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POSTOPERATIVE AUTOVACCINOTHERAPY FOR PATIENTS WITH GASTRIC CANCER AND EXPRESSION OF SOME PROTEINS IN TUMOR TISSUE

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Aim: **To study the efficacy of autovaccine in the treatment of gastric cancer and significance of molecular factors having prognostic values for disease outcome to evaluate its efficacy in clinical setting.** ***Patients and Methods:*** **150 patients with histologically proven adenocarcinoma of the stomach of stages II, III or IV were enrolled into study. 86 patients have been treated with autovaccine (AV) after operation. Expression of p53, Bcl-2, receptors of tyrosine kinase, vascular endothelial growth factor (VEGF), E-cadherin, α-catenin and β-catenin was determined in paraffin embedded tumor samples by means of immunohistochemical method with the use of respective monoclonal antibodies.** ***Results:*** **It was shown that application of AV has resulted in the increase of 3-year overall survival of patients having stage III of disease by more than 30%, but those having stage IV — only around 14%. The increase of 3-year overall survival of patients with metastases in lymph nodes ($N_{1–2}$) was observed also in more than 30%. It has been suggested the optimal phenotype for vaccine application: p53(+), EGFR(+), HER-2 neu (+), β-catenin (+), VEGF(+) and Bcl-2(+) with no dependence on E-cadherin and α-catenin presence.** ***Conclusion:*** **It was determined that the best effect of AV application is observed in patients with category $T_{3–4}$, poorly-differentiated tumors, metastases in lymph nodes ($N_{1–2}$), but without distant metastases (M_0). Gastric cancer patients with p53, EGFR, HER-2/neu, β-catenin, VEGF and Bcl-2-positive tumors are the favorable group for the treatment with AV in the adjuvant regime.**

Key Words: **gastric cancer, antitumor autovaccine, survival, p53, Bcl-2, EGFR, HER-2/neu, VEGF, E-cadherin, α-catenin, β-catenin.**

Gastric cancer is the second most common cancer and a leading cause of cancer-related death worldwide associated with poor response to the chemo- and radiotherapy [1, 2]. Therefore new approaches to the treatment of patients with gastric cancer (GC) may be promising [3–5]. Among them there is immunotherapy that is available for to-day: there are implemented the clinical use of immunomodulators, monoclonal antibodies and their conjugates as well anticancer vaccines which elicit specific antitumor immune response by means of tumor-associated antigens (TAA) and adjuvants [6, 7]. For clinical setting vaccines must be safe, effective against the identical tumor histological types and have stable properties [8].

At the same time it is known that disease outcome of patients diagnosed as having both the same stage of disease and tumor histological type may vary to a degree and advance of disease depends on the level of expression of the number of definite tumor proteins [9]. In our recent studies [10, 11] it has been shown the prognostic significance of the expression of Bcl-2 family proteins, receptors with tyrosine kinase activity, adhesion molecules and vascular endothelial growth factor (VEGF) for the patients with GC using both the immunocyto- and immunohystochemistry. In particular it was revealed that expression of p53 and VEGF is observed in diffuse type of carcinoma in patients with advanced disease and having poor survival time as a rule. Contrary it was shown that Bcl-2 expression in tumor on the early stage of disease is the marker of worse outcome. The expression in tumor both receptors of tyrosine kinase (EGFR and HER-2/neu) also testify the unfavorable prognosis for GC patients. The availability in tumor of E-cadherin is correlated with lymph node metastases free and observed on the early stages of disease. In tumor tissue of patients who have not distant metastases the expression of β-catenin was determined, and VEGF expression was clearly associated with stomach wall lesions. It was proved that expression of E-cadherin and α-catenin are the main features of intestinal type of GC on the early stages of disease and associated with favorable prognosis for patients with GC.

It should be mentioned that at present there are a lot of clinical data as to efficacy of autovaccines (AV) applied to prevent recurrence and metastasis in patients with GC [3, 5, 12, 13] but at the same time relationship between the vaccinotherapy efficiency and tumor molecular profile remains still not clear. The aim of this study was to evaluate the significance of molecular factors having prognostic values for disease outcome in patients with GC under application of the AV with assessment of its efficacy in clinical setting.

Patients. Tissue samples were taken from 150 patients with primary GC who have been treated in the "National Cancer Institute" (Ministry of Health of Ukraine, Kiev) between 1998 and 2007. The study included 86 patients with GC underwent AV after operation (subtotal resection or gastrectomy, group A) and 64 patients underwent operation only (group B). None of patients did not undergo preoperative radio- or chemotherapy. Adjuvant chemotherapy was not applied because of the rejection of patients to be treated or there were no indications to use it. All pa-

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Abbreviations used: AV – autovaccines; GC – gastric cancer; TAA – tumor-associated antigens; VEGF – vascular endothelial growth factor.

tients provided written consent to perform the study. Clinicopathological characteristics of patients with GC are presented in Table 1.

Table 1. Clinico-pathological characteristics of the GC patients

Indices		Group A (n = 86)	Group B (n = 64)
Gender	Male	48	35
	Female	38	29
TNM Stage	II	14	15
	III	34	20
	IV	38	19
Gastric wall invasion (T)	T_{1-2}	5	7
	T_3	42	38
	T_4	39	19
Lymph node metastasis (N)	N_0	37	26
	N_1	30	15
	N_2	19	23
Distant metastases (M)	M_0	74	60
	M_1	12	4
Grade of differentiation (G)	G_{1-2}	18	15
	G_3	36	25
	G_4	32	24

Autovaccine preparation and administration. Tumor tissue speciments have been obtained during operation and were snap frozen and stored at –20 °C until use. AV were prepared from autologous tumor cells and products of *Bacillus subtilis* B-7025 [14]. It is corresponded to International and State standards and is permitted to clinical apply in Ukraine (Certificate of State registration of immunological drug № 411/03-300200000, January 09, 2003).

The onset of vaccination was on the 10–14 day after operation provided as subcutaneous injections of 3 ml of AV (9.0–12.0 mg of proteins) and was performed triply with 7 day intervals. Revaccinations were repeated in one month and in six months at the same manner.

Immunohistochemical examination. Expression of p53, Bcl-2, EGFR, VEGF, HER-2/neu, E-cadherin, α- and β-catenin have been evaluated by means of immunohistochemical staining using specific monoclonal antibodies [15]: for p53, Bcl-2 — clones DO-7 and 124, respectively; for EGFR and HER-2/neu — clones 3B5 and E30, respectively; for E-cadherin, α- and β-catenin and VEGF — clones NCH-38, α-catenin-1, β-catenin-1 and VG1, respectively. All specific monoclonal antibodies belong to Dako Cytomation (Denmark). The visualization of immunohistochemical reaction was provided using Envision$^+$ kit and 3,3-diaminobenzidin (Dako Cytomation, Denmark) and hematoxylin staining. The results of immunohistochemical staining were interpreted as follows: tumors were considered as positive if the number of cells with nuclear staining for p53 was greater than 5% and if the number of cells with cytoplasmic reaction for other studied proteins was more then 20% [16].

Statistical analysis. All data were expressed as a mean ± SE. Differences between groups were examined for statistical significance using Student's *t*-test. $p < 0.05$ denoted the presence of a statistically significant difference.

It was found that application of AV has resulted in the increase of 3-year overall survival of patients having stage III of disease by more than 30%, but those having stage IV — only around 14% (Table 2).

Table 2. 3-year overall survival of GC patients according to TNM stage

TNM stage	3-year overall survival (%)		*p*
	Group A	Group B	
III	70.8 ± 5.6	39.4 ± 8.5	< 0.05
IV	40.9 ± 10.5	26.7 ± 11.4	

The increase of 3-year overall survival of patients with metastases in lymph nodes (N_{1-2}) was observed also in more than 30% (Table 3).

Table 3. 3-year overall survival of GC patients according to pN stage

pN stage	3-year overall survival (%)		*p*
	Group A	Group B	
N_0	81.2 ± 4.7	67.7 ± 8.4	
N_{1-2}	54.3 ± 6,5	23.1 ± 6.7	< 0.05

The 3-year survival rate of patients with well-differentiated tumors has been increased almost by 22%; and with poorly-differentiated — by 27% after AV application (Table 4).

Table 4. 3-year overall survival of GC patients according to grade of differentiation

Grade of differentiation (G)	3-year overall survival, %		*p*
	Group A	Group B	
G_{1-2}	77.4 ± 7.5	55.6 ± 11.7	
G_{3-4}	67.6 ± 4.5	40.4 ± 6.8	< 0.05

Moreover it was also observed the increase of 3-year survival rate among patients with tumors of T_3 and T_4 categories and who have been treated with AV by 22% and 42%, respectively.

Very important data were obtained after the analysis of 5-year overall survival of patients of groups A and B (Table 5). It is seen that every year more patients with GC are alive when they were treated with AV after surgical operation. But to provide statistical analysis as to the 5-year overall survival of patients with GC it needs further observations.

Table 5. 5-year overall survival of GC patients

Groups	1 year	2 year	3 year	4 year	5 year
Group A	86.6 ± 3.1*	75.5 ± 5.1*	71.4 ± 3.7*	64.6 ± 4.8*	51.6 ± 5.2
Group B	69.4 ± 6.2	52.2 ± 7.1	43.5 ± 6.3	40.1 ± 5.7	34.8 ± 5.1

*$p < 0.05$ compared with group B.

Analysis of the immunohistochemical investigation has shown, that patients with the T_3 and T_4 categories who have been treated with AV have more favorable prognosis (Table 6). Particularly, they had more higher α-catenin expression and lower expressions of p53 and VEGF in tumor tissue.

Table 6. Expression of molecular markers in gastric tumor and pT category (in %)

Marker	T_3		T_4	
	Group A	Group B	Group A	Group B
EGFR	26.47 ± 5.12	58.33 ± 8.45	14.50 ± 2.11	48.25 ± 7.74
VEGF	35.29 ± 5.38	26.13 ± 5.02	36.00 ± 3.25	50.00 ± 8.04
p53	72.35 ± 7.45	65.00 ± 8.68	62.90 ± 5.06	75.33 ± 10.12
Bcl-2	18.82 ± 3.12	25.25 ± 4.98	22.25 ± 2.48	25.00 ± 6.45
α-catenin	38.24 ± 5.50	24.66 ± 4.85	37.50 ± 3.38	23.33 ± 6.23
β-catenin	55.88 ± 6.33	33.33 ± 6.50	33.50 ± 3.02	26.00 ± 6.77
E-cadherin	47.06 ± 5.94	41.67 ± 6.89	55.50 ± 5.02	52.16 ± 9.89
HER2/neu	29.41 ± 5.22	35.33 ± 6.77	34.33 ± 3.31	25.00 ± 6.45

It was revealed the tendency for decrease of EGFR expression (decline to 19.0%), and increase both of Bcl-2 expression (up to 40 %) and VEGF (up to 69%) in tumor tissue of patients with diffuse type of GC.

It has been found that important features of tumor tissue of patients without metastases into lymph nodes are the absence of HER-2/neu, p53 and VEGF and the presence of E-cadherin and α-catenin expressions.

None the less it has been observed a significant increase of 3-year survival (by 31%) even among

patients with unfavorable prognosis (more higher p53 expression and more lower level of α-catenin) in group A with metastases into lymph nodes.

It was also shown that expression of studied factors have not been depended on tumor differentiation but we confirmed that well-differentiated gastric tumors may be characterized by the presence of E-cadherin, β-catenin, EGFR and HER-2/neu and also by the absence of VEGF, that is agree with literature data and is considered as the favorable prognosis.

Survival of such patients is one of the main criteria of cancer treatment efficiency. We have tried to establish the association between expression of the studied proteins and GC patients' survival rates. Particularly, we have shown that cells of tumor of patients with GC who lived less than a year were characterized by the presence of EGFR, Her-2/neu, VEGF, p53, Bcl-2, and by the absence of E-cadherin and α-catenin expressions.

It has been shown significant differences in overall survival of patients treated with AV (group A) and operation only (group B) (Table 6). It may prove that AV application in the treatment of patients with unfavorable immunophenotype (presence of mutant p53, β-catenin, tyrosine kinase receptors EGFR and HER-2neu) increases the chance to live more than one year compared to patients, who have been treated with operation only.

It has been suggested the optimal phenotype for vaccine application: p53(+), EGFR(+), HER-2neu (+), β-catenin (+), VEGF(+) and Bcl-2(+) with no dependence on E-cadherin and α-catenin presence.

We have seen that immunohistochemical evaluation of E-cadherin, α-catenin, VEGF, p53, Bcl-2 and Her2/neu is the most informative. It has been shown that AV application in the treatment of patients having stages III or IV significantly increased. But the best effect of AV application was observed in patients with GC in category T_{3-4} with metastases in lymph nodes (N_{1-2}), without distant metastases (M_0) and in poorly-differentiated tumors.

It is worth to note that our previous studies concerning the efficiency of AV application in the treatment of patients with colorectal [17] and lung [18] cancers have shown that it is also more preferably to use AV to treat the patients having stage III of the disease with metastases in lymph nodes.

Above presented data permitted to suggest that evaluation of the expression of molecular markers should be done before the application of AV in the treatment of patients with GC to predict unfavorable prognosis.

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