Exp Oncol 2013
35, 1, 37–40

Experimental ONCOLOGY

ACTIVITY OF THYMIDILATE "SALVAGE PATHWAY" ENZYMES IN HUMAN GASTRIC CANCER AND BLOOD SERUM: CORRELATION WITH TREATMENT MODALITIES

B.G. Borzenko[1,], E.M. Bakurova[1], Yu.A. Popovich[3], E.A. Sidyuk[3], A.Y. Popovich[2]*
[1]*Department of Chemistry, Maksim Gorky National Medical University, Donetsk, Ukraine*
[2]*Department of Oncology, Maksim Gorky National Medical University, Donetsk, Ukraine*
[3]*Donetsk Regional Anticancer Center, Donetsk, Ukraine*

A comparative study of enzyme activity features of thymidilate "salvage pathway" synthesis in blood serum and tissues of different age patients with gastric cancer ($T_{3-4}N_{0-x}M_0$) was carried out. *Aim*: To evaluate the diagnostic relevance of thymidilate metabolism enzymes activities and their association with tumor growth. *Methods*: Enzymes activities were determined by the radioisotope method and spectrophotometrically in tumor tissues and blood serum of 74 patients. *Results*: It was demonstrated that thymidine phosphorylase activity in gastric tumors is lower by 2.6 times as compared to non-neoplastic mucosa of resection margin. This being accompanied by decrease of its activity in the blood serum (from 47.9 ± 2.6 to 14.65 ± 2.4 nmol/min·mg, $p < 0.001$). An increase of thymidine kinase activity was revealed both in tumor tissues (more than 3.5 times) and in blood serum (from 3.9 ± 0,7 nmol/mg·h, to 6.8 ± 1.0 nmol/mg·h, $p < 0.01$). Changes in their activity in the postoperative period depended on the type of surgical procedure and tumor eradication. *Conclusion*: It could be suggested that control of individual dynamics of the enzymes activities in blood serum may be used as informative tool for monitoring of patients and treatment optimization.
***Key Words*: gastric cancer, thymidine phosphorylase, thymidine kinase, blood serum.**

Metabolic disorders associated with malignant transformation and tumor progression result from quantitative imbalance of enzymatic processes. One of the biological features of tumor cells is an opposite change in enzymes activity of anabolic and catabolic processes. Besides the intensity of cellular DNA synthesis and, thus, cell division, depends on the level of deoxythymidine triphosphate (dTTP, the key precursor for DNA synthesis). In human body DNA is synthesized following one of the two possible pathways. There are both de novo synthesis (from simple precursors) and "salvage pathway", for example, by recycling thymine which can be reincorporated into DNA. The intensity of "salvage pathway" is regulated by the activity of two enzymes — thymidine phosphorylase (TP; E C 2.4.2.4.) and thymidine kinase (TK; E C 2.1.2.1.) (Fig. 1). TK and TP also play an important role in thymidine homeostasis and thus in the synthesis of dTMP (a precursor of dTTP in DNA synthesis). TK has a key function in the synthesis of DNA and, thereby, in cell division as it is part of the unique reaction chain to introduce deoxythymidine into DNA. TK reflects proliferative activity. It is used as a proliferation marker in the diagnosis, a control of treatment and follow-up of malignant disease [1]. Thymidine phosphorylase (TP) is similar to the platelet-derived endothelial cell growth factor (PD-ECGF) and plays dual role in cell biology. A high expression is related to tumor angiogenesis and invasion, and therefore it is associated with a poor prognosis. It has been postulated that the angiogenic effect of PD-ECGF/TP is related to the enzymatic activity of TP, which catalyses the reversible phosphorolytic cleavage of thymidine (TdR) to thymine and 2´-deoxyribose-1-phosphate (dR-1-P) [2].

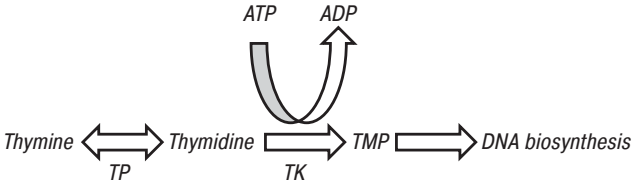

Fig. 1. Schematic representation of the thymidilate synthesis by the "salvage pathway"

Enzyme can also catalyze the interconversion of uracil, as well as several fluoropyrimidines. It has also an important pharmacological action. TP has a moderate of even negligible role in the activation of the antimetabolite 5-fluorouracill (5-FU) to fluoro-deoxyuridine-5´-monophosphate, but its phosphorolytic activity is essential for the activation of prodrug 5´-deoxy-5-fluorouridine (5´-DFUR, furtulon) to 5-fluorouracil. 5-FU is an intermediate in the activation of the prodrug capecitabine (Xeloda). The various complex interactions of TP/PD-ECGP give it an essential role in cellular functioning, and hence it is an ideal target in cancer chemotherapy.

Since last century, the 5-FU with other anticancer agents and prodrugs from group of fluoropyrimidines are widely used. For example: capecitabine (Xeloda), tegafur, furtulon (5´-deoxy-5-fluorouridine, 5´-DFUR), S-1 and other [3]. These drugs provide the basis for neoadjuvant and adjuvant chemotherapy in combination with various types of surgical procedures and different methods of polychemotherapy (PCT) (endolymphatic and intra-arterial chemotherapy).

This study evaluates the possibility of thymidilate metabolism biomarkers using in the clinical practice.

Received: February 7, 2013.
*Correspondence: E-mail: borzenko.bg@rambler.ru; 32023@mail.ru

Abbreviations used: 5-FU – 5-fluorouracil; dTTP – deoxythymidine triphosphate; PD-ECGP – platelet-derived endothelial cell growth factor; TK – thymidine kinase; TP – thymidine phosphorylase.

In addition, we investigate the individual enzymatic test as an estimate of anticancer treatment efficacy and prognosis of disease recurrence.

MATERIALS AND METHODS

Seventy-four adult patients, 25–70 year old, with gastric cancer were included in this study. Patients were staged in the accordance with TNM classification (the 6th edition) as $T_{3-4}N_{0-x}M_0$. Eighty-two non-cancerous patients of the same age without gastroduodenal zone pathology formed control group and also were examined. The study protocol was approved by Ethical Committee permission of M. Gorky National Medical University (Donetsk, Ukraine) for studies with human materials. The features of enzymes activity were studied in blood serum and tissues. Activity of tissue enzymes was determined in the surgically removed material in thirty tumors and in non-cancerous stomach mucosa (the mucosa margin of resection distant from the carcinoma had being as a control).

Survival retrospective analysis when different ways of chemotherapeutic agent's injections was made in 125 cases of inoperable gastric cancer.

Preparation of tissue and serum samples was realized by standard methods. Briefly, tissues were homogenized in 10 mM Tris-HCL buffer (pH 7.5) containing 0.1mM KCL, 2 mM sucrose. Samples were centrifuged at 5.000 g for 30 min and the supernatants were used for the enzyme assay. TK activity was determined by the radioisotope method as described previously [4]. Radioactivity of diethyl-amino-ethyl-cellulose paper disks (DEAE-cellulose disks paper "Limbro" (UK) was counted in a liquid scintillation SL-8 on a biological scintillation counter (CBS-2, Russia). TK activity was given in nanomoles of thymidylate formed within an hour per 1 mg of protein. TP activity was determined spectrophotometrically at 300 nm according to the amount of thymine formed [4]. From each supernatant, 0.02 ml of it was incubated with 50 mM potassium phosphate buffer and 10 mM thymidine ("Sigma", Germany) in a total volume of 1 ml. After incubation for 30 min at 37 ^{0}C, the reaction was terminated by boiling and adding 2.5 ml of 0.1 M NaOH. Activity was given nanomoles of thymine generated per 1 min/mg of protein. Protein concentration in biological material was determined using the method described by Lowry et al. [5].

Statistical analysis of results was performed using Medstat software package. On significance of differences was analyzed by parametric and nonparametric methods.

RESULTS AND DISCUSSION

The blood serum is the most available material for patients monitoring. We studied features of enzyme activity both in the tumor tissue and blood serum of patients with gastric cancer, and in the blood serum of the healthy persons (Table 1). There is evidence that TK demonstrates the highest level of activity in the blood of healthy persons at the age of 46–60 years. The relationship between parameters was assessed statistically using Spearman's rank correlation test, i.e., *rho* = 0.519, a positive correlation between the TK activity and age was shown. Therefore, we determined the possibility of TMP synthesis increasing by the "salvage pathway" in healthy individuals aged 46–60 years. However, an age-dependent activity increase is also characteristic of TP (the index of Spearman's rank correlation, i.e., *rho* = 0.874, a positive correlation between the TP activity and age). It is interesting to note that direct correlation between anabolism and catabolism enzymes activity (TK, TP) can regulate the rate of their pathway dTTP synthesis in healthy organism.

Table 1. The "salvage thymidilate synthesis" enzymes activity in serum in control group and gastric cancer patients at the different age

Age (years)	TK (nmol/mg·h)		TP (nmol/mg·min)	
	Control	GC	Control	GC
25–35	2.67 ± 0.34	-	36.16 ± 2.15	-
36–45	2.96 ± 0.51	5.98 ±1.10*	41.61 ± 3.12	35.26 ± 3.12
46–60	3.87 ± 0.71	6.78 ± 0.98*	47.88 ± 2.62	14.65±2.38***
61–70	3.05 ± 0.32	4.28 ± 0.89*	52.01 ± 2.89	17.32±2.28**

Notes: GC – gastric cancer; authentic differences versus normal value:*p < 0.05, **p < 0.01, ***p < 0.001.

It has been determined that in case of gastric cancer development the age-dependent property of TP activity was lost. A negative correlation between the TP activity in the blood serum and age was exposed (*rho* = — 0.189). In this case the increasing of TK activity was accompanied by decreasing of TP activity in the blood serum of patients with gastric cancer. This disorder may be one of the reason of dTTP synthesis increasing and higher rate of proliferation.

TK and TP activity changes in the blood serum of patients with gastric cancer demonstrate their activity peculiarities in tissues (Fig. 2). It has been found that TP activity was decreased in the tumor tissue up to 21.9 ± 8.6 nmol/mg·min in comparison with non-tumor mucose (57.82 ± 8.0 nmol/mg·min, p < 0.01) that was accompanied by decreasing of its activity in blood serum (up to 14.65 ± 2.4 nmol/mg·min, p < 0.001). The TK activity increase both in tumor tissues (more than 3.5 times) and in blood serum was determined.

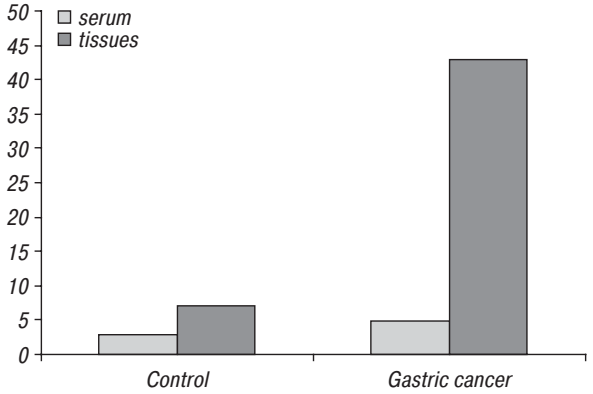

Fig. 2. The thymidine kinase activity in the blood serum of control group, patients with gastric cancer at the age of 46–60 years (nmol/mg·h)

According to the obtained findings as well as the results of our previous studies [4, 6] the activity of serum enzymes correlates with their activity in the tissues. Our findings comply with the results of other authors [7]. It is known that metabolic features of tumors can be due to their ability to express the embryonal forms

of the enzymes. In particular, it was shown the existence of several cellular forms of thymidine kinase: two mitochondrial TK isozymes — thymidine kinase A, which is localized mainly in their matrix, and thymidine kinase B — isozyme of the inner mitochondrial membrane. These proteins differ not only for physical and chemical properties, but also the sensitivity to chlorphynicole inhibition. A-isozyme is a predominant mitochondrial enzyme. It is determined in slowly proliferating tissues of adult animals and manifests a stable activity throughout its life. However, it is established that the major part of the enzyme in the cultures actively proliferating normal and tumor cells is localized in the cytoplasm. It has also been established that TK isozymes of the human of mitochondrial and cytoplasmic localization are expressed by different genes from chromosomes 16 and 17. Cytoplasmic thymidine kinase is named "embryonal" isozyme. It has different from mitochondrial protein physical and chemical properties — the isoelectric point, the sedimentation rate, electrophoretic mobility in polyacrylamide gel, specificity to phosphate donor [1]. It is TK "embryonic" that can phosphorylate thymidine for the synthesis of nuclear DNA, and its activity is directly correlating with the cellular proliferation rate. It has been determined that TK maximum activity of different incubation regimes has differed from physiological one (Fig. 3). As the maximum TK activity of blood serum of healthy people of different age is registered at 20th minute of incubation in the reaction medium, and two-stage activity increase at 10th minute is characteristic of TK blood of oncologic patients, and activity maximum is registered at 30th minute. The obtained data can be probably associated with of the "embryonal" isozyme expression increase, as it has been established that tumor isozyme is determined not only in tissues, but in patients' blood as well.

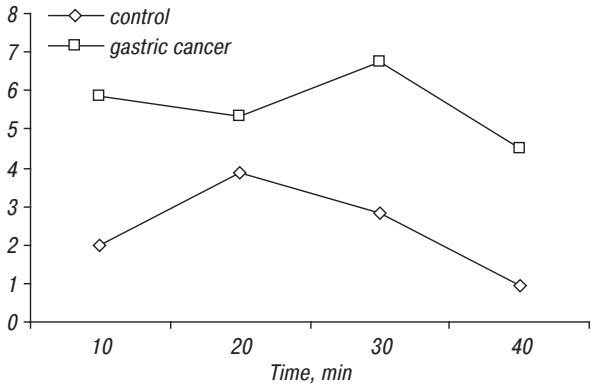

Fig. 3. Effect of different time of incubation on the thymidine kinase activity in the blood serum of control group, patients with gastric cancer at the age of 46–60 years (nmol/mg·h)

Different localization in the cytosol and in the nucleus is characteristic of TP as well, which may indirectly indicate the presence of isozyme forms too [8, 9]. The possibility of enzymatic tests using for diagnosis of recurrence, for individualization of chemotherapy was also studied. For this purpose the dynamics of TK and TP activity changes in the blood serum of patients with gastric cancer at pre- and post-operative periods was studied (the activity investigation was made every other 3 days within 14 days). Several groups were formed according to peculiarities of the enzymes activity (Table 2). They turned out to depend on the amount of tumor eradication, i.e., according to the type of surgical intervention (Table 2). Thus, it was registered the decrease of TK activity after radical surgery followed by reaching the normal value (2.1 ± 0.6 nmol/mg·h). TP activity was increased up to 38.3 ± 1.95 nmol/mg·min with reaching the normal level later.

At the same period following palliative operations the TK activity on day 6 after the surgical intervention began to increase (a dangerous symptom) and up to the end of the second week it exceeded the initial preoperative activity reaching 5.50 ± 0.45 nmol/mg·h. Simultaneously TP activity was decreased to the initial values. Thus, one can suppose that individual monitoring of TK and TP activity demonstrates the character of surgery and volume of tumor eradication.

Another aspect of the given study was to evaluate the effectiveness of different methods of chemotherapy according to the analysis of survival in groups of patients with unresectable gastric cancer treated with PCT scheme PF (5-fluorouracil and platinum). Out of 125 patients 45 patients took an endolymphatic infusions of chemotherapeutic agents, 50 — took the antitumoral agents by intra-arterial infusions and 30 patients had the prolonged intravenous PCT. It has been determined that survival rate of the patients with predominantly infiltrative form of tumor growth (T_4, infiltration of the surrounding organs, retroperitoneal spread) treated by endolymphatic PCT was 11.6 months that was higher than those of patients with the metastatic form of tumor growth (T_{3-4}, M_1) — 6.5; survival rate of patients treated by intra-arterial PCT — 12.4 vs. 4.5 months, and that of patients treated by intravenous PCT 8.9 and 8.8 months.

The present study suggests that the efficiency of PCT performed by different ways of the drugs administration may be associated with the character of tumoral process spread [10]. The recommendations for the different ways of 5-FU administration may be based on the previously stated peculiarities of pharmacokinetics [11]. It was demonstrated that the 5-fluorouracil maximum concentration in the tumor tissue by endolymphatic or intra-arterial infusion exceeds those after intravenous infusion and provides more prolonged effect on tumor cells. Besides, by endolym-

Table 2. The association between serum enzymes activity and volume of surgical intervention

Enzymes activity	The type of surgery					
	Radical		Diagnostic		Palliative	
	before	after 2 weeks	before	after 2 weeks	before	after 2 weeks
TK, nmol/mg·h	6.29 ± 1.47	2.09 ± 0.56*	6.43 ± 1.76	4.02 ± 0.45	4.17 ± 0.66	5.50 ± 0.45
TP, nmol/mg·min	15.28 ± 2.02	38.33 ± 1.95**	10.03 ± 2.41	11.43 ± 2.22	13.0 ± 1.88	14.52 ± 1.67

Note: authentic differences versus normal value: * $p < 0.05$, ** $p < 0.01$.

phatic administration there was noted a high selectivity of 5-FU accumulation in the tumor tissue, the parietal peritoneum and retroperitoneal lymph nodes [11]. It is important to note that intraoperative setting of permanent catheter according to techniques worked out in Donetsk Regional Anticancer Center allows to carry out up to 5 cycles of intra-arterial PCT (25 long-term intra-arterial injections of 5-fluorouracil) [12], that is not possible while dealing with traditional catheterization of gastric arteries according to Seldinger.

Obtained results allow to suggest that the control of individual dynamics of TK and TP activity in blood serum of gastric cancer patients may be useful as informative tool for monitoring of patients and treatment optimization.

REFERENCES

1. Brockenbrough JS, Morihara JK, Hawes SE, ***et al.*** Thymidine kinase 1 and thymidine phosphorylase expression in non-small-cell lung carcinoma in relation to angiogenesis and proliferation. J Histochem Cytochem 2009; **57**: 1087–97.

2. Bijnsdorp IV, de Bruin M, Laan AC, ***et al.*** The role of platelet-derived endothelial cell growth factor/thymidine phosphorylase in tumor behavior. Nucleosides, Nucleotides, Nucleic Acids 2008; **27**: 681–91.

3. Sam J, Lubner L, Noelle K, ***et al.*** A Phase II Study of Oxaliplatin, 5-Fluorouracil, Leucovorin, and High-Dose Capecitabine in Patients with Metastatic Colorectal Cancer. J Clin Colorectal Cancer 2010; **9**: 57–161.

4. Borzenko BG, Bakurova EM. Violation of the metabolism of DNA precursors in gastric mucosa as an indicator of likely malignancy ulcers of the body. Questions Oncol (St. Petersburg) 2007; **54**: 184–7 (In Russian).

5. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Proteins measurement with the Folin phenol reagent. J Biol Chem 1951; **193**: 265–75.

6. Borzenko BG, Kulchitskiy OK, Bacurova EM. Age aspects of the metabolism of thymidine monophosphate and possible ways of its control. Problems Aging Longevity 2002; **11**: 363–8 (In Russian).

7. Kubiak R, Miszczak-Zaborska E, Smolarek M, ***et al.*** The thymidine phosphorylase as the platelet-derived endothelial cell growth factor of endometrial cancer. Ginekol Pol 2009; **80**: 596–601.

8. Yu EJ, Lee Y, Rha SY, ***et al***. Angiogenic factor thymidine phosphorylase increases cancer cell invasion activity in patients with gastric adenocarcinoma. Mol Cancer Res 2008; **6**: 1554–66.

9. De Bruin M, Temmink OH, Hoekman K, ***et al.*** Role of platelet derived endothelial cell growth factor / thymidine phosphorylase in health and disease. Cancer Therapy 2006; **4**: 99–115.

10. Gamayunov ST, Slugarev VV, Terent'ev IG. Endolymphatic prevention of infectious complications and recurrence of intraabdominal recurrent of stomach cancer in the early postoperative period. Vestnik Limphology 2008; **2**: 23–4 (In Russian).

11. Stashkevich MA, Chomutov EV, Shatova OP, ***et al.*** Pharmacokinetics of fluorouracil in the tumor and normal tissue of patients with adenocarcinoma of the stomach. Oncology 2012; **14**: 19–23 (In Russian).

12. Sidyuk AV. Palliative combined treatment of stomach cancer with liver metastases. Ukr J Surgery 2012; **3**: 62–5 (In Russian).