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TRYPTOPHAN METABOLITES IN SERUM AND FECES OF PATIENTS WITH NON-SMALL CELL LUNG CANCER

DOI 10.25789/YMJ.2021.76.22

УДК: 616-006.699

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The kynurenine pathway of tryptophan metabolism (IDO1 / TDO2-KYN-AhR) plays an important role in carcinogenesis, tumor growth, the formation of the immunological microenvironment of tumors, and may be a potential target for the treatment of oncological diseases. Our objective was to study the content of tryptophan metabolites in blood serum and feces in patients with NSCLC for further estimation of possible correction of microbiomes` functional state and its role in the providence of the efficiency of treatment. The levels of tryptophan metabolites in blood serum and feces of 100 patients with NSCLC in comparison with 100 healthy donors were studied by HPLC-MS / MS. A number of differences were obtained: in the serum of patients, a statistically significant decrease in the levels of tryptophan, its` dominant metabolites of microbiotic origin - tryptamine, serotonin, downtrend for xanthurenic acid along with an increase of the levels of quinolinic acid (in tendency). In feces, the levels of indole in patients were higher than in healthy individuals, this may indicate the activation of the indole pathway of tryptophan metabolism in them or reflect a decrease in the absorption of these metabolites in the intestine. We consider that the decrease of tryptophan and its` dominant metabolites in the serum of patients with NSCLC, apparently, is not associated with an enhanced microbiotic synthesis in the intestine or the consumption of this amino acid with food, but may rather be due to the utilization of tryptophan by tumor cells, since its content in the feces of patients corresponds to that in healthy donors.

Keywords: non-small cell lung cancer, healthy donors, tryptophan metabolites, blood serum, feces.

Introduction. Recently the role of the regulatory system of cellular differentiation, carcinogenesis, tumor growth with enzymes of kynurenic pathway of tryptophan catabolism like IDO1, TDO2, KYN, its` metabolites and its` ligand AhR as key components was described. This system also plays an important role in immune regulation (activation of T regs, suppression of T-effectors and dendritic cells, participation in mucosal immunity) [8, 14]. At the same time, AhR activates PD1 expression in CD8+Tlymphocytes. Since application of immune checkpoint inhibitors (ICI), namely of PD-1/PDL-1 system, is known to be one of the most modern and promising approaches in immunotherapy of cancer, its` effect largely depends upon the patients` immune status. The source of AhR ligands are not only endogenous tryptophan metabolites but also metabolites of gut microflora, in particular, kynurenines of microbial origin [7] with their production depending upon phase metabolic state of bacteria [5, 6]. It was demonstrated that some kinds of malignant tumors (prostatic and breast cancer, glioma, lymphoma etc) produce a great amount of kynurenine and its` level correlates with unfavorable prognosis [12], because IDO1/TDO2KYN-AhR participates in immunosuppressive microenvironment formation which results in immune evasion. In gut IDO1/TDO2KYNAhR system seems to be one of the central regulators of microbiota/mucosal barrier interaction as well as a possible target for treatment of the diseases with its` impairment. Thus, through the IDO1/TDO2KYNAhR mechanism the double-component model macroorganism-microbiota is formed, which is able to transform in tumor-bearing organism to triple-component one: macroorganism-tumor-microbiota. The investigation of the system is likely to enable us to approach the solution of such problems as resistance to immune evasion of tumors and to the development of metabolic complications in tumor patients. Taking into account high incidence, severe course, insufficient effect of treatment and poor prognosis of non-small cellular lung cancer (NSCLC), active research is carried out all over the world to develop new methods of its` treatment based on biologic characteristics of the tumor, among which tryptophan metabolism plays an important role [1, 4, 11].

Our objective was to study the content of tryptophan metabolites in blood serum and feces in patients with NSCLC for further estimation of possible correction of microbiomes` functional state and its role in the providence of the efficiency of treatment.

Material and methods. Two groups were included in the research: group 1 was represented by 100 healthy donors (21 men and 79 women aged 23-70 years, Me 33,82) without any malignant or other chronic diseases; group 2 consisted of 100 patients (76 men and 24 women aged 36-81 year, Me 61,2) with verified NSCLC of various morphology and TNM stage. The I stage was diagnosed in 6%, II - in 22%, III - in 32%, IV - in 10% of the patients. Criteria of inclusion were the absence of application of antibiotics, pre- or probiotics during 3 months before the investigation and signed informed consent to participate in it. Criteria of exclusion for donors were age <18 years, for patients – tumors except NSCLC, severe comorbid disease, any gastrointestinal pathology, respiratory infections, psychosis, alcoholism, drug addiction, pregnancy, lactation. In all the persons included in the research samples of blood and feces were taken. Samples of serum prepared from blood and feces taken in tubes with ethanol were stored up to testing in Biobank at -20oC и -80oC respectively.

The quantitative analysis of Trp metabolites in blood serum and feces was carried out by high performance liquid chromatography with mass spectrometric detection (HPLC-MS/MS) using an Agilent 1200 liquid chromatograph (Agilent inc., USA) [2]. Chromatographic separation was performed using a Discovery PFP HS F5 analytical column (2.1*150 mm; 3 μm). The composition of the mobile phase: phase A - 0.1% solution of formic acid in dionized water; phase B - 100% acetonitrile for chromatography. The gradient of the mobile phase is from 1% B to 10% within 4 minutes, then up to 90% B by the 9th minute of the analysis. The flow rate of the mobile phase is 0.40 ml / min. For detection, a mass spectrometric detector based on an Agilent 6460 triple quadrupole (Agilent inc., USA) MRM and electrospray ionization was used. The resulting signal was processed using the Masshunter software (Agilent inc., USA). The concentration of metabolites was calculated by the internal standard method (2-hydroxynicotinic acid). Analytical standards were prepared using an artificial matrix containing bovine serum albumin and sodium chloride. The studied metabolites were added to the matrix and prepared according to the analysis method. For blood serum sample preparation, an internal standard (2-hydroxynicotinic acid) was added to 100 μL of serum, proteins were precipitated with acetonitrile, the supernatant was evaporated and re-

The content of tryptophan and its metabolites in serum and feces of healthy donors and patients with NSCLC (nmol/L)

Sample	The investigated metabolite	Number of patients with NSCLC	Number of patients in the control group	NSCLC patients Me [HQ …LQ]	Control group Me [HQ …LQ]	p-value	Corrected p-value
Serum	5-hydroxyindole acetate	97	95	63.8 [88.9 ... 39.1]	54.3 [67.2 ... 41.4]	0.035	0.322
	Anthranilic acid	97	95	13 [22.6 ... 10.6]	15.7 [20.9 ... 12.4]	0.075	0.644
	Indole-3-acrylate	98	96	7.4 [14.4 ... 4.2]*	14.5 [23.9 ... 8.3]	Менее 0.001	Менее 0.001
	Indole-3-acetate	99	96	1532 [2143 ... 984]	1793 [2249 ... 1353]	0.006	0.075
	Indole-3-butyrate	99	96	11 [15 ... 6.4]	10.4 [14.9 ... 7.8]	0.622	1.000
	Indole-3-carboxaldehyde	99	96	37.3 [53.7 ... 30.3]*	56.3 [79 ... 43.8]	Менее 0.001	Менее 0.001
	Indole-3-lactate	99	96	564 [713 ... 436]	662 [890 ... 518]	0.007	0.075
	Indole-3-propionate	99	96	405 [849 ... 230]*	870 [1363 ... 481]	Менее 0.001	Менее 0.001
	Kynurenine	99	96	2483 [3033 ... 2075]	2541 [2988 ... 2131]	0.950	1.000
	Kynurenic acid	98	96	14.1 [20.3 ... 10.8]	15.5 [19.2 ... 10.4]	0.960	1.000
	Xanthurenic acid	99	96	1.6 [2.7 ... 0.915]	2.7 [4.1 ... 1.3]	0.004	0.064
	Serotonin	99	96	414 [661 ... 264]*	648 [898 ... 500]	Менее 0.001	Менее 0.001
	Tryptamine	99	96	0.107 [0.163 ... 0.071]*	0.221 [0.337 ... 0.125]	Менее 0.001	Менее 0.001
	Tryptophan	99	96	18705 [22675 ... 15332]*	24062 [27806 ... 19792]	Менее 0.001	Менее 0.001
	Quinolinic acid	99	96	118 [179 ... 78.1]	93.7 [126 ... 69.4]	0.004	0.064
Feces	5-hydroxyindole acetate	97	95	0.109 [0.511 ... 0.043]	0.207 [0.529 ... 0.06]	0.191	1.000
	Anthranilic acid	99	96	0.106 [0.169 ... 0.078]	0.106 [0.149 ... 0.08]	0.816	1.000
	Indole	99	96	327 [595 ... 186]	243 [368 ... 157]	0.007	0.075
	Indole-3-acrylate	98	96	1.2 [2.3 ... 0.677]	1.6 [2.6 ... 0.815]	0.115	0.875
	Indole-3-acetate	99	96	6.1 [14.4 ... 2.8]	4.2 [9.1 ... 2.1]	0.018	0.180
	Indole-3-butyrate	99	96	0.447 [0.721 ... 0.284]	0.384 [0.677 ... 0.228]	0.153	1.000
	Indole-3-carboxaldehyde	99	96	1.7 [3.5 ... 1.1]	1.8 [3.3 ... 0.861]	0.596	1.000
	Indole-3-lactate	97	96	0.178 [0.361 ... 0.118]	0.196 [0.436 ... 0.128]	0.691	1.000
	Indole-3-propionate	99	96	3.6 [6.3 ... 1.5]	2.9 [7.1 ... 1.4]	0.836	1.000
	Kynurenine	99	96	0.184 [0.478 ... 0.106]	0.206 [0.379 ... 0.109]	0.938	1.000
	Kynurenic acid	99	96	3.3 [8.3 ... 1.2]	2.9 [7 ... 1.2]	0.439	1.000
	Xanthurenic acid	99	94	1.7 [4 ... 0.255]	1.1 [2.5 ... 0.256]	0.210	1.000
	Tryptamine	99	96	0.18 [0.924 ... 0.062]	0.117 [0.569 ... 0.055]	0.117	0.875
	Tryptophan	97	95	39.1 [81.3 ... 18]	38.5 [84.7 ... 20.1]	0.899	1.000
	Quinolinic acid	99	96	2.3 [4.6 ... 1.1]	2.6 [5.4 ... 1.4]	0.219	1.000

Note: * - statistically significant differences at $p \leq 0.05$ in comparison with the group of healthy donors.

dissolved in 10% methanol in water with the addition of ascorbic acid to prevent oxidation of analytes. Feces samples were lyophilized to a dry residue, then a sample of about 5 mg was extracted with 50% methanol in water with the addition of an internal standard and ascorbic acid. After centrifugation, the sample was analyzed by HPLC-MS / MS.

Statistical processing of the data was carried out by the methods of nonparametric statistics with the use of R programming language, version 4.1.0 and Rstudio softwear package, version 1.4.1717. The statistical significance of the differences of the mean values were represented by median (Me), higher (HQ) and lower quartile (LQ). Before intergroup comparison we performed the purification of the data from outliers which were accounted as doubtful. For calculation of statistically significant differences between values of the 2 groups Mann-Whitney criterion was used. For exclusion of the re-prediction errors the obtained p-values were corrected according to the Benjamini-Yekutieli method. Intergroup comparison was recognized as statistically significant when adjusted p was < 0.05 and the tendency - when adjusted p was from 0.05 to 0.1 (upward and downward trends).

Results and discussion. According to the obtained results in both groups the main – dominant - products of tryptophan metabolism, which present in serum and feces at the highest levels, were kynurenine, indole-3-acetate, indole-3-propionate, indole-3-lactate and serotonin (table).

In serum of patients with NSCLC the statistically significant decrease of tryptophan level in comparison with donors was found. Besides the lung cancer patients had statistically lower levels of tryptamine, serotonin, indole-3-propionate, indole-3-acrylate and indole-3-carboxaldehyde and tendency to decreased amounts of indole-3-acetate and indole-3-lactate; the majority of these metabolites are of microbial origin. Amongst the metabolites of kynurenine pathway upward trend of quinolinic acid level and downward – of xanthurenic acid level in serum were noted.

Assessment of tryptophan metabolites in feces of healthy donors and in NSCLC patients showed that uptrend of indole levels in the last group, but no statistically significant differences was found (table).

It is worth noting that indole is the dominant tryptophan metabolite in gut both in donors and in NSCLC patients. Unlike serum (in which the downtrend of the level of indole-3-acetate was registered), in feces it had no difference from the donors` values. Indole-3-acetate is able to be metabolized to quinolinic acid via anthranilic acid in enterocytes and immune cells of the gut, as the uptrend of quinolinic acid in patients` serum indicates.

Thus, in serum of NSCLC patients compared to the donor group the concentrations of almost all the tryptophan metabolites are decreased while the levels of NAD+ precursor quinolinic acid demonstrates an uptrend. Quinolinic acid is accounted to be toxic [3], it is known as an important regulator of proinflammatory cytokines` synthesis and is produced by macrophages in high amounts [10], evidently contributing to inflammatory microenvironment of the tumor.

The published data concerning the tryptophan metabolites in cancer patients are rather contradictory. Some authors describe statistically significant increase of both tryptophan and kynurenine levels [13], the other ones demonstrate the increase of kynurenine and decrease of tryptophan in cancer patients` serum [9]. It could be explained by different types of studied tumors as well as by their heterogeneity in cellular composition and metabolic peculiarities. Obviously the activity of microbiota may contribute in these differences, however it is not yet studied.

Conclusion. The decrease of tryptophan and its` dominant metabolites in serum of NSCLC patients is apparently related neither to impairment of their microbial synthesis in large intestine nor to lack of food consumption of the amino acid, but is due to utilization of tryptophan by tumor cells because its` content in feces of patients and donors is similar. Probably this indicates the activation of indole pathway of tryptophan metabolism or reflects the inhibition of their intestinal absorption in patients. Our data demonstrate that not only colorectal cancer but also tumors of anatomically distant organs, namely, lung cancer, may play a role of a tryptophan trap. High level of quinolinic acid may characterize the imbalance of tryptophan metabolism with the result of toxic metabolites` production in our cohort of patients.

The authors declare no conflict of interest.

Financing. The study was carried out within the framework of research work under the contract 0373100122121000031 for the implementation of research work under the project «Study of the IDO1 / TDO2-KYN-AhR system in the host-microbiota-tumor system in malignant neoplasms in order to develop a prodrug for their treatment», dated 01.06 .2021 with the Federal State Budgetary Institution «CSP» of the FMBA of Russia

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ARCTIC MEDICINE

DOI 10.25789/YMJ.2021.76.23

УДК 616-001.18:611.018.5-07(=1.571.56-81)

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BROWNING MARKERS IN ADULT INDIGENOUS RESIDENTS OF YAKUTIA IN CONDITIONS OF NATURAL COLD

In this work, the expression profile of markers of brown adipose tissue activity (CIDEA, PRDM 16), markers of browning of white adipocytes (HOXC9, Slc27A1) and marker of β-oxidation of fatty acids (Cpt1a) in 150 indigenous residents of Yakutia, miners of a diamond mining company, who were exposed to natural cold for 3 months, was analyzed in peripheral blood mononuclear cells. To determine the metabolic status, anthropometric data, glucose level and blood lipid profile of the subjects were evaluated.

Keywords: brown adipose tissue, cold, thermogenesis, browning, Yakutia, obesity.

Introduction. Obesity is characterized by an aberrantly increased amount of white adipose tissue resulting from dysfunctional regulation of the energy balance [13]. The modulation of energy consumption and expenditure is extremely complex and is the result of the integration of numerous neuroendocrine and environmental signals. Exposure in the cold is one of the main available stimulants that contribute to energy consumption by activating thermogenic pathways and thus ensuring survival in adverse temperature conditions [8, 12]. It is known that cold promotes beta-adrenergic stimulation through the sympathetic nervous system, which, in turn, induces thermogenesis, while activating brown adipose tissue (BAT) [7, 8, 12]. The activation of BAT promotes the oxidation of fats to produce heat, while an increased expression of the UCP1 protein is produced [8]. It is known that when stimulated by cold, white adipocytes can transdifferentiate into beige and brown-like adipocytes (a phenotype with increased expression of UCP1) in a process known as browning, leading to heat production [3]. It is important to note that during the browning process, the proliferation and differentiation of precursors of brown adipocytes also occurs, contributing to the growth of the population of heat-producing cells [14, 32]. A number of studies have shown that the activation of BAT in mouse models is able to prevent diseases such as obesity, type 2 diabetes and atherosclerosis [2, 6]. Thus, the study of the regulation of BAT was particularly important as a potential target for the treatment of obesity [14, 17]. It is known that adults have a different volume and amount of BWT, which decreases depending on age and BMI [11,33]. Studying the activation and browning of BZHT in humans is not easy due to several limitations. The most commonly used method available for this purpose is the study of the absorption of (18)F-FDG (2-deoxy-2-[18F]fluoro-d-glucose) by positron emission tomography-computed tomography (PET-CT), which, in addition to various technical limitations, is expensive and complex. In the search for alternative methods for assessing the activation of BAT in humans, we found the study of Palou and his colleagues, which was conducted on female rats, interesting. The results of this work showed that the expression of the regulators of the activity of BAT (CIDEA, Prdm16), browning of white adipose tissue (Hoxc9 and Slc27a1) and β-oxidation of fatty acids (Cpt1a) in both tissues correlates with the expression of the same markers in peripheral blood mononuclear cells (PBMC) when stimulated by cold [23]. The authors concluded that these genes can be considered suitable markers for assessing the activity of BAT in peripheral blood mononuclear cells (PBMC), avoiding the use of invasive procedures [23]. However, it was not clear whether the expression of these markers in the human PBMC is possible and whether it changes depending on exposure to cold. An earlier study conducted by us showed that adult indigenous residents of Yakutia exposed to cold showed greater beta-adrenergic activation and darkening of visceral fat depots compared to the comparison group living in thermoneutral conditions [12]. The aim of our study was to evaluate the expression of browning marker genes in the PBMC in cold-exposed adult indigenous residents of Yakutia compared with the control group, as well as to assess differences in metabolic status between the study groups.

Material and methods. The study was conducted in 2015 in the Verkhoyansk and Anabar districts of Yakutia in accordance with the guidelines of the Helsinki Declaration on the Ethical Treatment of People. The Protocol was approved by the Supervisory Board of the Ethics Committee of the YANC KMP (Protocol No. 46 of May 7, 2015).

Study participants. This study included 150 healthy male tunnellers of indigenous nationality engaged in open-pit diamond mining in the Anabar region of Yakutia (Polar zone, cold exposure group), and 29 healthy control subjects living in the city of Yakutsk (urban zone) in thermoneutral conditions. The subjects included in the cold exposure group spent an average of 8 hours a day working in the mine for 3 months (from

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