's tests. Survival curves were made using the Kaplan-Meier method, and the significance of the difference in survival rates was determined by the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model. All patients with lung cancer were divided into two groups according to their Cath K and Cyst C serum levels. The cut-off point was set at 23.35 pmol/l for Cath K, and 1.29 mg/l for Cyst C. Receiver-operating characteristics (ROC) curves were applied to find the cut-off level of Cath K and Cyst C. A value of $p < 0.05$ was considered to be the level of statistical significance.

Results

None of the prognostic parameters analyzed (Table 1) was correlated significantly with the serum Cath K and Cyst C levels ($p > 0.05$).

The levels of serum Cath K and Cyst C in patients with advanced NSCLC and healthy controls are shown in Table 2. The baseline serum Cyst C levels were significantly higher in patients with advanced NSCLC than in the control group ($p = 0.003$). Cath K levels were not significantly different from healthy control group ($p = 0.976$). Concentrations of Cath K and Cyst C did not differ markedly before and after chemotherapy of lung cancer ($p = 0.539$, $p = 0.751$). No correlation was found between Cath K and Cyst C concentrations and the histological type and staging of lung cancer. Patients in group IIIB had the same concentrations of Cath K and Cyst C as patients in group IV. However, the patients with T4-stage had lower level of Cyst C (after chemotherapy) than those with T2 ($p = 0.033$) (Fig. 1).

In the study group, PR (partial response) was reported in 16 patients (40%), stabilization (NC, no change) in 10 patients (25%), and PD (progressive disease) in 14 patients (35%). No correlation was found between the concentrations of Cath K and Cyst C and the effect of chemotherapy (Tables 3 and 4).

There was no correlation between serum levels of Cath K and Cyst C. A positive correlation was revealed between serum creatinine and serum Cyst C in patients who responded to chemotherapy (PR, NC) ($R = 0.535$; $p = 0.005$) (Fig. 2); between serum Cyst C and age of patients ($R = 0.456$; $p = 0.018$).

Table 1. Patients' characteristics.

| Parameter | n |
|---|-----------|
| No. of patients | 40 |
| Age, yr mean ±SD | 61.7±6 |
| Gender, male/female | 32/8 |
| Weight loss (10%), yes/no | 14/26 |
| Performance status, 0-1/2 | 29/11 |
| Histology, squamous cell/other | 17/23 |
| Stage, IIIB/IV | 21/19 |
| Haemoglobin (12g/dl), low/normal | 10/30 |
| Albumin (3.5 g/dl), low/normal | 4/36 |
| LDH (450 U/l), normal, elevated | 26/14 |
| Response to chemotherapy, yes/no | 26/14 |
| Serum creatinine concentration (mg/dl) | 0.99±0.29 |
| eGFR – MDRD (> 90ml/min/1.73 m ²) | 40 |
| Serum Procalcitonin (< 0.5 ng/ml) | 40 |

Abbreviations: n – number; SD – standard deviation; eGFR – MDRD – estimated Glomerular Filtration Rate according to Modified Diet in Renal Diseases

Table 2. Distribution of serum Cath K and Cyst C values in patients with NSCLC and healthy controls by Student's T – test for unpaired samples.

| Patients (n=40) mean ± SD | | Controls (n=15) mean ± SD | p |
|---------------------------|-------------|---------------------------|-------|
| Cath K (pmol/l) | 14.10 ± 4.2 | 14.04 ± 9.3 | 0.976 |
| Cyst C (mg/l) | 1.64 ± 0.5 | 1.06 ± 0.2 | 0.003 |

Abbreviations: n – number; SD – standard deviation

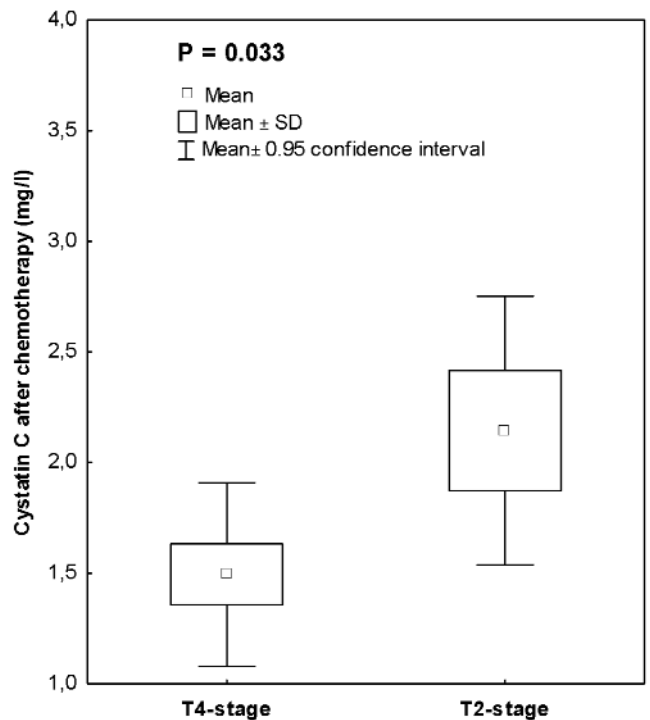


Fig. 1. The serum levels of Cystatin C after chemotherapy in groups of T4 and T2 (according to TNM) patients by Student's T – test for unpaired samples.

When all patients with lung cancer were divided into high and low groups using cut-off serum Cath K and Cyst C concentrations, the prognoses of high and low groups were not different. The cut-off serum concentrations were 23.35 pmol/l (Cath K) (Fig. 3), and 1.29 mg/l (Cyst C) (Fig. 4). The median survival of the study group was 11.3 months. Older age, weight loss,

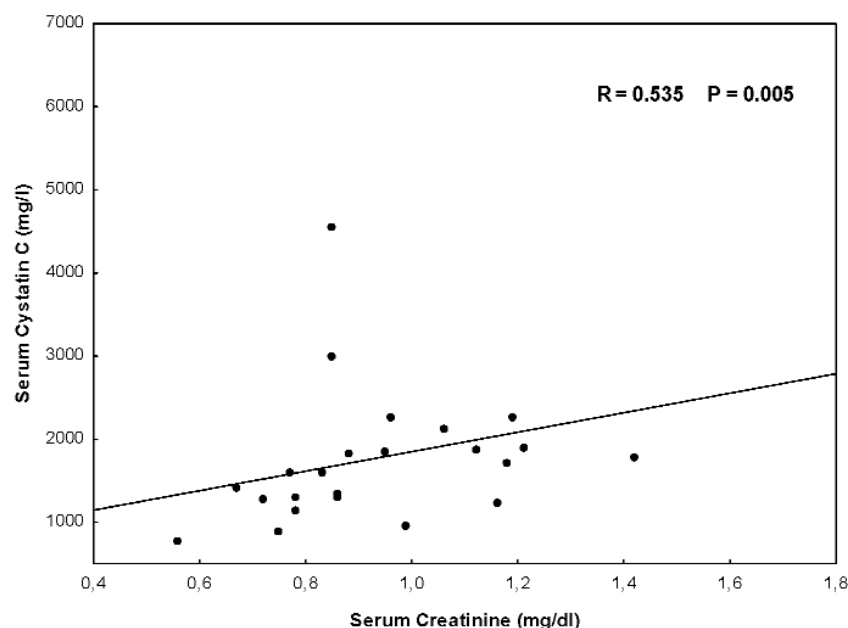


Fig. 2. Relationship between Serum Creatinine and Serum Cystatin C in patients who responded to chemotherapy (Partial Response + No Change).

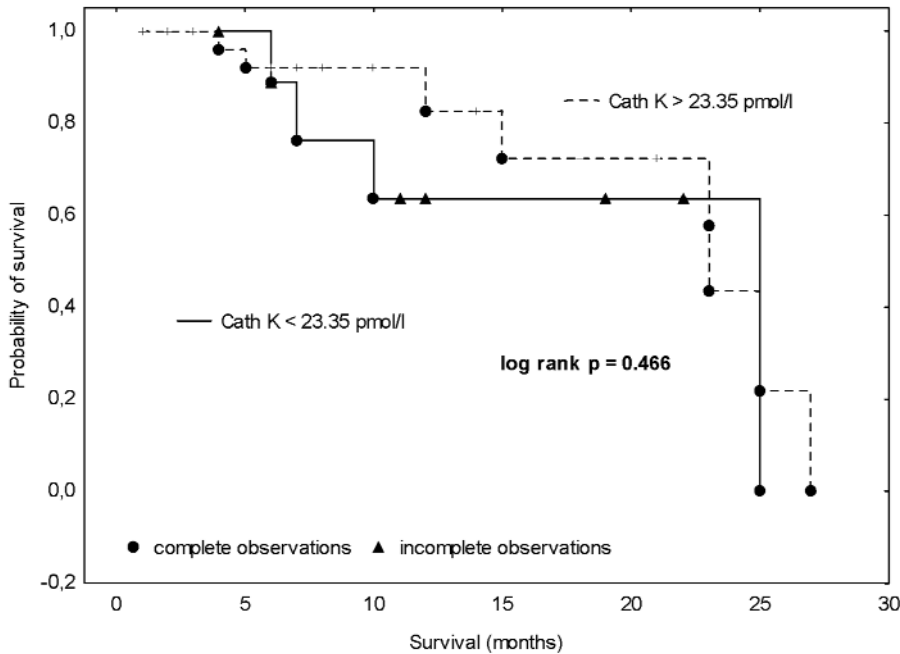


Fig. 3. Probability of survival for lung cancer patients in relation to serum Cath K levels (before chemotherapy)

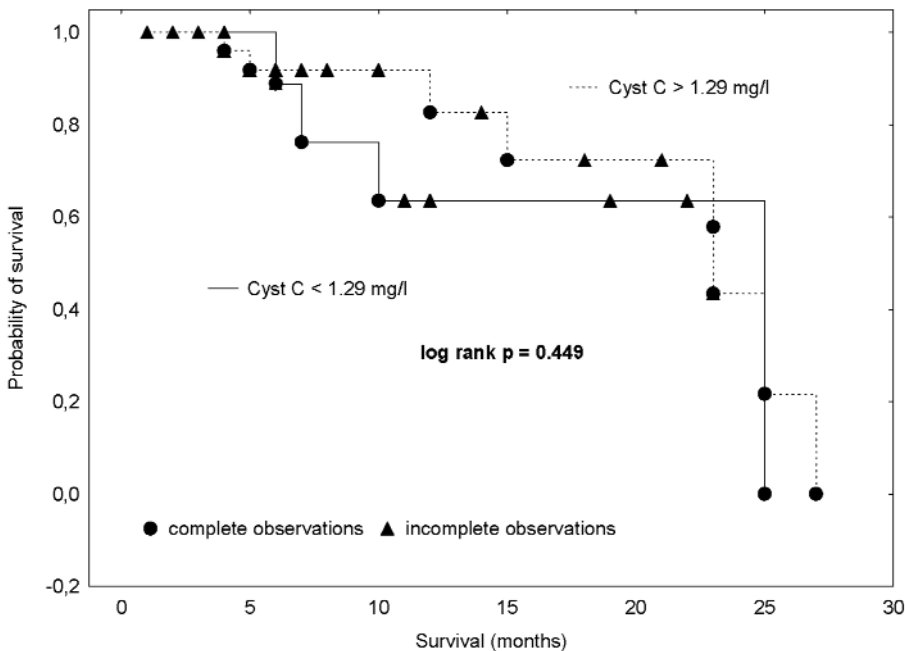


Fig. 4. Probability of survival for lung cancer patients in relation to serum Cyst C levels (before chemotherapy)

and performance status yielded a prognostic value (Table 5). Conversely, neither Cath K nor Cyst C levels were proved to be significant for survival.

ROC (Receiver Operating Characteristic) curve was analyzed to assess the effectiveness of serum Cath K and Cyst C determination in discrimination between NSCLC and controls (Fig. 5). The AUCs of Cath K and Cyst C were 0.485 and 0.588. ROC curves showed a poor clinical performance of Cyst C in the detection of NSCLC patients. The cut off values of Cath K and Cyst C were established at 8.72 pmol/l and 1.24 mg/l, respectively. There were no significant differences between the areas under the curves.

Discussion

Although cathepsin K has a well-defined function in inflammation, bone remodeling, macrophage activity, and lung fibrosis, its role in neoplastic growth has not been explained completely.

There have been no findings on serum concentrations of Cath K patients with lung cancer. Cath K expression in macrophages, in the lung by bronchial epithelial and alveolar cells, and in the stroma of NSCLC suggested higher serum concentrations of Cath K patients with lung cancer than in healthy people. According to Kleer *et al.* [13] Cath K plays a role in

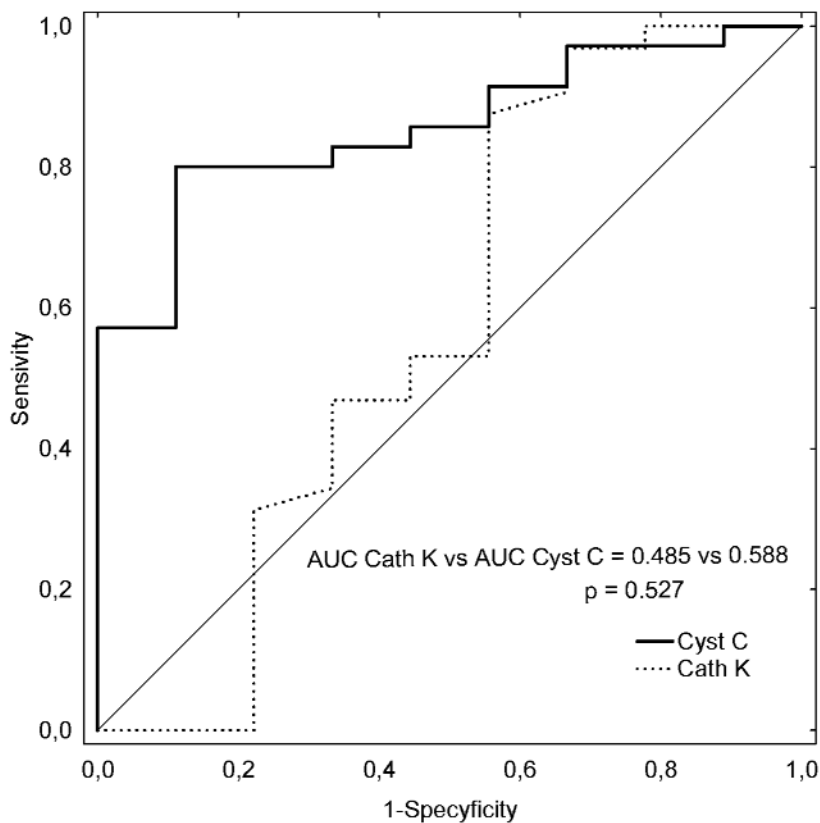


Fig. 5. ROC curves of Cath K and Cyst C (for discrimination between NSCLC and controls)

Table 3. Distribution of serum values of Cath K and Cyst C before and after chemotherapy, in patients with partial response or stable disease by Student's T – test for paired samples (subgroup analysis).

| Before chemotherapy mean ± SD | | After chemotherapy mean ± SD | p |
|-------------------------------|-------------|------------------------------|-------|
| Cath K (pmol/l) | 13.97 ± 3.8 | 13.60 ± 4.9 | 0.165 |
| Cyst C (mg/l) | 1.63 ± 0.5 | 1.68 ± 0.4 | 0.560 |

Abbreviations: n- number; SD- standard deviation

Table 4. Distribution of serum values of Cath K and Cyst C before and after chemotherapy, in patients with progressive disease by Student's T – test for paired samples (subgroup analysis).

| Before chemotherapy mean ± SD | | After chemotherapy mean ± SD | p |
|-------------------------------|-------------|------------------------------|-------|
| Cath K (pmol/l) | 14.31 ± 4.9 | 15.74 ± 4.4 | 0.615 |
| Cyst C (mg/l) | 1.66 ± 0.6 | 1.79 ± 0.4 | 0.792 |

Abbreviations: n- number; SD- standard deviation

tumor progression by promoting extracellular matrix degradation and angiogenesis as well as by increasing the invasiveness or neighboring tumor epithelial cells. Moreover, the relation between the enhanced secretion of Cyst C, an inhibitor of Cath K, and the invasive potential has been found in various cell lines [14].

Table 5. Influence of statistically significant prognostic factors and serum parameters on survival in advanced NSCLC patients

| Prognostic factor | p vaule |
|--------------------|---------|
| Age | 0.012 |
| Weight loss | 0.003 |
| Performance status | 0.005 |
| Serum Cath K | 0.721 |
| Serum Cyst C | 0.532 |

In this study we proved that patients with advanced NSCLC had similar Cath K concentrations to those in healthy people, but higher concentrations of Cystatin C. Our results are partially in accordance with Tumello's *et al.* findings [8]. These authors found higher Cyst C concentrations in patients with breast cancer, whereas Cath K concentrations were lower. In patients with prostate cancer, Cath K concentrations were the same as in healthy people [8]. It is known that in the intracellular environment, Cath K activity is inhibited by Cyst C and its expression by epithelial neoplastic cells is associated with an increase in the invasive potential [5,6]. It is likely that Cyst C and other antiproteases contribute to a decrease in Cath K concentration to the same or even lower levels when compared to healthy people.

In our study, the increased levels of Cyst C do not seem to be caused by an impaired kidney function as

none of these patients showed a clinically evident alteration in the renal functioning. The concentration of Cyst C was proved to correlate with the concentration of creatinine in serum of the study patients with partial remission or stabilization after treatment. No correlation was revealed in patients with progression, in whom cancer cells are the main source of Cyst C in serum. In these patients, the determination of cystatin C as GFR marker is not useful, which confirms Nakai's *et al.* studies [9]. These authors found that serum levels of cystatin C were not always a reliable marker of renal efficiency in patients with a malignancy.

Cystatin C is synthesized by all nucleated cells. It has been suggested that cystatin C might be overexpressed in tumor cells, resulting in increased circulating levels [15]. Moreover, cystatin C is thought to play a role in protein homeostasis in the extracellular compartment, inhibiting inappropriate hydrolytic degradation by proteinases released from dying cells [16]. Cystatin C may also be released from infiltrated inflammatory cells such as macrophages and neutrophils [17]. Higher serum concentrations of Cyst C were also determined in patients with ovarian cancer [18], colorectal cancer and melanoma [19] in comparison with healthy people. The results of our study carried out among patients with lung cancer are in agreement with findings mentioned above. However, the clinical usefulness of cystatin C determination in serum (as well as of Cath K) is poor, which was proved by means of ROC curves.

Findings on Cyst C activity in serum with regard to cancer staging are controversial. In our study, patients at the stage III B had identical concentrations to those of patients at the stage IV. The results of our study are confirmed by Dreilich's *et al.* studies [19] based on patients with esophagus cancer.

According to Werle and Kos [14,17] higher total concentrations of Cyst C found in sera of patients with lung, colorectal and melanoma cancer suggested the enhanced secretion of cystatin C from tumor cells, increasing at the same time the intracellular proteolytic potential of cysteine proteases. Moreover, increased levels of cystatin C in tumor tissues have been shown to correlate with favorable prognosis of cancer patients [20- 22]. High concentrations of Cyst C may inhibit proteases activity (including Cath K) and the growth of a tumor, which was confirmed by Strojan's *et al.* study [14]. They observed in patients with carcinoma of the head and neck, an inverse correlation between tumor cystatin C level and a more aggressive form of the disease. Our study is in accordance with the findings mentioned above. We indicated that patients with T2 – stage had higher concentration of Cyst C than patients with T4- stage. The low level of Cyst C in patients with T4-stage in our study may suggest a possibility of consumption or inactivation of Cyst C in the

course of tumor growth. Our study confirmed Nakai's *et al.* findings [9], demonstrating that in coloncancer sera, the level of Cyst C was lower in advanced stages than in early stages. On the other hand, Kos *et al.* found in the study group with colorectal cancer, the patients with high serum levels of Cyst C exhibited a higher risk of death (due to rapid growth of a tumor) than those with lower levels of an inhibitor [23]. In our study, no correlations were demonstrated between concentrations of Cyst C and Cath K, and survival time. Our observations are similar to Werle's *et al.* results of the studies in patients with NSCLC [17].

These various results obtained in studies of particular cancers may be due to the complexity of mechanisms influencing Cyst C concentration in serum. However, cysteine proteases (and also Cath K), and consequently their inhibitors, are involved in various physiological processes, including those which may act in an opposite way to a tumor's progression, such as apoptosis, activation of the T-cell immune response, as well as cell migration and seeding [24-27]. Thus, besides their concentration, the cell and tissue localization of Cyst C and Cath K could also make a critical switch between harmless and harmful.

Summarizing, it has been proved that cystatin C concentration is higher in patients with advanced NSCLC than in healthy people, whereas Cathepsin K concentration is comparable. Determination of Cathepsin K and Cyst C concentrations is not useful in monitoring therapy effects of NSCLC predicting survival time. Cyst C concentration decreases together with the growth of a tumor's size.

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