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Abstract

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THE STUDY OF THE ACTIVATION OF MTORC1 AND ITS SUBSTRATE P70S6K INVOLVED IN TYPE 2 DIABETES MELLITUS AND ONCOGENETIC PROCESSES

Introduction. Pathogenetic factors of diabetes may affect the activity of intracellular systems of oncogenesis and metabolism regulation, one of which is PI3K/Akt/mTORC1. Macrophages and lymphocytes are involved in the pathogenesis of diabetes and cancer. Detection of excessive activation of PI3K/Akt/mTORC1 components and substrates in these cells may indicate the need for additional correction of metabolic processes in patients with type 2 diabetes from the point of prevention of cancer. **The aim:** to study the activation of mTORC1 by determining the phosphorylation of PRAS40 and p70S6K1 in the leukocytes of patients with type 2 diabetes and cancer.

Materials and methods. The study included women from the following groups: control group, patients with type 2 diabetes, cancer patients, patients with both diseases. The content of phosphorylated PRAS40 (phospho-T246) and p70S6K1 (phospho-T389) was determined using laboratory kits ELISA KNO0421 and ELISA 85-86053 of Invitrogen (USA). The protein concentration in the lysate was determined using a BCA Novagen protein assay kit (USA). Measurements were performed on a microplate reader (Bio-tek Instruments, USA) at a wavelength of 450 nm.

Results. Significantly increased content of phosphorylated PRAS40 and p70S6K1 in leukocytes of patients with type 2 diabetes mellitus and cancer was detected. The number of positive phospho-PRAS40 tests in patients with diabetes was 83.3%, and in cancer patients - 66.7%. Was revealed the reduced content of phospho-PRAS40 in leukocytes of patients with a combination of diabetes and cancer.

Conclusions. The increased amount of phosphorylated PRAS40 and p70S6K1 proves the activation of the studied signaling pathway by diabetes mellitus type 2. Its decrease by cancer and diabetes can be explained by the possible competing effects of the proteins that affect upstream regulators of these kinases or them directly.

Key words: type 2 diabetes, signaling pathway, PRAS40, mTOR, p70S6K, cancer.

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ДОСЛІДЖЕННЯ АКТИВАЦІЇ MTORC1 ТА ЙОГО СУБСТРАТУ P70S6K, ЗАЛУЧЕНИХ ПРИ ЦУКРОВОМУ ДІАБЕТІ 2 ТИПУ ТА ОНКОГЕНЕТИЧНИХ ПРОЦЕСАХ

Вступ. Новітні дослідження довели вплив патогенетичних факторів цукрового діабету на активність внутрішньоклітинних сигнальних шляхів регуляції онкогенезу і метаболізму, одним з яких є PI3K/Akt/mTORC1. Макрофаги та лімфоцити беруть участь у патогенезі діабету та раку. Надмірна активація компонентів та субстратів PI3K/Akt/mTORC1 в цих клітинах може вказувати на необхідність додаткової корекції метаболічних процесів у хворих на діабет 2 типу з точки зору профілактики онкологічних захворювань. Мета: вивчити активацію mTORC1 шляхом визначення фосфорилювання PRAS40 та p70S6K1 у лейкоцитах хворих на діабет 2-го типу та рак.

Матеріали та методи. Жінки, залучені у дослідженні, були поділені на групи: контрольна група, пацієнти з діабетом 2 типу, хворі на рак та пацієнти з поєднанням раку і діабету. Вміст фосфорильованих PRAS40 (фосфо-T246) та p70S6K1 (фосфо-T389) визначали імуноферментним методом, використовуючи лабораторні набори ELISA KHO0421 та ELISA 85-86053 фірми Invitrogen (США). Концентрацію білка в лізаті визначали за допомогою набору для аналізу протеїнів BCA Novagen (США). Вимірювання проводилися на мікропланшетному зчитувачі (Bio-tek Instruments, США) при довжині хвилі 450 нм.

Результати. Виявлений достовірно підвищений вміст фосфорильованих PRAS40 та p70S6K1 у лейкоцитах хворих на цукровий діабет 2-го типу та рак. Кількість позитивних фосфо-PRAS40 проб у хворих на діабет становила 83,3%, а у хворих на рак - 66,7%. Виявлений знижений вміст фосфо-PRAS40 в лейкоцитах пацієнтів з поєднанням діабету і раку.

Висновки. Підвищений вміст фосфорильованих PRAS40 та p70S6K1 доводить активацію досліджуваного сигнального шляху при цукровому діабеті 2 типу. Зниження їх активації при поєднанні раку та діабету можна пояснити можливими конкуруючими ефектами білків, які впливають на регулятори цих кіназ або на них безпосередньо.

Ключові слова: діабет другого типу, сигнальний шлях, PRAS40, mTOR, p70S6K, рак.

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Introduction

Newest researches prove an increased risk of cancer in patients with diabetes mellitus (DM). Predisposition of patients with DM to cancer is realized through various pathological mechanisms, including obesity, cytokine imbalance, hyperinsulinemia, hyperglycemia and oxidative stress. These factors affect intracellular systems of regulation of cell`s survival and metabolism. Signal pathway PI3K/Akt/mTOR is one of these regulatory systems, its dysregulation leads to severe diseases such as cancer and type 2 diabetes (T2D) [1]. Insulin and insulin-like growth factor (IGF-1) are the main stimulators of this pathway in patients with DM. Hyperinsulinemia promotes the IGF bioavailability due to the decrease in the synthesis of IGF-binding proteins 1,2 (IGFBP1,2) in the liver, as well as through the indirect effects on growth hormone (GH) secretion [2].

mTOR (mammalian target of rapamycin) includes two complexes: mTORC1 and mTORC2. Raptor (regulatory-associated protein of mTOR) and PRAS40 (proline-rich Akt substrate 40kDa) are specific for mTORC1. mTORC1 is a highly

conserved serine/threonine protein kinase, which controls the cell growth and homeostasis, including protein synthesis, lipogenesis, glucose metabolism, autophagy, biogenesis of ribosomes and lysosomes, proliferation and survival in response to environmental signals such as amino acid levels, glucose, energy, oxygen, and growth factors [3]. Raptor is a scaffolding protein that mobilizes substrates for the mTOR kinase, interacting with their motifs (TOR signaling) [4]. PRAS40 is Akt1-1 substrate (AKT1S1) and component of the mTORC1/p70S6K signaling pathway [5]. PRAS40 is both a substrate and a negative regulator of mTORC1 and is phosphorylated by Akt, Pim-1 (T246) and mTORC1 (S183/S212/S221). Phosphorylation causes binding of PRAS40 to the protein 14-3-3-ζ, to the dissociation of PRAS40 and raptor and, respectively, the activation of mTORC1 [5, 6, 7]. The increased level of PRAS40 phosphorylation was detected by several types of tumors [1].

Activated mTORC1 phosphorylates and changes functional activity of S6K-protein kinase (p70S6K1), which is responsible for protein synthesis in ribosomes and regulates growth, proliferation, apoptosis, cell's survival, as well as metastasis and invasion of cancer cells [8]. Phosphorylation of p70S6K1 through activated mTOR enhances of insulin resistance [5].

The composition of leukocytes includes several types of cells that play an essential role in the development of pathological conditions: cancer, diabetes and its complications [9, 10, 11].

Taking into account the effects of pathogenetic factors of T2D on the activity of the signal path, the determination of content of phosphorylated kinase PRAS40, p70S6K may have an additional value for determining oncological risk in patients with T2D.

Our aim was to study the mTORC1 activation by determining the phosphorylation of its inhibitor PRAS40 and downstream substrate p70S6K1 in leukocytes of patients with T2D and cancer.

Materials and methods. The clinical trial was carried out in accordance with the guidelines of the Helsinki Declaration (1975) and its revised version of 1983. All patients signed an informed consent for further diagnostic and research work. During the study 66 women were examined. All examined patients belonged to the Caucasian race, age was in range from 46 to 72. The bases for the study were: Precarpathian Oncology Center and Regional Clinical Hospital in Ivano-Frankivsk.

All the patients involved were women in order to avoid the inaccuracies during calculations and as two of the oncological diseases described in this study have gender specifics. Women were divided into groups: I – healthy (control group) (n = 12), II – patients with T2D (n = 18), III – patients with cancer without T2D (n = 18), IV – women with combination of cancer and T2D (n = 18). Patients were grouped accordingly to age and BMI. All patients with T2D used different kinds of therapy, including antidiabetic pills, insulin or their combinations. In the investigation were involved women with endometrial, breast and colorectal cancer. Blood collection was carried out before special anti-tumor therapy: chemotherapy, hormonal therapy or radiotherapy.

Immediately after collection blood was centrifuged at RT in the 15 ml conical Falcon™ tubes using Histopaque 1077 (Sigma, USA) as a substrate, collected lymphocytes were washed in PBS and frozen at -80°C until further use. For determinations of phospho-PRAS40 (phospho-T246) and phospho-p70S6K1 (phospho-T389) amount ELISA kits KHO0421 and 85-86053 respectively (both from Invitrogen, USA) were used. The studies were carried out in triplets. The cells were lysed in the extraction buffer with inhibitors of proteases and phosphatases from the kit. The protein concentration in the lysate was determined using Novagen (USA) BCA protein assay kit. The measurements were carried out on a microplate reader (Bio-tek Instruments, USA) at a wavelength of 450 nm. The OD values of samples obtained are located on the calibration curve satisfactorily coinciding with a theoretical curve that indicates no scattering of the data. DM compensation was assessed by determining the level of HbA1c by the method of ion-exchange chromatography, using the BIO-RAD D-10 analyzer, the BIO-RAD (USA) reagents. PRAS40 was determined in units, according to the study guide; the level of p70S6K1 was determined in conventional units, depending on the amount of protein in the blood cell lysates.

Analysis of the data was carried out using Statistica 12.0 (StatSoft Inc., USA), One-Way ANOVA program. The data are presented in tables as $x \pm SD$ ($x \pm$ standard deviation). Differences between the values in the control and experimental groups were determined using the Student's t-test. Values of $P < 0.05$ were considered as significant.

The research protocol was approved by the Ethics Committee at the Ivano-Frankivsk National

Medical University (protocol № 97/17 of 19 October 2017). Written informed consent was obtained from each study participant.

Results. The results confirm the frequent clinical cases of cancer in people aged over 55 years on the background of obesity. HbA1c levels in II and IV groups of patients were higher than 7.5% **(Table 1).**

Table I – Characteristics of age, BMI and HbA1c in patients involved in the study (x ± SD)

Indexes	I group (n = 12)	II group (n = 18)	III group (n = 18)	IV group (n = 18)
Age (years)	60.0 ± 30.71	57.50 ± 1.17	59.2 ± 2.19	58.5 ± 1.93
BMI (kg/m^2)	30.22 ± 1.13	32.12 ± 1.49	32.47 ± 0.93	30.2 ± 1.84
HbA1c (%)	-#	7.68 ± 1.66	- #	8.20 ± 0.73

* – the difference from the control group is significant,

P < 0.05; # - HbA1c for patients without diabetes was not determined

The amount of phosphorylated PRAS40 significantly increases in leukocytes of patients of group II with T2D and of group III with cancer, but decreases in patients with combination of diabetes and cancer compared to control group (P < 0.05) (Fig.1.). Level of phospho-PRAS40 in women of group IV was significantly decreased in comparison to patients from other experimental groups (P < 0.05) **(Figure 1).**

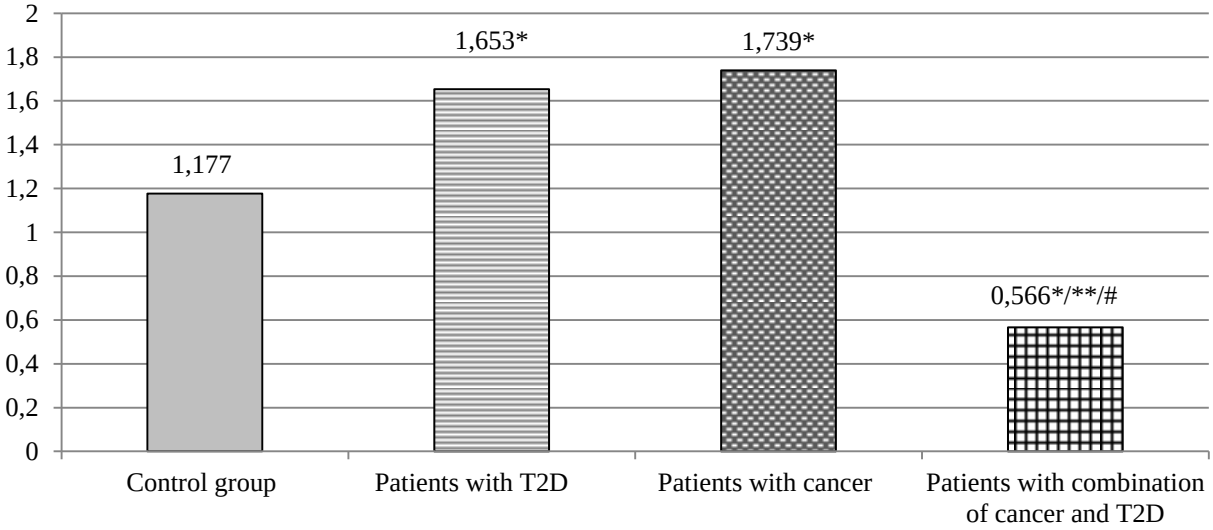

Figure 1 – Content of phospho-PRAS40 in patients with T2D and cancer: * – the difference from the control group is significant, P < 0.05; ** – the difference from the group patients with T2D is significant, P < 0.05. # – the difference from the group patients with cancer is significant, P < 0.05

The number of phospho-PRAS40-positive samples in patients with T2D was 83.3%, and in patients with cancer – 66.7%.

According to the results of study, significant increase in the p70S6K1 phosphorylation was detected in groups of patients with T2D and with cancer compared to control group (P < 0.05) (Fig. 2). Level of phospho-p70S6K1 in women of group IV was significantly decreased compared to patients from the other experimental groups (P < 0.05), but not significantly, compared to control group **(Figure 2).**

Significant difference in the activity of kinases PRAS40 and p70S6K in the PBMC between patients with different types of cancer within groups III and IV was not observed (P > 0.05).

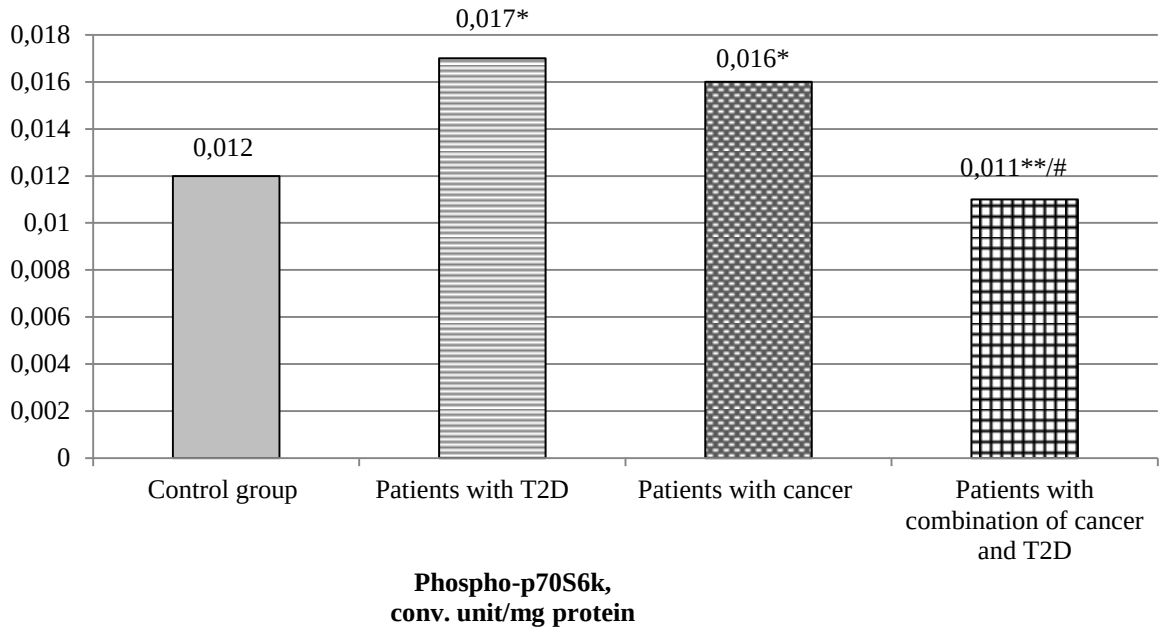

Figure 2 – Content of phospho-p70S6K1 in patients with T2D and cancer: * – the difference from the control group is significant, P < 0.05; ** – the difference from the group patients with T2D is significant, P < 0.05. # – the difference from the group patients with cancer is significant, P < 0.05

Discussion. Increased level of phospho-PRAS40 in most of the patients of group II with T2D and patients of group III with cancer confirms the mTORC1 activation by these diseases. It is known that tissues of patients with T2D are characterized by enhanced activity of mTORC1 and its substrate p70S6K, resulting in phosphorylation of IRS-1 (S307 and other residues), impairment of insulin signaling and, consequently, insulin resistance [12, 13, 14]. Increased level of phosphorylated PRAS40 in patients with T2D, besides possible changes due to disease, is probably determined by the ratio of complex effects of metformin and insulin, which were taken by patients. Metformin lowers mTORC1 activity but on the other hand improves insulin signaling. Insulin activates mTORC1 via the signaling cascade of PI3K/Akt/mTORC1 [14] and inhibits the activation of AMPK by metformin [15], which in its turn inhibits mTORC1. The final result of the interaction of these drugs and the signaling mechanisms that they induced, obviously, is the enhanced phosphorylation of the mTORC1 inhibitor – PRAS40.

Although the implementation of the insulin signal in the cell occurs through phosphorylation and sequestration of PRAS40, silencing of the AKT1S1 gene promotes the degradation of the IRS-1 in skeletal muscle via proteasome activation, which leads to an impairment of the IRS-1/Akt signaling pathway that regulates glucose transport into the cells. Excessive expression of PRAS40 inhibits proteasome activation and increases the stability of IRS-1 [16], which leads to an increase of insulin sensitivity. Hyperexpression of PRAS40 improved signaling of insulin in the heart and liver of mice on a high-fat diet [17]. Also, AKT1S1 knockout reduces the phosphorylation of mTORC1 substrates in certain cell types, indicating the importance of PRAS40 for PI3K/Akt/mTORC1 signaling through unclear mechanisms [18, 19, 20]. Knockout of PRAS40 in primary human skeletal muscle cells reduced insulin-mediated phosphorylation of Akt by 50%, as well as that of Akt substrates GSK-3 (Glycogen synthase kinase-3) by 40% and TSC2 (Tuberous Sclerosis complex 2) by 32% [13]. The latter fact testifies to the negative regulation by PRAS40 of its own phosphorylation. In addition, the activity of mTORC1 is positively regulated via PRAS40 phosphorylation by mTORC1, which leads to amplification of the signal. Consequently, the role of PRAS40 is not limited to negative regulation of the mTORC1 activity. Apparently, it performs more complex functions in insulin signaling.

Hyperactivation of mTORC1 is often observed by sporadic cancers. Several types of tumors demonstrated an increase in the level of PRAS40 phosphorylation [21], which is associated with enhanced activity of kinases such as Akt, Pim-1 and

mTORC1. The intensification of the translation caused by aberrant activation of mTORC1 leads to an increase in the cell size and proliferation, two common cancer features, and the search for mTORC1 inhibitors is considered a promising approach for the cancer treatment [6, 22]. From this perspective, the increased activity of mTORC1 in leukocytes is of interest because it may serve as an additional diagnostic marker of the disease.

Some of the PRAS40 functions, such as regulation of the nucleolar stress response, proteasome activity and cell survival, suggest that PRAS40 may be involved in the progression of malignant tumors. The phosphorylated PRAS40-T246 may also be a biomarker for predicting the susceptibility to inhibitors of Akt in cancer patients [1, 23, 24].

It was assumed that in the leukocytes of the patients of group with both cancer and diabetes, an additive effect on phosphorylation of PRAS40 would be observed. Therefore, somewhat unexpected was a decrease in the amount of phospho-PRAS40 below the control level (Figure 1). Consequently, in patients of the latter group, the activity of mTORC1 and p70S6K1 in leukocytes may be depressed, in comparison with control and, especially, with groups of patients with diabetes and cancer (Figure 2).

The decrease in PRAS40 phosphorylation in leukocytes in a group of patients with cancer and diabetes may be explained by competition for common signaling mechanisms. Also, antagonistic interaction between the two main cascades controlling proliferative processes – PI3K/Akt and MAPK – is not excluded. It has been shown that the MAPK/ERK signaling pathway can also activate mTORC1, both by phosphorylation of TSC2 and PRAS40 [6]. It is also known that an excess of insulin by diabetes can stimulate proliferative processes and malignant transformation through the Ras/MAPK/ERK1/2 cascade [25]. Thus, PRAS40 phosphorylation in leukocytes largely determines activity of mTORC1 and p70S6K1.

These data are confirmed by the results of the study of p70S6K1 phosphorylation (T389) and activation. The pattern of the PRAS40 phosphorylation in general coincides with the phosphorylation of the mTORC1 substrate – downstream protein kinase p70S6K1.

The composition of leukocytes includes monocytes/macrophages (up to 11% of the total amount of leukocytes) and lymphocytes (up to 40%) involved in the processes of cellular and humoral immunity. Macrophages and lymphocytes are involved in the pathogenesis of diabetes, diabetic atherosclerosis, formation of insulin resistance as well as immune response to cancer and tumor maintenance. The signaling pathway PI3K/Akt/mTORC1 activity in leucocytes plays an important role in these processes [10, 11, 26]. It has been shown that this cascade is responsible for the differentiation of immune cells involved in tumor recognition, clearance and mechanisms of cancer escape from immunological surveillance [9]. In leucocytes mTOR regulates the IL-12 synthesis and secretion, which plays an important role in the activities of natural killer cells and T-lymphocytes [27]. The activation of mTOR/S6K signaling upon NKG2D/DAP10 receptor complex stimulation promotes cancer progression through an enhanced energetic metabolism [28]. Targeting mTORC1 is a promising strategy in cancer therapy [22].

Thus, the present study showed that phosphorylation of PRAS40 is generally consistent with the activation of mTORC1, as measured by phosphorylation of p70S6K1. The increasing of the levels of phospho-PRAS40 and phospho-p70S6K1 in patients with cancer and in patients with T2D is a sign of mTORC1 activation for both types of diseases. The decrease of phospho-PRAS40 in patients with a combination of T2D and cancer may be explained by the involvement of other intracellular regulatory systems of oncogenesis and metabolism that inhibit mTOR signaling.

Conclusions

The increased amount of phosphorylated PRAS40 and p70S6K1 proves the activation of the studied signaling pathway by DM2. Its decrease in case of presence of both diabetes and cancer can be explained by the possible competing effects of the proteins that affect upstream regulators of these kinases or them directly. The exact explanation of the occurrence of this peculiarity requires the further study of the changes of intracellular processes by these diseases.

Prospects for future research

Future research will be related to the study of the effect of antidiabetic drugs on the activity of the signaling pathway PI3K/Akt/mTOR.

Conflict of interest

The authors declare no conflict of interest.

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