Exp Oncol 2022
44, 2, 163–168

Experimental ONCOLOGY

SERUM ANTIBODIES AGAINST GROEL AS AN ADDITIONAL RISK BIOMARKER OF BIOCHEMICAL RECURRENCE AFTER RADICAL PROSTATECTOMY

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***Background*: The level of heat shock protein 60 (Hsp60) is elevated in tumor cells compared with normal prostate epithelium. Hsp60 is involved in tumor growth, invasion, and metastasis and is considered as a biomarker for cancer diagnosis and prognosis. *Aim*: To study the level of antibodies against prokaryotic homolog of human Hsp60 (GroEL) in prostate cancer (PCa) patients as an additional risk marker for the prediction of biochemical recurrence after radical prostatectomy (RP). *Patients and Methods*: A total of 55 patients with localized and locally advanced PCa, who had undergone RP between July 2013 and May 2014 were enrolled. Level of antibodies to GroEL and human Hsp60 was determined by enzyme-linked immunosorbent assay before surgery. Serum samples of blood donors with low reactivity to GroEL and human Hsp60 were used as controls. The relationship between IgG antibodies against bacterial Hsp60 and human Hsp60 and clinicopathological features were analyzed. The biochemical recurrence (BCR) free survival rate was estimated by the Kaplan — Meier method. The univariate and multivariate Cox regression models were used to evaluate the risk factors of BCR-free survival rate. *Results*: There were significant differences in anti-GroEL IgG levels between control and PCa patients while no significant differences in anti-human Hsp60 IgG levels between control and PCa patients were detected. During the follow-up period, 40/55 (72.7%) patients developed BCR. The time from surgery to BCR was from 18 to 72 months. Elevated IgG antibodies against bacterial Hsp60 in patients who had undergone RP were associated with early occurrence of biochemical relapse and lower 5-year BCR-free survival rate respectively ($p < 0.001$). The multivariate analysis indicated that IgG to GroEL (hazard ratio = 2.465; 95% confidence interval: 1.311–4.634, $p < 0.05$) could be independent prognostic factor in the patients who had developed BCR. *Conclusion*: Elevated levels of IgG antibodies against GroEL before surgery can predict early occurrence of BCR after RP and can serve as an additional independent risk biomarker of a BCR after RP.**
***Key Words*: biochemical recurrence, heat shock protein 60, GroEL, radical prostatectomy, prostate cancer.**

DOI: 10.32471/exp-oncology.2312-8852.vol-44-no-2.17827

Prostate cancer (PCa) ranks as the second most frequently diagnosed cancer in males worldwide and places fifth in mortality among males with tumors, its burden is expected to grow to 2.2 million of new cases and 720,000 of new deaths in 2040 [1]. Radical prostatectomy (RP) is a principle treatment option for patients with localized cancer and it gives a good chance of a recovery. However, the incidence of recurrence after radical surgery remains high. Up to 30% of men who had undergone RP relapse with rising serum prostate-specific antigen (PSA) level. The known predictive criteria such as Gleason score, preoperative PSA levels, and clinical TNM stage alone and in combination are associated with outcome but do not accurately predict which individuals will relapse [2]. The search for new additional serum or tissue predictors of cancer progression would help to identify patients at a risk of a recurrence and to optimize treatment.

Heat shock protein 60 (Hsp60) and anti-Hsp60 antibodies are considered as possible biomarkers for the diagnosis and prognosis of cancer [3–5]. Hsp60 is a conservative, highly immunogenic protein. It is involved in folding, refolding, transport, and degradation of proteins, regulation of cell growth and differentiation, apoptosis, intercellular signaling [6]. However, the demonstrated cross-reactivity between human Hsp60 (hHsp60) and a prokaryotic homolog of human Hsp60 (GroEL) creates dissonance in the clear definition of the role hHsp60 in systemic diseases [7].

The level of Hsp60 is elevated in tumor cells as compared with normal tissue in patients with ovarian cancer [8, 9], myeloid leukemia [10], urinary bladder cancer [11, 12], breast cancer [13, 14], Hodgkin's disease [15], pancreatic carcinoma [16], osteosarcoma [17], brain tumors [18], uterine cancer [13], colorectal cancer [19, 20], PCa [21–24], exocervical cancer [25], and adrenal cancer [26]. The elevated levels of anti-Hsp60 antibodies were detected in patients with breast cancer [13, 14, 27, 28], osteosarcoma [17], colorectal cancer [20], ovarian cancer [29], squamous cell carcinoma of oral cavity [30], and PCa [31].

The expression level of Hsp60 correlates strongly with the high Gleason score and is also associated with the high level of initial PSA and lymph node involvement [32]. Patients with overexpressed Hsp60 in RP specimens have a significantly shorter biochemical recurrence (BCR) free time, and high

Submitted: May 18, 2022.
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Abbreviations used: BCR – biochemical recurrence; ELISA – enzyme-linked immunosorbent assay; GroEL – prokaryotic homolog of human Hsp60; hHsp60 – human Hsp60; Hsp60 – heat shock protein 60; o.d.u. – optical density units; PCa – prostate cancer; PSA – prostate-specific antigen; RP – radical prostatectomy.

Hsp60 level is considered as an independent predictor of biochemical relapse [33].

In addition, the data suggest a strong correlation between GroEL expression and serum anti-hHsp60 antibodies level [7]. This fact creates the necessity to investigate diagnostic and prognostic properties not only of hHps60 but also of its prokaryotic homolog.

The aim of the study was to assess the level of antibodies against GroEL in PCa patients as a probable additional risk marker for the prediction of BCR after RP.

MATERIAL AND METHODS

Study population. The study included 55 patients with localized, locally advanced PCa who had undergone RP between July 2013 and May 2014. Before surgery, patients were stratified into risk groups using D'Amico criteria: level of preoperative PSA, clinical TNM stage, and biopsy Gleason score.

The study protocol was approved by the Ethics Commission of the State Institution "Institute of Urology of National Academy of Medical Sciences of Ukraine", and written informed consent was obtained from all patients.

The pre-surgery examination included general clinical laboratory tests, prostate biopsy with morphological verification, determination of a PSA level, ultrasound scanning, magnetic resonance imaging or computed tomography of the lower pelvis. All patients had undergone open or laparoscopic RP. The lymphadenectomy was performed for patients classified as high-risk accord D'Amico criteria. The RP specimens including prostate, seminal vesicles, and bilateral pelvic lymph nodes were examined microscopically after routine preparation. The follow-up period included a digital rectal examination and measurement of a PSA level at 4 weeks and quarterly after surgery during the first year and then each 6 months. A BCR was defined as the elevation of a PSA level > 0.2 ng/ml in two consequent measurements.

Enzyme-linked immunosorbent assay (ELISA). Serum levels of anti-Hsp60 antibodies were determined by a modified ELISA before performing RP. Recombinant proteins GroEL *E. coli* (Abcam, UK) and human Hsp60 (hHsp60) (Sigma-Aldrich, USA) were used as antigens. Low reactive to GroEL (n = 70) and hHsp60 (n = 23) serum samples of blood donors without any family history of cancer were used as controls [34].

The 96-well plates were coated with 10 mg/ml recombinant protein (GroEL or hHsp60) in the phosphate-buffered saline overnight at 4 °C. After washing, the wells were blocked with phosphate-buffered saline Tween-20 at 37 °C for 1 h. After washing, the serum samples were added in a 1:50 dilution and were incubated overnight at 4 °C. Anti-human IgG antibodies conjugated with horseradish peroxidase were used for the detection of bound antibodies. All reactions were developed with 0.5 mg/ml 2.2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (Sigma-Aldrich, USA) in 0.1 M citrate buffer (pH 4.35) containing 0.03% H_2O_2 for 15 min. Absorbance (optical density) at 405 nm was read using a microtiter plate reader (LabSystems Multiskan, USA). The serum was considered as Hsp60-positive in case if the optical density in a 1:50 dilution exceeded the average value of the optical density in control by 2 standard deviations (M + 2 SD). All serum samples were examined for the presence of anti-GroEL and anti-hHsp60 antibodies in 4 replicates.

Statistical analysis. SPSS version 22.0 (IBM SPSS Statistics 22.0) was used for statistical analysis. Continuous variables are expressed as the median (range) and categorical variables are expressed as numbers (percentage). Differences in continuous variables between groups were compared using a one-way ANOVA test. Differences of categorical variables between groups were compared by chi-square test. The 5-year BCR-free survival rate was estimated by the Kaplan — Meier method and differences between subgroups were analyzed by the Log-rank test. Univariate analysis was performed to assess the prognostic relevance of clinicopathological factors. The multivariate Cox regression proportional hazard model was used to evaluate the significant variables in univariate analysis. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The clinicopathological features of the patients are presented in Table 1. The groups of low and intermediate risk were combined.

During the follow-up period (42–93 months) 40/55 (72.7%) patients had developed a BCR, 11/40 (20.0%) of them were in a low and intermediate-risk group, 29/40 (55.7%) high-risk group. Time from surgery to a BCR was 18–72 months.

Levels of IgG anti-GroEL and anti-hHsp60 antibodies were detected in all serum samples investigated by ELISA. There were significant differences in anti-GroEL IgG levels between control and PCa patients (0.210 ± 0.078 against 0.553 ± 0.314 optical density units (o.d.u.), $p < 0.001$). There were no significant differences in anti-hHsp60 IgG levels between control and PCa patients (0.142 ± 0.073 against 0.109 ± 0.112 optical density units, $p > 0.05$).

Before surgery, the elevated levels of IgG anti-GroEL antibodies were detected in 35/40 (90.9%) patients with BCR, 11/11 (100%) of them were in the low and intermediate-risk group and 24/29 (82.7%) in the high-risk group. There was a significant difference in anti-GroEL IgG levels between PCa patients with BCR, PCa patients without BCR and the control group (Fig. 1). However, there was no significant difference in anti-hHsp60 IgG antibodies levels between PCa patients with or without BCR.

The relationship between clinicopathological characteristics and biochemical relapse is presented in Table 1. There was no significant difference in age, clinical and RP specimens TNM stage, biopsy and

Table 1. Baseline characteristics of PCa patients according to BCR

Variables	Total (n = 55)	BCR (n = 40)	No BCR (n = 15)	p
Age, years (range)	64 (52–74)	64 (53–74)	64.5 (52–74)	0.935
T stage (%)				1.0
T1c–2a	8 (14.5%)	4 (10%)	4 (26.6%)	
T2b	8 (14.5%)	8 (20%)	-	
T2c–T3	39 (71%)	28 (70%)	11 (73.4%)	
T4	-	-	-	
Biopsy Gleason score (%)				0.395
⩽6	29 (52.7%)	22 (55%)	7 (46.6%)	
7	13 (41.8%)	13 (32.5%)	8 (53.4%)	
⩾8	3 (5.5%)	5 (12.5%)	-	
Preoperative PSA level, ng/ml (range)	12.3 (4.7–194.7)	12.4 (4.7–194.7)	12 (4.7–72)	0.297
<10	18 (32.7%)	13 (32.5%)	4 (26.6%)	
10–20	21 (38.2%)	14 (35%)	8 (53.4%)	
⩾20	16 (29.1%)	13 (32.5%)	3 (20%)	
RP specimens T stage (%)				0.932
T1c–2a				
T2b	6 (10.9%)	3 (7.5%)	3 (20%)	
T2c–T3	9 (16.5%)	8 (20%)	1 (6.6%)	
T4	39 (71%)	27 (67.5%)	11 (73.4%)	
	2 (3.6)	2 (5%)	-	
RP specimens Gleason score (%)				0.866
⩽ 6	28 (50.9%)	22 (55%)	6 (40%)	
7	24 (43.6%)	16 (40%)	8 (53.4%)	
⩾ 8	3 (5.5%)	2 (5%)	1 (6.6%)	
RP specimens lymph nodes (%)				0.399
N0	44 (80%)	31 (77.5%)	13 (86.6%)	
N1	11 (20%)	9 (22.5%)	2 (13.4%)	
D'Amico risk group (%)				0.132
Low risk	8 (14.5%)	3 (7.5%)	4 (26.6%)	
Intermediate risk	7 (12.8%)	8 (20%)	-	
High risk	40 (72.7%)	29 (72.5%)	11 (73.4%)	
Anti-hHsp60, optical density units, 405 nm (range)	0.178 (0.031–0.629)	0.184 (0.047–0.629)	0.160 (0.031–0.567)	0.800
< 0.285	16 (29.1%)	11 (27.5%)	5 (33.3%)	
⩾ 0.285	39 (70.1%)	29 (72.5%)	10 (66.7%)	
Anti-GroEL, optical density units, 405 nm (range)	0.539 (0.041–1.209)	0.866 (0.257–1.209)	0.228 (0.041–0.422)	0.001
< 0.366	17 (31%)	5 (12.5%)	12 (80%)	
⩾ 0.366	38 (69%)	35 (87.5%)	3 (20%)	

pathological Gleason score, preoperative PSA, lymph node involvement, d'Amico risk groups, and level of IgG to hHsp60 between a group of BCR and the BCR-free group ($p > 0.05$). However, we have found a significant difference in the level of IgG to GroeL between the two groups ($p < 0.001$).

In this cohort, the 1-, 3-, and 5-year BCR-free survival rates were 100%, 93%, and 35% respectively (Fig. 2, *a*). The 1-, 3-, and 5-year BCR-free survival rates in patients with elevated anti-GroEL IgG (> 0.366 o.d.u.) were 100%, 85%, 15%, in comparison with 100%, 95%, and 87% in patients with anti-GroEL IgG ⩽ 0.366 o.d.u. ($p < 0.001$) (Fig. 2, *b*).

Univariate and multivariate Cox analyses were performed to identify the factors that could affect the BCR-free survival of PCa patients (Table 2). In the univariate analysis, only IgG to GroEL level was a significant prognostic factor ($p < 0.05$). The multivariate analysis indicated that IgG to GroEL (hazard ratio = 2.465; 95% confidence interval: 1.311–4.634, $p < 0.05$) was an independent prognostic factor in the patients who developed BCR.

It is considered that high levels of anti-Hsp60 antibodies are associated with several chronic and autoimmune processes [35, 36]. The elevated levels of antibodies against bacterial Hsp60 is an indication of the presence of infection diseases in the past [37]. The question of the role of infectious agents in PCa has been widely debated in the scientific literature over the past several decades [38]. In particular, the risk of PCa was elevated in males who had any sexually

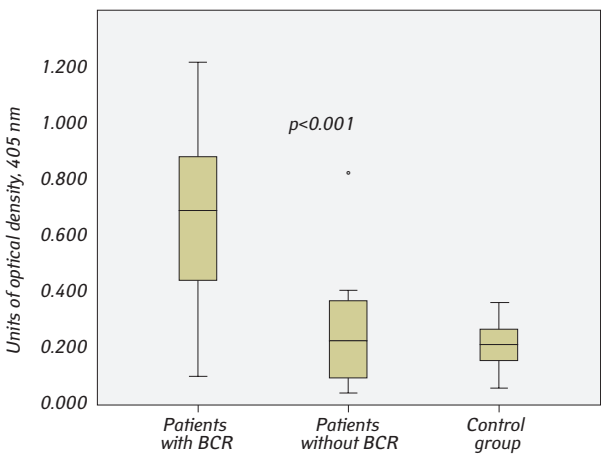

Fig. 1. Levels of IgG against GroEL in PCa patients and healthy individuals

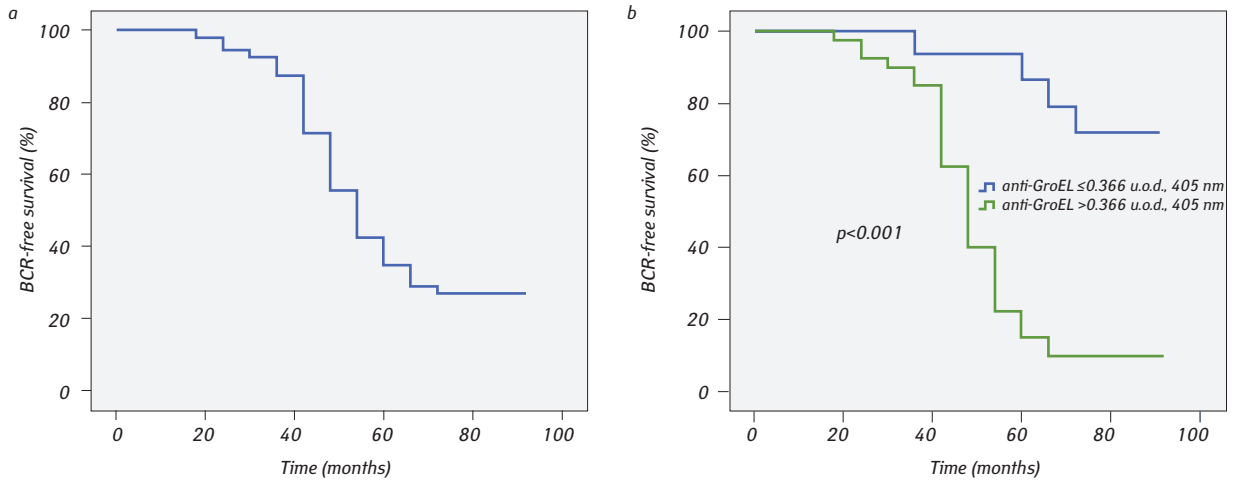

Fig. 2. BCR-free survival in PCa patients: *a* — Kaplan — Meier BCR-free survival plot for all PCa patients under study; *b* — Kaplan — Meier BCR-free survival plots for the groups with different preoperative anti-GroEL IgG levels

Table 2. Univariate and multivariate analysis of BCR-free survival in PCa patients

Variables	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% confidence interval	*p*	Hazard ratio	95% confidence interval	*p*
Age, years (> 62 *vs* ⩽ 62)	1.197	0.629–2.278	0.583			
T (⩾ T2c *vs* T1c-T2b)	0.931	0.473–1.832	0.835			
Biopsy Gleason score (⩾7 *vs* ⩽6)	0.996	0.534–1.857	0.988			
PSA, ng/ml (⩾20 *vs* <20)	0.733	0.378–1.421	0.357			
D'Amico risk group (high *vs* low + intermediate)	0.802	0.400–1.606	0.532			
Lymph nodes (positive *vs* negative)	0.626	0.297–1.317	0.217			
IgG against hHsp60, optical density units 405 nm (> 0.285 *vs* ⩽ 0.285)	0.969	0.514–1.827	0.923			
IgG against GroEL, optical density units 405 nm (> 0.366 *vs* ⩽ 0.366)	2.548	1.364–4.760	<0.001	2.465	1.311–4.634	0.005

transmitted infections or only gonorrhea in their lifetime [39].

Hsp60 has apoptotic dualism, it regulates IL-8 and TGF-β production and their release in cancer cells. The overexpression of Hsp60 is associated with increased expression of anti-apoptotic proteins Bcl-xl and Bcl-2 and decreased expression of Bax [13, 40–42]. Hsp60 is able to promote metastasis [43]. Hsp60 is involved in immune response, it enhances the activation of cytotoxic T cells through activated macrophages, the chaperone boost secretion of pro-inflammatory cytokines by macrophages and dendritic cells and it acts as a costimulator of the human regulatory T cells $CD4^+CD25^{int}$ and $CD4^+CD25^{hi}$ [10, 42–44].

This pilot study investigated serum reactivity to Hsp60 in PCa patients before surgery as an additional risk marker of a BCR after RP. It was found that initial PSA level, biopsy Gleason score, clinical TNM stage, and RP specimens Gleason score with TNM did not demonstrate accurate potential predicting the relapse in rising serum PSA postoperatively.

Although the number of patients in our study was not large enough, we have demonstrated that elevated preoperative levels of IgG against bacterial Hsp60 in patients were associated with earlier biochemical relapse and lower 5-year BCR-free survival rate, respectively.

Human Hsp60 (the target antigen) can be recognized not only by antibodies against hHsp60 but also by the cross-reactivity of antibodies against bacterial Hsp60 presented in serum samples of PCa patients. We suggest that the elevated levels of IgG antibodies against bacterial Hsp60 before surgery can predict the early occurrence of BCR after RP and can serve as an additional independent risk biomarker.

The role of anti-Hsp60 antibodies in malignant transformation is still largely unknown. Whether anti-Hsp60 antibodies are only the markers of earlier BCR onset or could affect cancer cell viability remains to be clarified. Nonetheless, the further study of bacterial and human Hsp60 as diagnostic and prognostic biomarkers in PCa patients, who will receive RP, seems to be promising.

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РІВЕНЬ СИРОВАТКОВИХ АНТИТІЛ ДО БІЛКІВ ТЕПЛОВОГО ШОКУ 60 ЯК ДОДАТКОВИЙ БІОМАРКЕР РОЗВИТКУ БІОХІМІЧНОГО РЕЦИДИВУ ПІСЛЯ РАДИКАЛЬНОЇ ПРОСТАТЕКТОМІЇ

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***Стан питання*:** Рівень білка теплового шоку 60 (БТШ60) підвищений у пухлинних клітинах у порівнянні з клітинами нормального простатичного епітелію. БТШ60 залучений до пухлинного росту, інвазії та метастазування і розглядається як діагностичний та прогностичний біомаркер онкологічних захворювань. ***Мета*:** Дослідити рівень антитіл до прокаріотичного гомолога білка теплового шоку 60 (ПГБТШ60) у хворих на рак передміхурової залози (РПЗ) на предмет можливості використання цього показника як додаткового предиктора розвитку біохімічного рецидиву (БР) після радикальної простатектомії (РПЕ). ***Матеріали та методи*:** У дослідження включено 55 хворих на клінічно локалізований та місцево-розповсюджений РПЗ, яким у період з червня 2013 р. до травня 2014 р. було виконано РПЕ. Рівень антитіл до ПГБТШ60 та людського БТШ60 (лБТШ60) визначали до хірургічного втручання за допомогою імуноферментного аналізу. Зразки сироватки крові пацієнтів з низькою реактивністю до ПГБТШ60 та лБТШ60 було використано як контрольні. Досліджувалася залежність між клініко-патологічними показниками та рівнем IgG проти ПГБТШ60 та лБТШ60. Ступінь біохімічної безрецидивної виживаності оцінювали за допомогою методу Каплана — Меєра. Одно- та багатофакторна модель регресії Кокса застосовувалася для визначення факторів ризику впливу на біохімічну безрецидивну виживаність. ***Результати*:** Різниця рівнів анти-ПГБТШ60 IgG між групою хворих на РПЗ та контрольною була статистично достовірною (0.210 ± 0.078 проти 0.553 ± 0.314 одиниць оптичної щільності відповідно, $p < 0{,}001$), в той час як достовірності різниці в рівнях анти-лБТШ60 антитіл IgG між цими групами не виявлено. Протягом періоду спостереження (42–93 міс) у 40 з 55 хворих відмічено розвиток БР. Час від хірургічного втручання до розвитку БР становив 18–72 міс. Підвищення рівнів анти-ПГБТШ60 IgG у хворих, яким виконували РПЕ, асоціюється з раннім виникненням БР та нижчим показником 5-річної біохімічної безрецидивної виживаності ($p < 0{,}001$). Багатофакторний аналіз продемонстрував, що рівень IgG до ПГБТШ60 (відношення ризиків = 2,465, 95% довірчий інтервал: 1,311–4,634, $p < 0.05$) може розглядатися як незалежний прогностичний фактор розвитку БР. ***Висновки*:** Підвищення рівня IgG антитіл проти ПГБТШ60 перед хірургічним втручанням є предиктором раннього розвитку БР після РПЕ та може виступати в ролі додаткового незалежного біомаркера ризику виникнення БР після РПЕ.

***Ключові слова*:** білок теплового шоку 60, БТШ60, біохімічний рецидив, радикальна простатектомія, рак передміхурової залози.