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COMBINED USE OF HAEMOSTATIC SYSTEM INDICES FOR EVALUATION OF UPPER RESPIRATORY TRACT CANCER PROGRESSION

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Aim: **To analyze whether comprehensive assessment of haemostatic system components, in particular, indices of coagulation and fibrinolytic systems along with functionally related proteins, could be indicative of upper respiratory tract (URT) cancer progression.** ***Materials and Methods*****: Indices of coagulation and fibrinolytic systems along with functionally related proteins, in particular, trypsin-like amidolytic activity, trypsin-like proteolytic activity, thrombin-like amidolytic activity, elastase-like amidolytic activity, fibrinolytic activity, potential amidolytic plasmin activity, content of fibrinogen, antithrombin III, α_1-proteinase inhibitor, and α_2-macroglobulin, and prothrombin time were evaluated in blood plasma of patients with URT cancer of II (n = 10) and III (n = 25) stages with the use of routine biochemical methods.** ***Results:*** **For both groups of patients with URT cancer there have been shown notable differences for the majority of the studied indices, especially the indexes of proteolytic activities, from these of healthy donors, and in the case of URT cancer of III stage they reached statistical significance. In contrary, the changes in the content of antithrombin III, α_1-proteinase inhibitor, and α_2-macroglobulin were insignificant. In both groups of patients significant increase of fibrinogen content has been registered, while the content of soluble fibrinogen didn't change. Also, in both groups of patients there a significant increase of potential activity of plasminogen was documented, while clot lysis time was significantly increased only in patients with III stage URT cancer. Multifactorial analysis of haemostatic system indices evidenced for efficacy of their combined use for evaluation of URT cancer progression risk.** ***Conclusion:*** **Combined use of fibrinogen and α_2-macroglobulin content and the level of amidolytic thrombin-like activity could serve as an indicator of URT cancer progression.**

Key Words: **upper respiratory tract cancer, prognosis, haemostatic system.**

The raising cancer incidence is among the most serious medical and social problems of our time. According to the Cancer Register of Ukraine, the annual mortality from malignant processes is close to 100 thousands cases, and the annual morbidity reaches 160 thousands cases (0.2 and 0.32% of the country population, respectively). 7.5–8% of cancer cases are related to the cancer of upper respiratory tract (URT), which incidence has increased by 1.6 times during last 10 years [1]. As it is commonly accepted, the early detection of a disease is a key to its effective therapy. However, cancer is usually detected by its late clinical manifestations, and no universal index for early cancer detection has been found so far [2]. However, according to the data of some researchers, not a single index but a combination of several cancer-related biochemical alterations in components of haemostatic system (HS), could be considered promising [3].

It is well known that HS consists of two oppositely directed enzymatic sub-systems, which provide the formation of fibrin clot and its lysis. The majority of enzymatic HS components are trypsin-like proteinases that are synthesized as inactive pro-enzymes with their following processing into active forms via high-selective enzymatic cleavage. In turn, these activated proteinases are activators for other pro-enzymes, pro-factors and are under strict control of high-selective protein inhibitors [4, 5]. Disturbances of this highly regulated systems lead to the disturbance of various physiological processes that are aligned with numerous diseases. Malfunction of proteolysis significantly disturbs both fibrin clotting and fibrinolysis, complement and kinine systems [6, 7], and causes tissue damage and uncontrolled tumor growth [8–12]. Cancer cells are known to produce proteolytic enzymes, which affect haemostasis as well as promote tumor invasion and metastasis [13, 14]. Non-functional proteolysis plays a prominent role in post-operative complications, such as thrombosis and bleeding, recurrence and metastasis [15–17]. It should be underlined that a number of HS components is directly involved into cancer development [18–20]. Therefore, in the present work we aimed to analyze whether comprehensive assessment of HS components, in particular, indices of coagulation and fibrinolytic systems along with functionally related proteins, could be an informative tool for prediction of disease progression in patients with URT cancer.

MATERIALS AND METHODS

Patients. In the study, 10 patients with primary laryngeal cancer of II stage and 25 patients with URT cancer of III stage were enrolled. The patients were cured in SI "O.S. Kolomiychenko Institute of Otolaryngology of National Academy of Medical Sciences of Ukraine" in 2008–2010. The patients underwent surgical treatment, those with URT cancer of III stage

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Abbreviations used: α_1IP – α_1-proteinase inhibitor; α_2M – α_2-macroglobulin; AT-III – antithrombin III; BAPNA – N_α-benzoyl-D,L-arginine para-nitroanilide; FA – fibrinolytic activity; HS – haemostatic system; p-NA – para-nitroaniline; PRA – trypsin-like proteolytic activity; PT – prothrombin time; URT – upper respiratory tract.

after surgery were treated by radiotherapy. Peripheral blood was taken 1 day before the operation. The control group consisted of 24 healthy donors. The study has been performed in accordance with ethics rules for biomedical research; all patients have given an informed consent for the participation in the study.

Biochemical measurements. The samples of platelet-delpeted citrate plasma were obtained by centrifugation of blood at 1200 g for 20 min.

Trypsin-like amidolytic activity was determined by extinction of para-nitroaniline (p-NA) formed by splitting of chromogenic substrate — N_α-benzoyl-D,L-arginine para-nitroanilide (BAPNA) using spectrophotometry at 383 nm and was expressed in nmol of p-NA per 1 ml of plasma per 1 min [4].

Trypsin-like proteolytic activity (PRA) was determined by enzymatic cleavage of protamine with following evaluation of arginine-containing peptides soluble in 20% trichloroacetic acid and was expressed in nmol of soluble arginine per 1 ml plasma per 1 min [4].

Thrombin-like amidolytic activity was determined by extinction of p-NA formed by splitting of chromogenic substrate — Tos-Gly-L-Pro-L-Arg-para-nitroanilide (Chromozym TH) using spectrophotometry at 405 nm and was expressed in nmol of p-NA per 1 ml of plasma per 1 min [21].

Elastase-like amidolytic activity was determined by extinction of p-NA formed by splitting of chromogenic substrate — Suc-L-Ala_3-para-nitroanilide using spectrophotometry at 410 nm and was expressed in nmol of p-NA per 1 ml of plasma per 1 min [22].

Fibrinolytic activity (FA) was evaluated by euglobulin method and expressed as time (min) of fibrin clot formation and dissolution [23].

Potential amidolytic plasmin activity was determined by extinction of p-NA formed by splitting of chromogenic substrate — H-D-Val-L-Leu-L-Lys-para-nitroanilide (S-2251) using spectrophotometry at 405 nm and was expressed in nmol of p-NA per 1 ml of plasma per 1 min [24].

The content of fibrinogen was determined by the method of Belitser et al. [25].

Prothrombin time (PT) was evaluated by duration (s) of plasma coagulation in the presence of thromboplastin and calcium chloride [26].

The content of antithrombin III (AT-III) was determined by Abildgaarg method [21] and was expressed as % of the norm.

The contents of α_1-proteinase inhibitor (α_1IP) and α_2-macroglobulin (α_2M) were determined by inhibition of BAPNA lysis with or without soybean trypsin inhibitor [4].

Statistical analysis of the data was performed using Statistica program. The data were presented as M ± m. The differences between the groups were analyzed using Student's t-criterion. The difference was considered significant if $p < 0.05$.

RESULTS AND DISCUSSION

The main indices of plasma coagulation system of the patients and healthy donors are presented in Table 1. The most significant changes were recorded in the patients with URT cancer of III stage: the contents of fibrinogen, AT-III, and level of amidolytic thrombin-like activity increased by 1.8-, 1.2- and 1.6-fold, respectively. Also, in this group PT was significantly higher than in healthy donors. In the patients with laryngeal cancer of II stage increased PT and fibrinogen content are noted, whereas the level of amidolytic thrombin-like activity was close to that in control group. In both groups of the patients the content of soluble forms of fibrin wasn't different from the normal level. We have conclude that pre-treatment levels of fibrinogen, AT-III, and amidolytic thrombin-like activity in the group of patients with III stage of malignancy were significantly higher than in patients with II stage as well as in control group. These differences may be evaluated as the evidence for the dependence of these haemostatic indices of the stage of disease.

Table 1. Indices of clotting system of blood plasma of patients with URT cancer

Groups of patients	Content of fibrinogen, g/l	Dissoluble fibrin, mg%	Prothrombine time, s	Content of AT-III, %	Amidolytic thrombine-like activity, nmol p-NA/(min·ml)
Patients with URT cancer of III stage (n = 25)	4.0 ± 0.3 p < 0.001	4.0 ± 0.3	29.0 ± 1.0 p < 0.001	119.0 ± 6.6 p < 0.02	16.0 ± 3.0 p < 0.05
Patients with URT cancer of II stage (n = 10)	3.1 ± 0.2 p < 0.001	4.0 ± 0.8	28.0 ± 1.4 p < 0.02	107.0 ± 5.0	10.2 ± 2.0
Healthy persons (control group) (n = 24)	2.2 ± 0.1	4.3 ± 0.4	23.5 ± 0.8	100.0 ± 2.7	9.6 ± 1.0

Note: statistically significant differences with control group are marked by corresponding p value.

It is known that cancer cell secretion of proteolytic enzymes causes the destruction of intercellular matrix thus creating favorable conditions for tumor invasion. The activity of proteases are dependent both on the level of their production and of their blocking by specific inhibitors [27, 28]. The levels of activity of proteolytic enzymes and the content of protease inhibitors (α_2M and α_1IP) in blood plasma of the patients are presented in Table 2. According to these data, in the group of patients with UPT cancer of III stage the levels of PRA and elastase-like amidolytic activity are significantly higher than the corresponding levels in healthy donors, while the content of α_2M is significantly reduced. PRA in patients with UTR cancer of II stage was also significantly increased, but lower than in the patients with stage III of the disease. The elastase-like amidolytic activity in patients with UTR cancer of II stage just tended to be increased in comparison to the control group, while the content of α_2M was reduced in contrary to that in patients with III stage. The level α_1IP in patients with II stage of URT cancer wasn't different from its reference value.

Table 2. Characteristics of PRA, elastase-like amidolytic activity and contents of their inhibitors in blood plasma of patients with URT cancer

Groups of patients	PRA, nmol Arg/(min·ml)	Amidolytic elastase-like activity, nmol p-NA/(h·ml)	Content of α_2M, g/l	Content of α_1IP, g/l
Patients with URT cancer of III stage (n = 25)	78.0 ± 4.0 p < 0,001	14.0 ± 1.7 p < 0.02	1.5 ± 0.1 p < 0.001	2.5 ± 0.2 p < 0.05
Patients with URT cancer of II stage (n = 10)	72.5 ± 5.1 p < 0.01	10.3 ± 1.5	1.6 ± 0.2	1.8 ± 0.2
Healthy persons (control group) (n = 24)	55.5 ± 3.2	9.2 ± 1.0	2.00 ± 0.08	2.00 ± 0.08

Note: statistically significant differences with control group are marked by corresponding p value.

Both total FA and potential activity of plasminogen are exclusively important indicators of the state of fibrinolytic system. The process of plasminogen activation into plasmin plays a key role in fibrin clot lysis, but could be involved in tumor development by non-functional activation of matrix proteinases with following direct or mediated destruction of extracellular matrix [29, 30]. As one may see (Table 3), there is a significant slowdown of FA in patients with URT cancer of III stage. In both groups of patients a statistically significant increase in the potential activity of plasminogen was noted.

Table 3. Indices of fibrinolytic system of blood plasma of patients with URT cancer

Groups of patients	Clot lysis time, min	Potential plasmin-like amidolytic activity, nmol p-NA/(min·ml)
Patients with URT cancer of III stage (n = 25)	263.0 ± 12.0 p < 0.02	0.76 ± 0.06 p < 0.001
Patients with URT cancer of II stage (n = 10)	238.0 ± 6.0	0.74 ± 0.10 p < 0.05
Healthy persons (control group) (n = 24)	237 ± 5	0.57 ± 0.02

Note: statistically significant differences with control group are marked by corresponding p value.

According to the data of postoperative clinical observation of the patients with UTR of III stage, in 3–12 months after surgical removal of primary tumor, 10 patients developed relapse or lymph node metastases, and 15 patients were in remission. Pre-treatment indices of haemostasis system of these patients are represented in Table 4. We can see that pre-treatment levels of PRA, thrombin-like amidolytic and elastase-like amidolytic activities, the contents of fibrinogen and α_1IP were significantly higher in both groups of patients compared to the group of healthy persons. Contrary, the level α_2M as well as FA were considerably lower.

Could all these data be considered useful for the evaluation of UTR cancer progression? Combined use of the studied indices allowed create an effective approach based on evaluation of pre-treatment level of amidolytic thrombin-like activity, the content of fibrinogen and α_2M. At the same time, the levels of amidolytic elastase-like and PRA remain valuable indicators of the general condition of the patients, but they were less informative in regard of prognosis of disease course in post-treatment period. That's why it seems reasonable to use an additional index accounting the differences between the thrombin-like activity and contents of fibrinogen and α_2M of each patient from their normal levels ([Fg], [Thr] and [α_2M]). The formula for calculation of such index (let's name it "index H") is as follows:

$$H = [Fg][Thr] / [\alpha_2 M].$$

By calculation of individual parameters of the patients with URT cancer using this formula with following use of the methods of variation statistics for both groups of patients, the average value of H index for the group of patients with complications was 6.35 ± 1.67 *vs* 2.65 ± 0.53 for group patients in remission ($p < 0.05$).

In conclusion, the results of combined use of pre-treatment indices of HS and functionally related proteins of blood plasma in patients with II and III stages of URT cancer evidence on association of these indices with the disease progression. The level of thrombin-like amidolytic activity, α_2M and fibrinogen contents in blood plasma of the patients with URT cancer of III stage could be used as valuable index for cancer recurrence and metastasis at post-treatment period.

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Table 4. Pre-treatment indices of the groups of patients with URT cancer of III stage with post-operative complications and patients in remission

Groups of patients	PRA, nmol Arg/(min·ml)	Amidolytic elastase-like activity, nmol p-NA/(h·ml)	Content of α_1IP, g/l	Content of α_2M, g/l	Amidolytic thrombine-like activity, nmol p-NA/(min·ml)	Content of fibrinogen, g/l	Clot lysis time, min
Patients with relapses of disease or metastasis (n = 10)	81.0 ± 7.2 p < 0,01	14.8 ± 2.3 p < 0.05	2.5 ± 0.2 p < 0.05	1.40 ± 0.10 p < 0.001	20.0 ± 3.9 p < 0.02	4.6 ± 0.4 p < 0.001	278 ± 20
Patients in remission (n = 15)	75.7 ± 3.5 p < 0.001	11.5 ± 1.5	2.10 ± 0.15	1.70 ± 0.10 p < 0.05	12.1 ± 2.7	3.5 ± 0.2 p < 0.001 p1 < 0.05	249 ± 9
Healthy persons (control group) (n = 24)	55.5 ± 3.2	9.2 ± 1.0	2.00 ± 0.08	2.00 ± 0.09	9.6 ± 1.0	2.2 ± 0.1	237 ± 5

Note: p – the difference is significant compared to control group; p_1 – the difference between the indices of patients with post-operative complications and patients in remission is significant.

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