LOCAL ANTITUMOR IMMUNE RESPONSE IN PATIENTS WITH SKIN MELANOMA: THE RELATIONSHIP WITH THE EFFICIENSY OF INTERFERONTHERAPY

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INTRODUCTION

Assumptions about the leading role of immunological mechanisms in the regression of melanoma, which is indirect evidence of severe limfoid cells infiltration of the primary tumor, there were quite a long time [1-3]. In particular, it was shown that the recurrence of the disease is less common in patients with severe local lymphoid plasmocytic reaction in the stroma of the tumor, detected in 37% of cases of primary localized skin melanoma [2]. Study Clemente C.G. et al. [3], held on a large clinical material, it was found that infiltration limfoidcells primary skin melanoma is independent favorable prognostic factor.

It is now recognized that the predominant cell population of the inflammatory infiltrate in the stroma of skin melanoma is represented by T-lymphocytes, and the presence of $CD8^+$ cells in the parenchyma it is an important predictor of better survival of patients [4]. However, whether this fact is evidence of the elimination of tumor cells, or a sign of inhibition of tumor growth remains controversial.

Later it was shown that not only the primary tumor, but metastatic lesions and secondary bear evidence of immunological control their growth. Based on the analysis of the transcriptional profile of genes of innate and adaptive immune response to allocate options for metastatic melanoma, with an immunologically active and inactive ("asymptomatic") phenotypes [5-7]. Biopsies of metastatic lesions in these patients in the dynamics of immunotherapy showed transition profiles of transcription factors from the immunologically inactive to active [6]. The authors of the cited work suggested that in the later stages of the disease expression of melanoma differentiation antigens, due to the activation of immune-related transcription factors that support the dedifferentiation of tumor cells [7]. Detection of

interferon regulatory factor 1 (IRF-1) in these samples is correlated with better patient survival [8, 9]. Furthermore, in tumors of patients who responded to immunotherapy showed signs of severe inflammatory reactions background activation interferon stimulating genes (ISGs) and induction of γ-interferon (γ-IFN). Involvement of T lymphocytes into inflammatory due to chemokine production, which in turn correlates with an intensive infiltration of the tumor parenchyma $CD8^+$ T lymphocytes [10].

Thus, according to most researchers, limfoid cells infiltration and/or induction of transcription factor genes of the immune response indicating the activation of antitumor immune defense, which correlates with a favorable prognosis in patients with skin melanoma.

These aspects of the relationship of the tumor and the organism with the systemic immune reactions is now being given special attention because of the findings of the prognostic value of various immunological parameters in patients with malignant tumors and the development of standardized approaches to their determination [11-13]. Therefore discusses the inclusion criteria immunological parameters in cancer staging [14, 15] and in some cases the use of immuno-dependent criteria (irRC) for evaluating immunotherapy applications [14].

This communication is devoted to the study by immunohistochemical (IHC) method of localization of lymphocytes and their phenotypic characteristics and severity of tissue infiltration of the primary tumor in order to clarify the prognostic value of these parameters with adjuvant interferon therapy in patients with primary localized melanoma.

SUBJECTS AND METHODS

The study included 12 patients with melanoma IB-IIC stage disease (mean age (57,7 ± 3,7) years) who were treated at the Department of tumors of the skin and soft tissues of the National Cancer Institute in 2009-2012. Patients received standard therapy, including surgery (wide excision of the primary tumor) and adjuvant α-IFN therapy (Laferobion subcutaneously 3 MIU 3 times a week for 12 months). Relapse

during the first year after removal of the primary tumor was seen in this study as an adverse course of the disease, accordingly, the absence of relapse as favorable. All patients provided written informed consent to participate in the study, which was approved by the local Ethics Committee.

Characteristics of patients according to gender, age, location and histopathological features of the primary melanoma (average thickness of the tumor Breslow (3,1 ± 0,4) mm), with the course of the disease is presented in the table.

Table. Characterization of patients

Indicators		Number of patients, course of the disease (favorable / unfavorable)
Gender	Female	8 (5/3)
	Male	4 (3/1)
Age	31-50 years	3 (1/2)
	> 50 years	9 (7/2)
Tumor localization	Extremities	7 (4/3)
	Body	5 (4/1)
Histological type of tumor	Epithelioid	8 (4/4)
	Spindle-cell	1 (1/0)
	Mixed	3 (3/0)
Ulcerated tumor	Yes	8 (4/4)
	No	4 (4/0)
Thickness of the tumor Breslow, mm	1,1-2,0	2 (1/1)
	2,1-4,0	8 (7/1)
	> 4,0	2 (0/2)
Total patients		12 (8/4)

IHC study of the primary tumor was performed on material obtained in the surgical treatment of patients. Operating material was fixed in buffered 10 % formalin solution (pH 7,4) and compacted in paraplast using of histoprocessor Histos-5 ("Milestone", Italy). From the paraffin blocks were prepared histological sections 5 microns in thickness with a microtome Microm NM325 ("Thermo Scientific",

Germany). Sections were stained with hematoxylin and eosin. IHC reactions were carried with antibodies against human antigens: polyclonal CD3, monoclonal CD8 (clone C8/144B), CD4 (clone 4B12), CD20cy (clone L26), CD56 (clone 123C3), CD45RO (clone UCHL1) ("Dako", Denmark) in accordance with the manufacturer's recommendations, as well as CD45RA (clone ALB11) ("Beckman Coulter", USA), HLA-DR (clone G46-6 (L243)), CD25 (clone M-A251), CD95 (clone DX2) ("Becton Dickinson", USA). To visualize the products of IHC reaction were used detection system EnVision™ FLEX ("Dako", Denmark) using as the chromogen 3-amino-9-ethylcarbazole (Mono AEC; "DBS", USA). Sections were stained with hematoxylin Gill. As a positive control tissue samples with positive reactivity, and for the negative control procedure was performed without the use of primary antibodies.

The resultant preparations were studied and photographed using a Nikon Eclipse 80i microscope with camera DS-5SMc/L2. Evaluation of the content of labeled cells in the preparations made semi-quantitatively: "0" – no, "1+" – single cells, "2+" – 10-30 cells, "3+" – more than 30 cells in the field of view, expressing the results in arbitrary units from 0 to 3.

Statistical processing was performed using the program Excel (MS Office 2003, XP) and STATISTICA 6,0 ("StatSoft Inc.", USA). Results of the study were presented as median with minimum and maximum values. To determine the significance of differences in the groups of indicators used Mann-Whitney test. Differences were assessed as reliable at $p < 0,05$.

RESULTS AND DISCUSSION

Among the studied leukocyte antigens (CD3, CD8, CD4, CD20, CD56, CD45RA, CD45RO, CD95, CD25, HLA-DR) in the IHC detection of immune inflammation in the tissue of the primary melanoma was confirmed by expression of only 5 (CD3, CD8, CD45RA, CD45RO, CD20) (Fig. 1).

Fig. 1. Population structure of cells of lymphoid infiltration of primary skin

melanoma

As can be seen, in all cases, the cells of limfoid cell infiltrate into the stroma parenchyma of melanoma have been presented by T-lymphocytes ($CD3^+$) and only in 3 of 12 cases, in addition to $CD3^+$-cells, B-lymphocytes are identified ($CD20^+$). Furthermore, all T-cells express exclusively coreceptor CD8, allowing these cells to include a subpopulation of cytotoxic T lymphocytes (CTL). Most CTL localized in the tumor stroma (respectively, 2,10 (1,25; 2,50) versus 0,62 (0; 1,40) cond. u. in its parenchyma, $p < 0,05$). Thus parenchymal infiltration melanoma $CD8^+$ lymphocytes were detected in 9 of 12 patients.

$CD8^+$ subpopulation of T lymphocytes has been presented as naive T-cells expressing an isoform of tyrosine phosphatase CD45RA, and immunological memory cells $CD45RO^+$. A detailed analysis showed that the tumor stroma in all the cases studied, there are both naive $CD8^+$ lymphocytes and cells of immunological memory (respectively, 2,28 (1,33; 2,88) and 2,00 (1,67; 2, 86) cond. u.). In contrast, melanoma parenchyma in 8 out of 12 cases infiltrated $CD45RA^+$ lymphocytes, where as the $CD45RO^+$-cells were found in only 4 of 12 patients (respectively 0,57 (0; 2,00) and 0 (0; 1,00) cond. u.). It is important to note that in these four cases were found as $CD45RA^+$-, and $CD45RO^+$-cells, which does not preclude the expression of both isoforms of tyrosine phosphatase on $CD8^+$ T lymphocytes during immune inflammation in primary skin melanoma.

The obtained results do not contradict the previously described data [3, 6, 9], indicates the predominance of $CD8^+$ lymphocytes among lymphoid cell infiltration in the tissue of the primary melanoma. Identification phenotypic differences of these cells suggests that the formation of local antitumor immune responses can occur not only due to the activation of naive CTL in the tumor microenvironment with its subsequent infiltration of the parenchyma, but also generate a melanoma-specific CTL from the effector T cell immunologic memory.

Currently, the infiltration of the primary tumor $CD8^+45RO^+$ lymphocytes seen in some malignant tumors as one of the most important prognostic indicators

associated with a favorable course of the disease [8, 9, 13].

In this connection was a retrospective analysis of the expression of the most informative leukocyte antigens described above, in primary melanoma tissues, depending on the efficacy of IFN therapy in such patients in the adjuvant setting. Given that the conditions of application of α-IFN during the first year after excision of the primary melanoma progression of the disease reported in 4 of 12 patients for data analysis were divided into two groups: with favorable and unfavorable course of the disease (Fig. 2).

Fig. 2. Population structure of lymphoid cells infiltrate the primary skin melanoma in patients with favorable and unfavorable course of the disease on the background of adjuvant IFN therapy

As can be seen from the data presented in Figure 2, the intensity of infiltration (total share of stained cells in the stroma and parenchyma of the tumor) primary

melanoma different populations of lymphocytes ($CD3^+$-, $CD8^+$-, $CD45RA^+$-, $CD45RO^+$- and $CD20^+$-cells) in patients with a favorable and unfavorable course of disease against the background of IFN therapy is not significantly different ($p > 0,05$). Draws attention to expressed tendency to increase the proportion of $CD8^+$- and $CD45RA^+$-cells in the inflammatory infiltrate in patients with a favorable course of the disease (Fig. 3), as well as a small proportion and, consequently, low information detection of B-lymphocytes in the context of their prognostic value in primary skin melanoma.

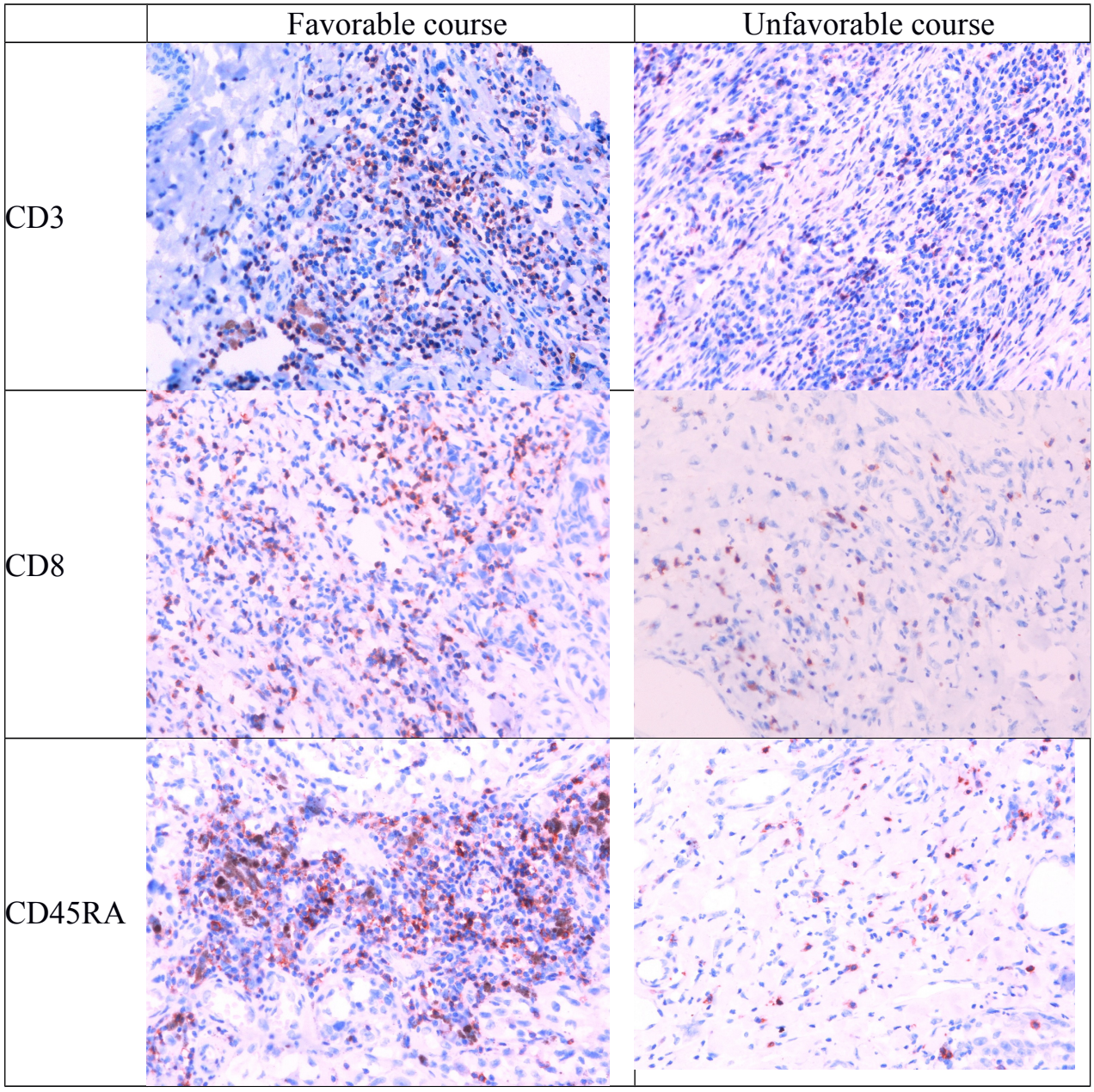

Fig. 3. Representation of $CD3^+$-, $CD8^+$- and $CD45RA^+$-lymphocytes in the

stroma of melanoma patients with favorable and unfavorable course of the disease. IHC reaction, hematoxylin. Microphotograph, the lens 20, the ocular 10.

Therefore, an analysis of the subpopulation of T lymphocytes infiltrating separately stroma or parenchyma of the primary tumor. It was found that patients with a favorable course of the disease, unlike patients with progression of melanoma, recorded more pronounced infiltration of the tumor stroma $CD45RO^+$-cells (respectively, 2,10 (1,67; 2,86) and 1,85 (1,67; 2,00) cond. u., $p < 0,05$), and parenchyma – $CD8^+$ lymphocytes (respectively 1,0 (0; 1,4) and 0,07 (0; 0,57) cond. u., $p < 0,05$).

These results suggest that increasing the proportion of CTLs, and / or effector memory T cells among the lymphocytes of the immune inflammation in patients with primary localized melanoma is associated with a favorable course of the disease against adjuvant IFN therapy.

It can be assumed that the immunological memory cell sensitized to the antigens of melanoma in the early stages of tumor growth capable of forming a local anti-tumor immune response plays a key role in the prevention of the disease recurrence. The clinical significance of immunological phenomenon of accumulation $CD8^+$- and $CD45RO^+$-cells in tumor tissue has been established previously with other malignant tumors [9, 13, 16].

In addition to the results obtained can be reduced as described previously [17], clinical and laboratory criteria for prediction of disease progression in patients with primary localized skin melanoma, based on the detection of changes in indicators of systemic immunity on the background of adjuvant IFN therapy. In our opinion, these immunological criteria in combination with the evaluation of the local anti-tumor immune response will help determine the individual characteristics of the disease, to predict the response of an organism to immunotherapy and thus optimize treatment strategy in this category of patients.

The further development and improvement of this approach in predicting the course of disease in patients with malignant tumors associated with harmonization in

the laboratory diagnostics. In this way, it is important to overcome the inherent difficulties caused by the use of different reagents in laboratories, features qualitative and semiquantitative account of the intensity of limfoid-cells infiltration, as well as different criteria for choosing its localization in tumor tissue. Development of the standard laboratory protocol will promote wider introduction of immunological criteria in the laboratory and diagnostic complex in oncology.

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