ORIGINAL PAPER

Statistical correlations between peripheral blood lymphocyte subpopulations and tumor inflammatory infiltrate in stage I of skin melanoma

MARIANA COSTACHE[1)], MONICA NEAGU[2)], ANGELA PETRESCU[2)], CAROLINA CONSTANTIN[2)], GINA MANDA[2)], CAMELIA DOINA VRABIE[3)], MARIA WALLER[2)], C. S. PETRESCU[2)], OLGA SIMIONESCU[4)]

[1)]*Department of Pathology, "Carol Davila" University of Medicine and Pharmacy, Bucharest "Victor Babeş" National Institute of Pathology, Bucharest University Hospital, Bucharest*

[2)]*"Victor Babeş" National Institute of Pathology, Bucharest*

[3)]*Department of Pathology, "Carol Davila" University of Medicine and Pharmacy, Bucharest "Victor Babeş" National Institute of Pathology, Bucharest "Sf. Ioan" Hospital, Bucharest*

[4)]*Department of Dermatology, "Carol Davila" University of Medicine and Pharmacy, Bucharest; "Colentina" Hospital, Bucharest*

Abstract

The article presents statistical correlations of immune cell parameters investigated in patients diagnosed with skin melanoma stage I. Recent data indicate a suppressed immune response, probably sustained by immune-regulating molecules expressed or shed from the tumor. These molecules block an efficient immune response and thus the tumor develops. All the molecules that are part of the tumor escape mechanisms can be targets for immune-mediated anti-tumor agents. We try to find the significance of some immunohistochemical markers (UCHL1, CD4, CD8) in tumoral inflammatory infiltrate and to establish the statistical correlations between molecular markers and tumor grade and stage. The studied parameters were: CD3+, CD4+, CD8+, CD56+16+ and CD19+. The statistical results were performed with SPSS *v.* 15.0. We demonstrate that a CD4+ on-going immune response is elicited in the investigated patients. We found a possible compensatory mechanism between T-lymphocytes and NK-cells and also between the antibodies generating cells and the natural cytotoxic cells. We are confident that these statistical correlations between clinical, immunological and immunohistochemical data can be useful in the disease management and personalized immune-therapy.

***Keywords*:** melanoma, stage 1, CD4+, CD8+, T-cell, immunotherapy, statistics.

Introduction

Melanoma one of the most aggressive forms of human cancer [1], although represents only 4% of all skin cancers, it accounts for 80% of skin cancer deaths and it is placed second after adult leukemia in terms of potential productive life-years loss [2, 3]. The lifetime risk increased steadily in the last 80 years from 1/1500 in 1935 to 1/50 in present, increasing at a rate of 3–5% per year, faster than any other cancer [4]. With no sensitive tools available to monitor therapy and follow-up, the above-mentioned statistics reflect the unpredictable pattern of recurrence of melanoma, as well as its resistance to treatment by radiation and chemotherapy [5]. Reports regarding cellular dysfunction associated with melanoma showed that patients with advanced melanoma had severe CD4+ and CD8+ T-cell lymphopenia associated with reduced T-cell proliferation [6]. The lower proliferation capacity registered in melanoma patients could be assigned to Tregs (T-regulatory cells) (CD4+CD25+ lymphocytes) [6]. Tregs suppress the immune responses, both the development and effector functions of tumor-specific T-cells [7], and can be a target for therapeutic intervention [8].

In therapy, immune-related markers were best put in use. IFN-alpha is one of the most used immunotherapy agent and the only approved by FDA. This therapy has a recently explained mechanism. It was shown in bulk PBMC that *in vitro* IFN-alpha exposure induced rapid and strong up-regulation of the DC-maturation markers CD80+, CD86+, and CD83+. Moreover, autonomous memory CD4+ T-cells proliferated when exposed to IFN-alpha-treated monocytes. These findings strongly point out that IFN-alpha promptly generates non-dendrite APCs able to stimulate memory immune responses [9].

The melanocyte performs several functions: synthesis of melanin granules, growth and maintenance

of dendrites; it is sensitive to hormones or neurotransmitters stimuli that induce the translocation melanin granules within the cytoplasm and then to adjacent cells that have phagocytic capacities [10]. Several conditions, genetic or non-genetic factors, can induce the neoplastic transformation of the melanocyte the leading to the development of skin melanoma. All these intra and extracellular events are subject for biomarkers finding that can be relevant for melanoma development [2, 11].

In the presented article, we show our preliminary results regarding immune cells detected in blood circulation or at tumoral site in a group of skin melanoma patients diagnosed in stage I. Therefore, circulating and tumor-resident immune cells were investigated in order to evaluate their potency as candidate biomarkers for skin melanoma. Several lymphocyte subpopulations involved in the anti-tumor immune response were analyzed by flow cytometry and by immunohistochemical characterization of the leukocyte infiltrates into the tumoral tissue.

Material and Methods

Patients' group

Forty-one patients diagnosed according to *American Journal of Critical Care* supported by the *American Cancer Society* (AJCC) were included after their informal consent in the tested group. All the patients were subjected to blood withdrawal and circulating immune cells identified using the IVD standard MultiTEST IMK Kit (Becton Dickinson). Patients were analyzed immediately after surgery without any additional therapy.

Immunophenotyping

Immunophenotyping of peripheral blood was assessed using BD MultiTEST IMK kit for CD3 FITC/CD8 PE/CD45/PerCP/CD4 APC and CD3 FITC/CD16+CD56 PE/CD45 PerCP/CD19 APC subpopulations and analyzed by flow cytometry (flow cytometer FACScan, Becton Dickinson). Results are expressed as [%] of the stained subpopulation.

Immunohistochemistry

After surgical removal of the skin tumor, the sample has been processed for standard histopathological diagnosis and immunohistochemical investigation of tumoral and peritumoral inflammatory infiltrate. The immunohistochemical staining was performed with the antibodies presented in Table 1.

Table 1 – ***Monoclonal antibodies and conditions in which they were used for immunohistochemical staining of inflammatory infiltrate***

Antibody	Source	Dilution	Clone
CD45RO	DAKO, Glostrup, Denmark	1:50	UCHL1
CD8	DAKO, Glostrup, Denmark	1:50	CD8/144B
CD4	Novocastra	1:20	NCL-L-CD4-368

Statistics

We presents the statistical results [12, 13] for a group of 41 patients diagnosed in stage I (mean age = 57.27 years) in order to evaluate the statistical significance of the peripheral blood lymphocyte subpopulations in correlation with the clinical stage. For statistical analysis we have used: normality tests for data (represented by histograms and Q–Q plots), correlations and regressions. The statistical results and graphics were performed with SPSS *v.* 15.0 for Windows.

Results

Peripheral lymphocyte subpopulations

Analyzing the mean value of the circulating CD3+, CD4+, CD8+, CD56+16+ and CD19+ in patients diagnosed in stage I only CD4+, CD8+ and CD19+ had mean values outside the normal standard values (Table 2).

Table 2 – ***Mean values for the peripheral percentages of investigated subpopulations in comparison to normal ranges***

Characteristics (mean value)	Stage I [%]	Normal ranges [%]
CD3+	69.22	67–76
CD4+	47.8	38–46
CD8+	21.63	31–40
CD4+/CD8+ ratio	2.6	0.9–1.5
CD56+/16+	12.2	10–19
CD19+	17.83	11–16

Figures 1 and 2 show that there are few individual values outside the normal ranges.

Calculating the correlations between the circulatory immune cells (Table 3 [12–14]) a strong correlation was found between the [%] of peripheral CD3+ and the patients age.

It is already reported in the literature that the peripheral CD3+ is modified in relation to the normal ageing process [15]. The good correlation found between CD3+ and CD4+, and their mean values indicate that an anti-tumoral response mediated by CD4+ is on-going.

The statistics has pointed out an inverse correlation between CD3+ and CD16+ that can be explained by compensatory mechanisms between the T-mediated immune response and the NK-cytotoxicity. The inverse correlation found between circulatory CD4+ and CD8+ is somewhat to be expected as the suppressor mechanisms sustained by CD8+ T-cells is counterbalanced by the activation of T-helper lymphocytes. The inverse correlation found between B-cells and NK, namely CD19+ and CD16+ is as well postulated to the existence of a compensatory immune mechanisms between the antibody generating cells (B-cells) and the natural cytotoxicity cells (NK-cells). The obtained correlations between the CD4 and CD8 and their ratio was to be expected.

The regression curves [14, 16] presented in Figure 3, show that the stated correlations are linear (type $y = ax + b$), and that the values obtained for the equations coefficients of these curves (Table 4) have a good statistic significance $p<0.05$ or very good $p<0.01$. We have found a normal distribution of the values (Figure 1) for CD4+, CD16+ and acceptable for CD3+, CD8+ and CD19+. The normal distribution of ages shows that our patients group was selected accordingly.

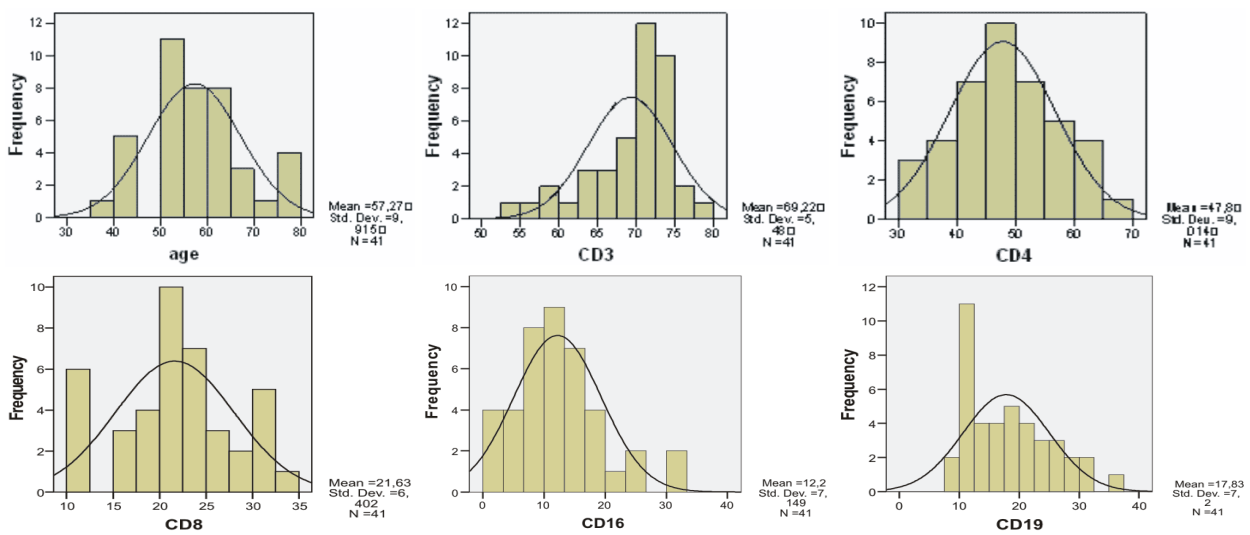

Figure 1 – ***Distribution patterns for studied parameters.***

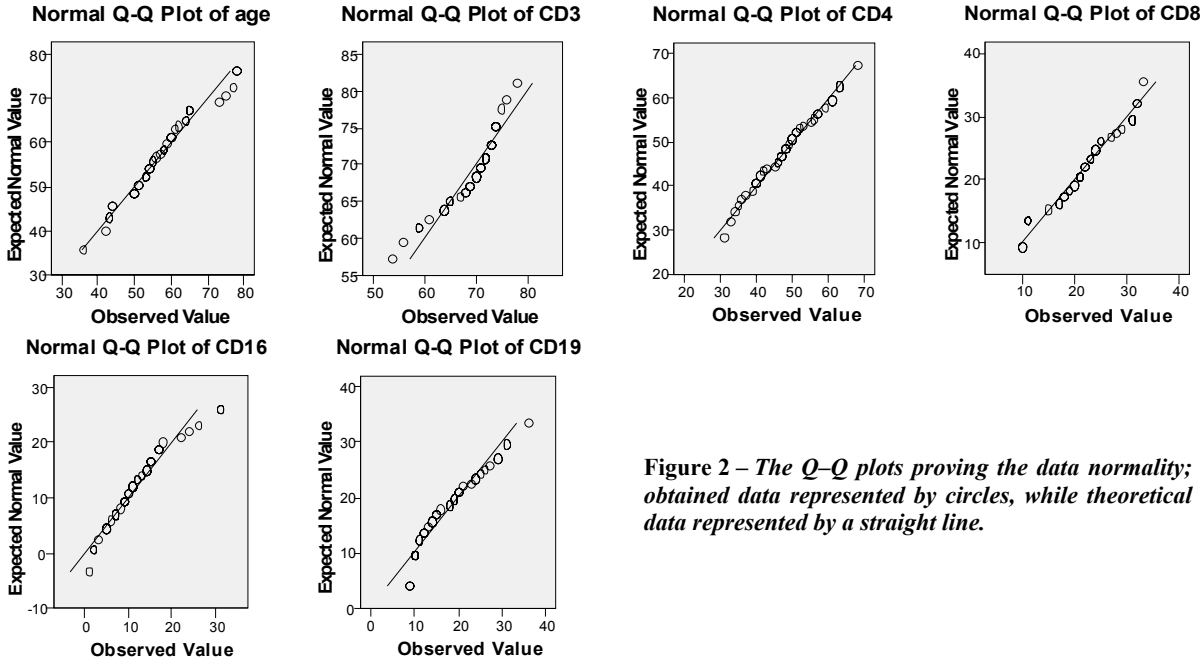

Figure 2 – ***The Q–Q plots proving the data normality; obtained data represented by circles, while theoretical data represented by a straight line.***

Table 3 – ***The values for the correlations***

Correlations		age	CD3	CD4	CD8	CD4CD8	CD19	CD16
age	Pearson Correlation	1	,318*	,275	-,075	,223	-,168	-,100
	Sig. (2-tailed)		,043	,082	,642	,161	,293	,534
	N	41	41	41	41	41	41	41
CD3	Pearson Correlation	,318*	1	,732**	-,142	,390*	-,298	-,450**
	Sig. (2-tailed)	,043		,000	,377	,012	,058	,003
	N	41	41	41	41	41	41	41
CD4	Pearson Correlation	,275	,732**	1	-,739**	,864**	-,148	-,394*
	Sig. (2-tailed)	,082	,000		,000	,000	,357	,011
	N	41	41	41	41	41	41	41
CD8	Pearson Correlation	-,075	-,142	-,739**	1	-,892**	,046	,086
	Sig. (2-tailed)	,642	,377	,000		,000	,776	,594
	N	41	41	41	41	41	41	41
CD4CD8	Pearson Correlation	,223	,390*	,864**	-,892**	1	-,101	-,211
	Sig. (2-tailed)	,161	,012	,000	,000		,529	,185
	N	41	41	41	41	41	41	41
CD19	Pearson Correlation	-,168	-,298	-,148	,046	-,101	1	-,695**
	Sig. (2-tailed)	,293	,058	,357	,776	,529		,000
	N	41	41	41	41	41	41	41
CD16	Pearson Correlation	-,100	-,450**	-,394*	,086	-,211	-,695**	1
	Sig. (2-tailed)	,534	,003	,011	,594	,185	,000	
	N	41	41	41	41	41	41	41

*. Correlation is significant at the 0.05 level (2-tailed). **. Correlation is significant at the 0.01 level (2-tailed).

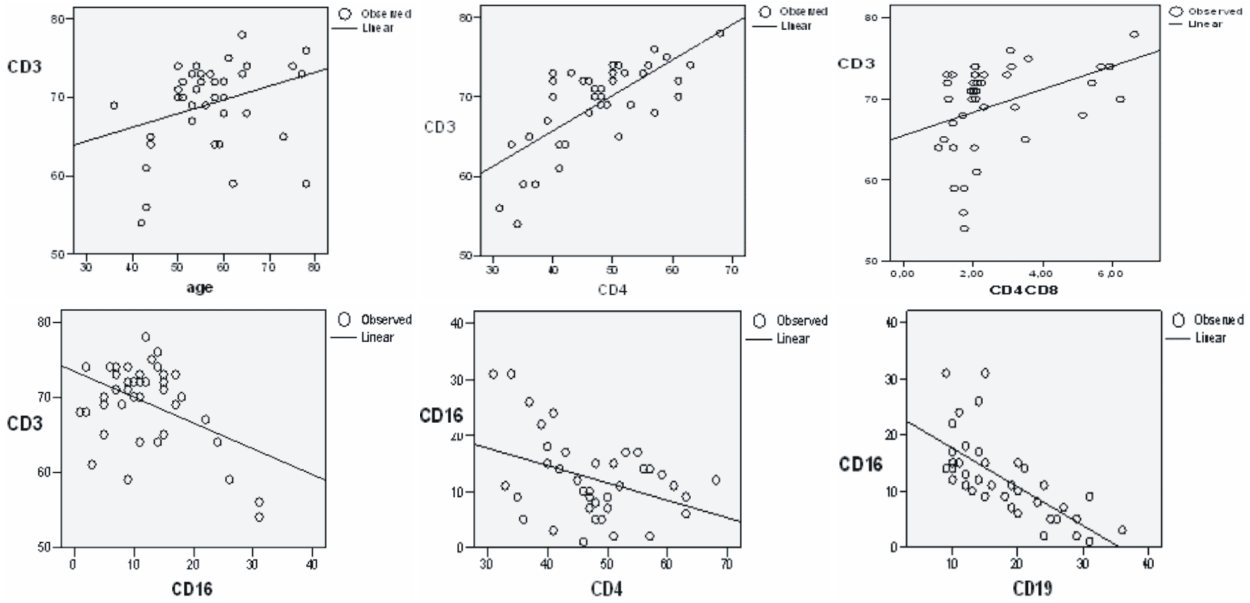

Figure 3 – ***Regression curves.***

Table 4 – ***The equations for the regression curves, describing the relationship between parameters***

Parameters in relationship	Equation	*p*-values for equation coefficients
CD3 and age	y = 0.176 x + 59.147	for a, $p<0.05$; for b, $p<0.0001$
CD3 and CD4	y = 0.445 x + 47.956	for a, $p<0.0001$; for b, $p<0.0001$
CD3 and ratio	y = 1.42 x + 65.553	for a, $p<0.05$; for b, $p<0.0001$
CD3 and CD16	y = -0.345 x + 73.423	for a, $p<0.005$; for b, $p<0.0001$
CD16 and CD4	y = -0.312 x + 27.129	for a, $p<0.05$; for b, $p<0.0001$
CD16 and CD19	y = -0.69 x + 24.503	for a, $p<0.0001$; for b, $p<0.0001$

The Q–Q plot (Figure 2) presents the values pairs containing the calculated *percentiles.* The upper and lower value for the for the sequences of values (x_1, x_2, ..., x_p and y_1, y_2, ..., y_p) are known as the two quartiles, namely the upper and lower quartiles. We have used them in order to verify normality of data [14].

The Q–Q plot compares the obtained with theoretical data. The theoretical data has a normal distribution with same mean and standard deviation. If the variable distribution is normal, then the graph has a linear trend.

In Figure 2 a normal distribution of values for the variables: age, CD4 (excellent), CD8, CD19, CD16 and CD3 is demonstrated (Figure 2).

This obtained normality is encouraging to use further methods and models offered by the statistical analysis [17, 18].

Immune cell tumoral infiltration

At the histopatologically examinated cases, we had observed that the peri- and intratumoral inflammatory infiltrate (Figures 4 and 5), consisted mainly of T-lymphocytes, UCHL1-positive (Figures 6–8).

The finding is an argument for the cell-mediated defense mechanism, which exists in the majority of cutaneous malignant tumors. The stromal inflammatory reaction is represented by lymphocytic infiltrates with or without melanophages, situated “in band-like” under the lesion. We found few T-lymphocytes positive for CD4 (Figures 9 and 10) and CD8 (Figures 11 and 12).

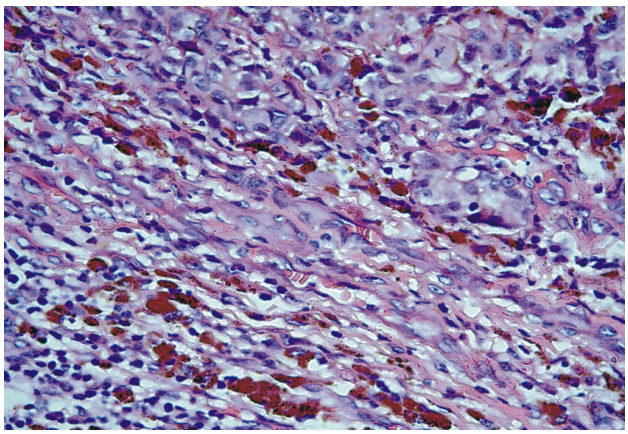

Figure 4 – ***Nodular melanoma; dermal inflammation contains numerous melanophages (HE stain, ob. 40×).***

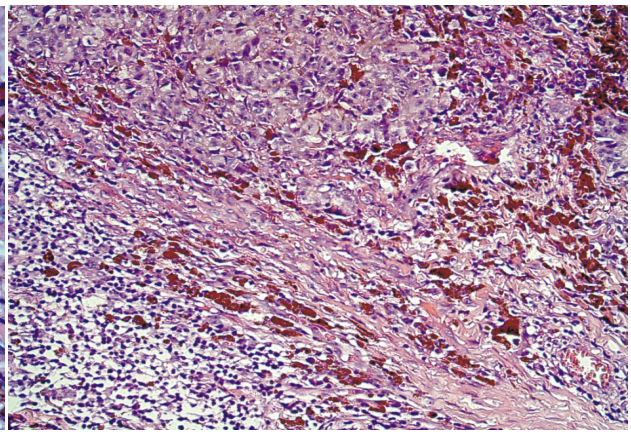

Figure 5 – ***Nodular melanoma; dermal inflammation contains numerous melanophages (HE stain, ob. 20×).***

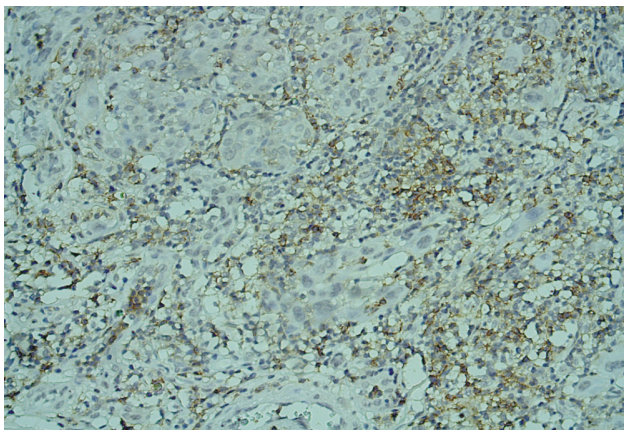

Figure 6 – ***Nodular melanoma (UCHL1-positive, ob. 20×).***

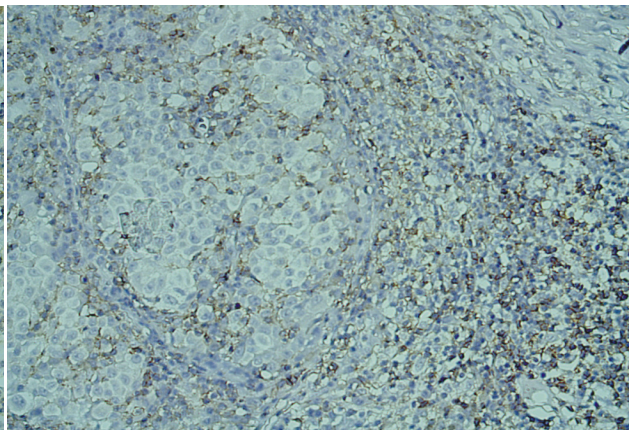

Figure 7 – ***Nodular melanoma (UCHL1-positive, ob. 20×).***

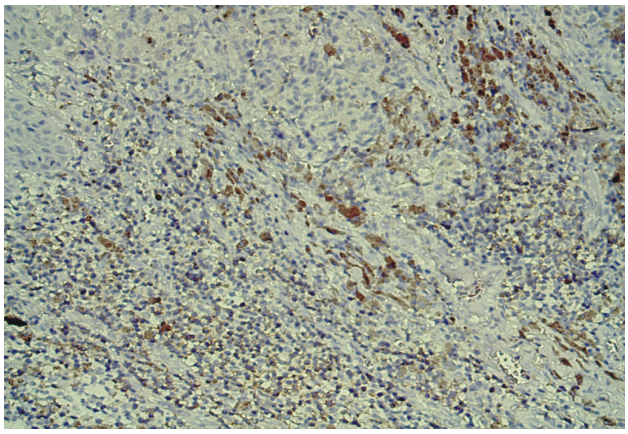

Figure 8 – ***Nodular melanoma (UCHL1-positive, ob. 20×).***

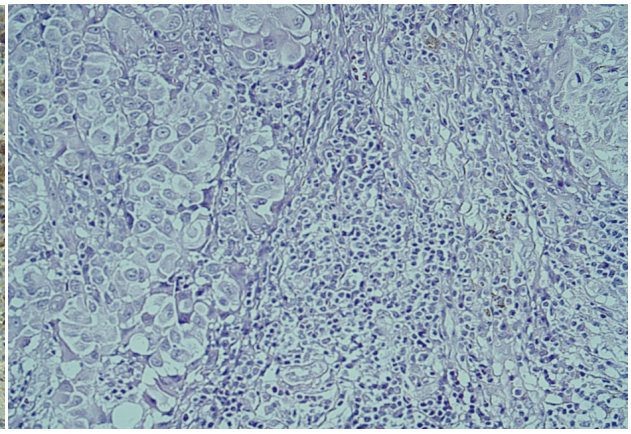

Figure 9 – ***Nodular melanoma (CD4-positive in few lymphocytes, ob. 20×).***

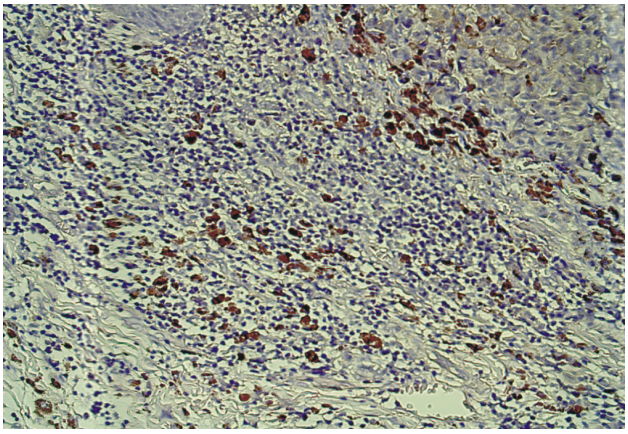

Figure 10 – ***Nodular melanoma (CD4-positive, ob. 20×).***

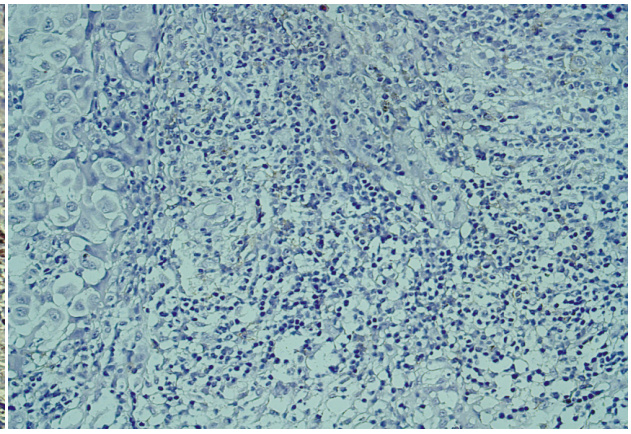

Figure 11 – ***Nodular melanoma (CD8-positive in few lymphocytes, ob. 20×).***

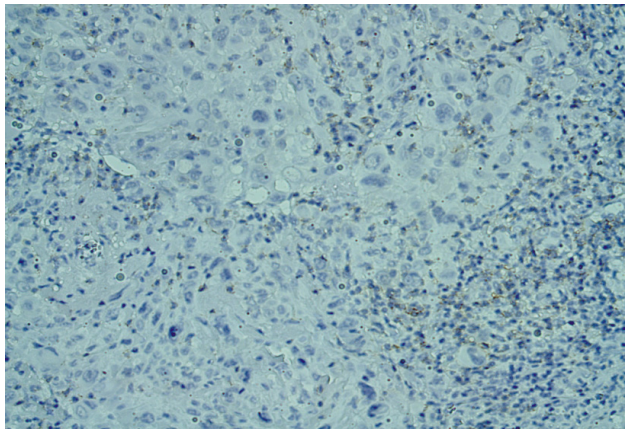

Figure 12 – ***Nodular melanoma (CD8-positive, ob. 40×).***

In addition, variable quantities of T-lymphocytes infiltrate were found in the examined tumors, it is known that this infiltrate is reduced in the invasive and aggressive forms of melanoma, resulting from the proliferative capacity of the tumor. The inflammatory reaction is well represented when the tumor is in the first three stages of Clark invasion.

When the tumor is ulcerated, in the inflammatory infiltrate can be identified plasma cells.

We observed that the presence of a dense lymphocytary inflammatory infiltrate peri- and intra-tumoral is associated with a better prognostic [19]. The inflammatory infiltrate diminishes but it does not disappearing completely with the tumor growing in size.

Discussion

The stages made by Søndergaard K and Schou G [20] based on the quantity of the inflammatory infiltrate are the following:

- 0: inflammatory reaction is absent;
- 1: few small groups of lymphocytes;
- 2: bigger groups of lymphocytes;
- 3: discontinuous band encircling the tumor, which is smaller than 0.5 mm in size;
- 4: continuous "band-like" of infiltrate encircling the tumor.

The existence of an inflammatory infiltrate was associated with the presence of lymph node metastasis. Weissmann A *et al.* [21] and Mascaro JM *et al.* [22] found a high concentration of plasma cells infiltrate in 29 cases from 132 studied malignant melanoma. Its presence was correlated with the increase in size of the tumor, the location and the presence of the ulceration predicts an unfavorable prognosis.

IFN treatment was demonstrated to significantly prolong relapse-free survival of patients with stage IIB–III melanoma. A multi-parameter quantification of patients showed that serum concentrations of IL-1alpha, IL-1beta, IL-6, IL-8, IL-12p40, IL-13, granulocyte colony-stimulating factor, monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein (MIP)-1alpha, MIP-1beta, IFN-alpha, TNF-alpha, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and TNF receptor II were found to be significantly higher in patients with resected high-risk melanoma compared with healthy controls. These patients subjected to IFN-alpha2b therapy had a significant decrease of serum levels of immunosuppressive and tumor angiogenic/growth stimulatory factors (VEGF, EGF and hepatocyte growth factor) and increased levels of antiangiogenic IFN-gamma inducible protein 10 (IP-10) and IFN-alpha [23].

Some 10 years ago, patients treated with IFN-alpha2b were monitored by evaluating peripheral lymphocytes. Thus, the number of peripheral lymphocytes decreased during the treatment for all patient groups. In addition, the percentages of CD8+ and CD20+ lymphocytes were higher in stage IV patients than in stage III. A significant increase in the percentage of CD20+ cells, mostly B-lymphocytes, was observed in the stage III over time but not in stage IV patients [24].

An immune-related antigen that seems to be blamed for an inefficient anti-tumor immune response in melanoma is the cytotoxic T-lymphocyte antigen-4 (CTLA-4). Given the described role of CTLA-4 in inhibiting the immune response, a specific therapy was established. This therapy uses anti-CTLA-4 antibody – currently in phase III clinical trials [25] – and was demonstrated to rapidly restore immune dysfunctions. Therefore, the effectors and memory CD4+ and CD8+ T-cell pool is restored along with TCR-dependent T-cell proliferation, T-cells becoming entirely resistant to Treg-mediated suppression. Progression-free and as well overall survival was directly correlated with reviving reactive memory T-cells found to be anergized in stage IV melanoma [6].

The vaccination-type treatment in melanoma patients was the best beneficiary of immune-related markers. Recently in a phase I/II trial for melanoma vaccine comprising six melanoma-associated peptides (MAGE proteins, MART-1/MelanA, gp100, and tyrosinase) patients follow-up was performed using the *in vitro* proliferation of CD4+-lymphocytes. After vaccination, a good response was marked by an increased proliferation of T-cells to relevant peptides in 81% of patients [26]. The field is intriguing due to its dual relation regarding melanoma development. The immune-related markers can be utilized to scan the immune reactivity of patients to their own tumor, but due to the known induced immune suppression, the immune system can also be a target for therapy.

There is an extensive literature that focuses on skin melanoma biomarkers; only in 2008, the scientific attention was drawn upon several new biomarkers. In spite of the constant consideration of the matter, there is still an imperative need for precise staging and disease characterization that could lead to new, rational and personalized approaches to treatment [11]. Probably, research has reached a moment, when skin melanoma should be considered as a complex disease in "both time and space" terms [27]. Despite rapid advances in the fields of molecular and cell biology, it is still widely debated how neoplastic cells progress through carcinogenesis and acquire their metastatic ability. If "cancer is a dynamic and a hierarchical system" [27], we should look for markers that change when normal tissue becomes abnormal and/or markers that reflect alteration at different levels, starting from intracellular level, cell, tissue and organ.

Conclusions

This article has highlighted the possible links between the values of immune cell population in stage I of skin melanoma. Our approach can be used in a future panel of markers with improved prognostic value and eventually treatment guidelines.

We found statistically significant correlations between the circulatory immune cells in stage I of skin melanoma. Thus, CD4+-based on-going immune response was shown.

We found that there is a possible immune compensatory mechanism between the T-mediated immune

response and the NK-cytotoxicity and also between the antibody generating cells (B-cells) and the natural cytotoxicity cells (NK-cells).

Peri- and intra-tumoral inflammatory infiltrate was predominantly represented by T-lymphocytes (UCHL1-positive).

In the invasive and aggressive forms of melanoma, the infiltrate is reduced, this results from the proliferative capacity of the tumor.

The inflammatory reaction is well represented when the tumor is in the first three stages of Clark invasion and is associated with a better prognostic.

When the tumor is ulcerated, plasma cells can be identified in the inflammatory infiltrate.

CD4 and CD8 were positive in few T-lymphocytes.

We are confident that these statistical correlations between clinical, immunological and immunohistochemical data can be useful in the disease management and personalized immune-therapy.

Acknowledgement

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Corresponding author

Mariana Costache, MD, PhD, Department of Pathology, "Victor Babeş" National Institute of Pathology, "Carol Davila" University of Medicine and Pharmacy, 8 Eroilor Sanitari Avenue, 050474 Bucharest, Romania; Phone/Fax +4021–318 07 62, e-mail: m_costache_dermatopat@yahoo.com, angelap@vbabes.ro

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