Հայաստանի Գիտությունների Ազգային Ակադեմիա
Национальная Академия Наук Армении
National Academy of Sciences of Armenia

Հայաստանի Կենսաբանական Հանդես
Биологический Журнал Армении
Biological Journal of Armenia

• Փորձարարական և տեսական հոդվածներ •Экспериментальные и теоретические статьи•
•Experimental and theoretical articles•

Biolog. Journal of Armenia, 2 (68), 2016

THE RELATIONSHIP BETWEEN ARGINASE ACTIVITY AND CHANGE OF POLYAMINES QUANTITY IN HUMAN BLOOD SERUM DURING BREAST CANCER

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Cancer cells may differ from their normal counterparts in the activities of certain enzymes. That difference may act as a useful biological marker of malignancy in particular tumors. Arginase (EC 3.5.3.1) hydrolyses L-arginine into urea and L-ornithine (precursor for polyamines). The enzyme activity as a test for cancer diagnosis and treatment is suggested. The main goal of the study is not only to reveal a new tumor marker, but also to clarify new relationship mechanisms between Arginase activity and change of polyamines quantity in healthy and cancer cells, which will allow influencing metabolic different processes of cancer cells.

Arginase – polyamine – breast cancer – diagnosis and treatment

Քաղցկեղային բջիջները կարող են տարբերվել իրենց առողջ անալոգներից որոշակի ֆերմենտների ակտիվությամբ: Այդ կենսաբանական ցուցանիշը կարող է ծառայել որպես մարկեր տվյալ ուռուցքի չարորակության գնահատման համար: Արգինազը (EC 3.5.3.1) կատալիզում է L-արգինինի հիդրոլիզը L-օրնիթինի (պոլիամինների նախանյութ) և միզանյութի առաջացումը: Ենթադրվում է, որ արգինազի ակտիվության արժեքը կարող է կիրառվել որպես դիագնոստիկ թեստ քաղցկեղի ախտորոշման և բուժման նպատակով: Աշխատանքի հիմնական նպատակը ոչ միայն նոր ուռուցքային մարկերի բացահայտումն է, այլ նաև արգինազի ակտիվության և պոլիամինների քանակական փոփոխության փոխկապակցվածության նոր մեխանիզմների պարզաբանումը առողջ և քաղցկեղային բջջում, ինչը թույլ կտա միջամտել քաղցկեղային բջիջների նյութափոխանակային տարբեր պրոցեսներին:

Արգինազ – պոլիամին – կրծքագեղձի քաղցկեղ – ախտորոշում և բուժում

Раковые клетки отличаются от здоровых аналогов активностью некоторых ферментов. Этот биологический индикатор может служить маркером для оценки качества опухоли. Аргиназа (ЕС 3.5.3.1) катализирует гидролиз L-аргинина в L-орнитин (предшественник полиаминов) и мочевину. Предполагается, что уровень ферментативной активности аргиназы может быть использован в качестве теста для диагностики и лечения рака. Наша основная цель заключается не только в открытии нового опухолевого маркера, но и в изучении новых механизмов взаимосвязи между аргиназной активностью и изменением количества полиаминов в здоровых и раковых клетках, которые позволят выяснить некоторые метаболические процессы в раковых клетках.

Аргиназа – полиамины – рак молочной железы – диагностика и лечение

Great medical and biological efforts are continually invested into understanding of cancers pathology and finding new methods for its diagnosis and treatment [1]. Cancer cells may differ from their normal counterparts in the activities or concentration of certain enzymes. The application of measures correlating the activities of such enzymes may lead to elucidation of therapeutic approaches to cancer [14]. Arginase (EC 3.5.3.1) hydrolyses L-arginine into urea and L-ornithine (polyamines precursor) [6, 10]. Our goal to investigate Arginase activity in blood serum during different stages of breast cancer, suggesting the enzyme activity as a test for cancer diagnosis and treatment [7, 11, 16]. Currently are shown rapid growth of polyamines (spermine, spermidine, putrescine) quantity in blood serum and urine during malignant tumors in different organs [15]. As polyamines are essential for cell growth, one of the mechanisms by which polyamines accelerate tumor growth is through the increased availability of this indispensable growth factor [17]. In addition, polyamines seem to accelerate tumor invasion and metastasis not only by suppressing immune system activity against established (already existing) tumors but also by enhancing the ability of invasive and metastatic capability of cancer cells [12]. Currently are actual research works about the clarification of Arginase isoforms function in polyamines biosynthesis [5, 8]. Polyamines which are putrescine, spermidine and spermine are alkaline aliphatic amines, which electrostatically interact with macromolecules and modulate their biosynthesis, cell proliferation and apoptosis [9, 17]. Cancer cells produce proteases to destroy the surrounding matrix, and produce proteins to create new vessels. Hypoxic cancer cells lose their adhesion characteristics and have enhanced capacity for migration. Polyamines synthesized by cancer cells are transferred to cancer cells under hypoxic conditions that have increased capacity for polyamine uptake and decreased intracellular polyamine synthesis. Increased polyamine uptake by immune cells results in decreased production of tumoricidal cytokines and the amount of adhesion molecules, and these eventually attenuate the cytotoxic activities of immune cells [12]. The levels of Arginase activity and polyamines quantity in malignant tissues were reported by several scientists to be increased compared with healthy tissues [7, 15, 16]. However, besides the limited number of these studies, none of authors traced the relation between Arginase isoenzymes activity and biological behavior of tumors and polyamines quantity. Therefore, the present study was designed not only to determine Arginase activity levels in cancer tissues but also to correlate them with biological behavior of this tumor. There are two distinct isoforms of Arginase: Arginase I or ureotelic Arginase and Arginase II or nonureotelic Arginase, which have similar enzymatic features, but different cell localization and tissue distribution, amino acid sequence structure (53% is the same) and radically different *pI* value [10, 13]. The previous studies of our laboratory were to reveal the role of Arginase isoenzymes in polyamines biosynthesis in rats. It was shown that the necessary amount of ornithine for the biosynthesis of polyamines is provided via Arginase II [3, 4]. Before coming to the above mentioned conclusion we have identified two important facts. The kinetic studies of ureotelic and nonureotelic Arginases have shown that AII has a special stereospecific part which bind polyamines in contrast to ureotelic Arginase. It was shown proportionate decrease of polyamines amount during inhibition of nonureotelic Arginase with N^G-hydroxy-L-arginine.

Materials and methods. This study is performed with blood serum of patients with breast cancers who were hospitalized in the National Center of Oncology aft. V.A.Fanarjyan. The most common system used to describe the stages of different types of cancers is the American Joint Committee on Cancer TNM system. Arginase activity is determined in blood serum of 7 healthy individuals and patients with breast cancer (28 patients, I-III stages, 37-72 years old).

*Separation and purification of Arginase isoenzymes****.*** The method of Kossman (1966) was used with some modifications. On the blood serum 0,2M Glycine buffer, pH 9,5 was added. In the column (2,5×50 cm) containing Sephadex G-150 the blood serum - 0,2M Glycine buffer mixture was added. The column was balanced with Na-phosphate buffer (pH 7,2) and 40 fractions each one of 4 ml were collected. 4 ml of high-molecular-weight protein fraction after gel-filtration is passed through the column CM-cellulose (1.5x35 cm), balanced against 0.005 M Tris-HCl buffer, pH 7.2, elution gradiented with the same buffer gradual increasing of molarity from 0.05 to 0.25 M KCl, elution speed is 24 ml/h, was collected 32 fractions each one of 4 ml.

*Archibald's method for Arginase activity determination****.*** The method of Van Slyke and Archibald (1946) was used with some modifications. In test-tube is added 1.5ml 0.2M Glycine buffer, 0.5ml blood serum, 0.2ml 5µM $MnCl_2x4H_2O$, 0.4ml 50µM L-arginine. Enzyme catalysisis was stopped with 1ml 20% trichloroacetic acid. In supernatant is determined the final product of the catalysis which is urea. Add in a test-tube 2.5ml acidic mixture (3 parts of concentric H_3PO_4, one part of concentric H_2SO_4, 0.237g $MnSO_4xH_2O$, 1.7ml 0.1M $FeCl_3$, 398ml distilled water), 1ml supernatant, 0.25ml 3% DAMO (diacetyl monoxime) and boil it in water bath 45 minutes. The intensity obtained yellow color measure with spectrophotometer in 487nm (Genesys 10, USA) [2]. Activity of enzyme is evaluated with the received urea, in micromoles for 1ml fresh tissue.

*Dansylation and thin layer chromatography (TLC) analysis****.*** The method of Seiler (1970) and Khan (2006) was used with some modifications as follows. Tissues were extracted in 0.2M cold $HC1O_4$ at a ratio of about 100 mg/ml $HC1O_4$. After extraction for 1 h in an ice bath, samples were pelleted at 11.500g x 20 min in +4°C. 200 µl of $HClO_4$ extract were mixed with 400 µl of dansyl chloride (5 mg/ml in acetone), and 200µl of saturated sodium carbonate were added. After brief vortexing, the mixture was incubated in darkness at room temperature overnight. Excess dansyl (5-(Dimethylamino) naphthalene-1-sulfonyl chloride) was removed by reaction with 100 µl (100mg/ml) of added proline, and incubation for 30 min. Dansylpolyamines were extracted in 0.5 ml benzene, and vortexed for 30s. Up to 50µl of dansylated extract were loaded on the preadsorbent zone of silica gel plates, and the chromatogram was developed for about 1h with chloroform/triethylamine (25:2, v/v) solvent system. The R_F values were calculated using the formula, R_F = distance traveled by solute spot/distance traveled by solvent front. The dansylpolyamine bands were scraped, eluted in 2 ml ethyl acetate, and quantified in 505 nm (Genesys 10, USA) [5, 15].

Statistical Analysis. Results are expressed as means ± SD and means ± SE. Results are examined by Student's t-test (single sample) using Statistica software (StatSoft 10.0).

Results and Discussion. Arginase activity was determined in blood serum of patients with breast cancer in different stages: 10 cases were stage I (56±9, $T_1N_0M_0$, 1-2 cm), 10 cases had stage II (48±11,$T_{1-2}N_1M_0$,2-2.7 cm), 8 cases had stage III (56±12, $T_{2-3}N_{1-3}M_0$,4.3-5.4 cm) (tab. 1).

Table 1. Demographic characteristics of patients with breast cancers

Patients	Sex	Age	Cancer	Stage	TNM
7	Female	50±8	-	-	-
10		56±9	breast	I	$T_1N_0M_0$
10		48±11	breast	II	$T_{1-2}N_1M_0$
8		56±12	breast	III	$T_{2-3}N_{1-3}M_0$

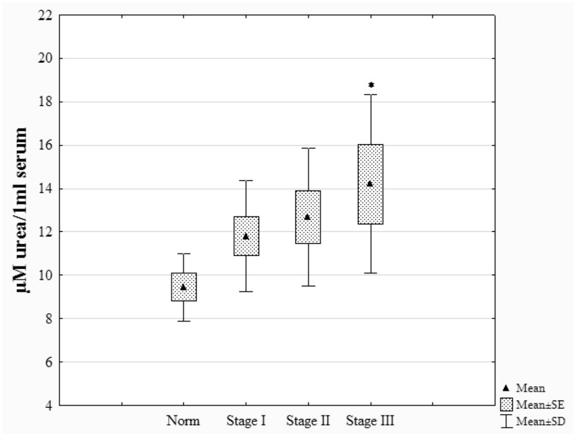

Fig. 1. The change of Arginase activity in womens' serum during different stage of breast cancer ($p<0.001$). Breast cancer group patients: 10 cases were stage I ($T_1N_0M_0$), 10 cases had stage II ($T_{1-2}N_1M_0$), 8 cases had stage III ($T_{2-3}N_{1-3}M_0$), * - $p<0.05$.

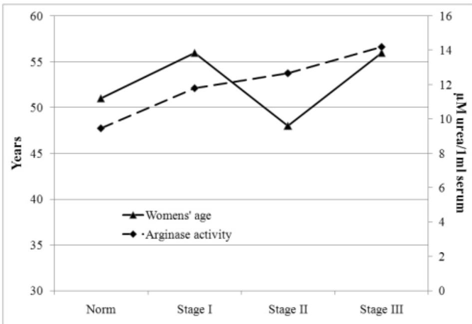

Fig. 2. The correlation between Arginase activity, cancer stage and human age in blood serum at breast malignant tumors

Our studies have shown that in women breast cancer group of stage I activity of serum Arginase was increased by 28.8%, in group of stage II by 36.1% and group of stage III by 48.4% comparing to the healthy women group (fig. 1).

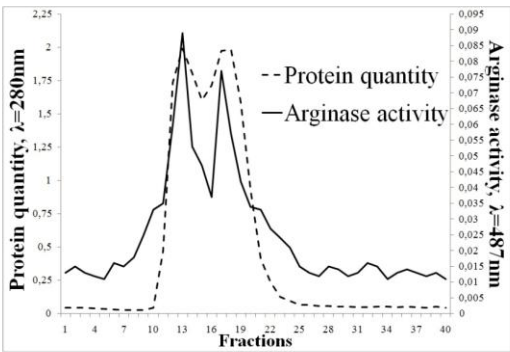

Fig. 3. The spectrum of Arginase isoenzymes and protein quantity after gel-filtration (Sephadex G-150) in blood serum of healthy patients (n=5, p<0,05).

Studies have shown that there is no correlation between human age and Arginase activity changes: consistent pattern for stage 1-3 does not change (fig.2). Consequently, the more advanced the breast cancer, the higher the level of serum Arginase activity. It has been reported that the mean activity of Arginase is high in the early stages and higher in the advanced states of the malignant group compared to those of the normal ones.

We have tested the Arginase activity and protein quantity spectrum in blood serum of patients with breast cancer (3 patients, II stage) through gel filtration (Sephadex G-150) and ion-exchange chromatography (CM-cellulose). The spectrum of Arginase isoenzyme and protein quantity in blood serum of healthy patient after gel-filtration (Sephadex G150) we have two peaks, respectively in protein fractions number 13^{th} and 17^{th} (fig. 3). Both fractions were separated with CM-cellulose. After ion-exchange chromatography of 13^{th} fraction, we have two peaks for protein quantity and arginaes isoenzyme (fig. 5).

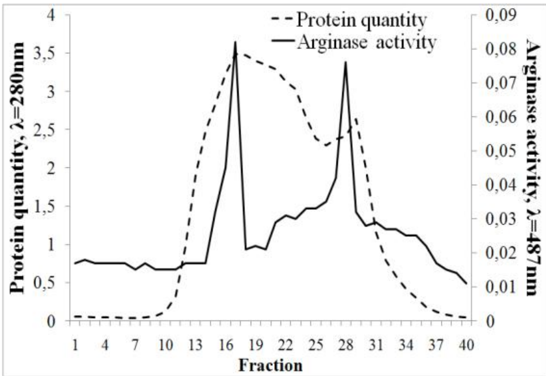

Fig. 4. The The spectrum of Arginase isoenzymes and protein quantity after gel-filtration (Sephadex G-150) in blood serum of patients with breast cancer (n=5, p<0,05).

In blood serum of patients with breast cancer after gel-filtration (Sephadex G150) we have two peaks, respectively 17^{th} and 29^{th} in protein fractions of protein quantity and Arginase isoforms spectrums (fig. 4). In contrast to the standard peaks are shifted to the right. In 17^{th} and 29^{th} fractions Arginase activity was increased respectively by 43.9% and 33.5%. The 17^{th} high molecular fraction was separated by CM-cellulose.

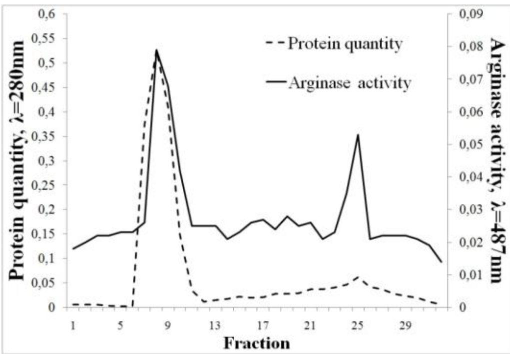

Fig.5. The spectrum of Arginase isoenzymes and protein quantity after ion-exchange chromatography in blood serum of healthy patients (fraction N13, CM-Cellulose, during fractions 2, 9, 15, 21, 27-0.05M, 0.1M, 0.15M, 0.2M and 0.25M KCl respectively, n=5, p<0,05).

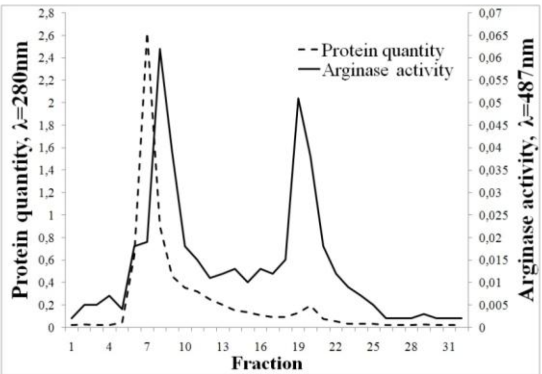

Fig.6. The spectrum of Arginase isoenzymes and protein quantity after ion-exchange chromatography in blood serum of patients with breast cancer (fraction N17- number one peak, CM-Cellulose, during fractions 2, 9, 15, 21, 27 0.05M, 0.1M, 0.15M, 0.2M և 0.25M KCl respectively, n=5, p<0,05).

After ion-exchange chromatography we have two peaks in spectrum of Arginase isoenzyme and protein quantity, respectively in 7^{th} and 20^{th} fractions. Comparing to the standards the peaks are curved to the left. In contrast to the healthy patients Arginase activity in the 7^{th} and 20^{th} fractions Arginase activity of blood serum is increased respectively by 5.2% and 18.6% (fig. 6). These data show that after partial purification of enzymes with ion-exchange chromatography Arginase activity in fractions of patients with malignant tumors is also increased. It should be mentioned that also is changed the spectrum of Arginase activity and protein quantity.

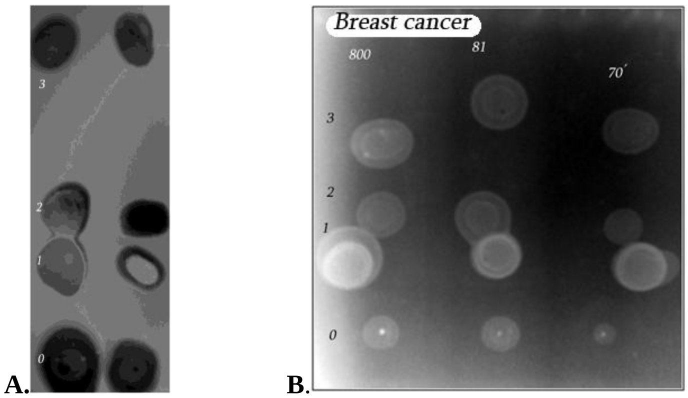

Fig.7. A. Quantitative and qualitative analysis of blood serum of a healthy (A) and breast cancer patient (B) through thin layer chromatography (A, RFSPM – 0.29, RFSPD – 0.45, RFPUT – 0.82). B – I stage (70`, RFSPM – 0.3, RFSPD – 0.42, RFPUT – 0.67), II stage (81, RFSPM – 0.33, RFSPD – 0.42, RFPUT – 0.75) and III stage (800, RFSPM – 0.32, RFSPD – 0.44, RFPUT – 0.65). The quantity of polyamines is presented in nM polyamine in 50µl of experimental solution (n=5, p<0,05, 0 – origin, 1-SPM, 2-SPD and 3-PUT).

For the next stage, we have performed quantitative analysis for polyamines through thin layer chromatography in blood serum of healthy patients and patients with cancer (fig.7). The results show that polyamines quantity is increased in blood serum of patients with cancer, and the increase is corresponding to the growth of the stage of the disease (tab. 2).

Table 2. The change of polyamines quantity in blood serum of healthy patients and patients with cancer (the quantity of polyamines is presented in nM polyamine in 50µl of experimental solution), M±m, n=5, p<0,05).

Stage of desease	Polyamins	Polyamine quantity (nM polyamine in 50 µl of experimental solution)
0	PUT	12,2±2,3
	SPD	9,6±1,4
	SPM	14,3±2,4
I	PUT	17,2±3,1
	SPD	20,4±2,9
	SPM	23,6±3,8
II	PUT	20,8±2,7
	SPD	24,3±3,2
	SPM	27,4±3,7
III	PUT	22,9±2,1
	SPD	25,6±3,3
	SPM	34,8±4,2

The increase of total polyamines quantity compared with standard is 69.5%, 101% and 131%, respectively in I, II and III stages. The increase of polyamines quantity coincides with the increase of Arginase activity, what shows the correlation between them during the disease.

The practical importance of our work is through the change of nonureotelic Arginase activity, we can influence on polyamines quantity, thus influencing on cancer cell's metabolism. In our studies, a significant increase in blood serum Arginase activity could be important for the early diagnosis of mentioned cancer and for their treatment. Our results will serve for structure-based drug design, because we suggest that Arginase inhibition may have some protective effects on different types of cancers development as it inhibits ornithine levels, precursors of polyamines, and also polyamines levels. The synthesis of new inhibitor for Arginase, which will be harmless for organism, will allow us to change the course of cancer. Our further investigations will be directed to answer to the mentioned question.

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Received on 27.01.2016