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ORIGINAL ARTICLE

The value of tumor infiltrating lymphocytes as prognostic factor for lymph node status and survival amongst patients with cutaneous malignant melanoma

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Summary

Purpose: *Tumor infiltrating lymphocytes (TILs) in cutaneous malignant melanoma are classified as brisk, non-brisk or absent. Numerous studies suggest the presence of TILs, especially brisk, are associated with a lower rate of lymph node metastasis and with an improved overall survival (OS). Our purpose was to assess the value of TILs as a prognostic factor for the lymph node metastasis and survival in completely resected pT3 stage malignant melanoma patients.*

Methods: *We included a number of 114 patients with pathological pT3 cutaneous malignant melanoma, treated exclusively in our institution, between 2000-2015. Correlations of clinical and pathological factors with lymph node status and OS were analyzed.*

Results: *A brisk infiltrate was present in 60% of the patients, whereas 40% presented a non-brisk infiltrate or absent TILs. In univariate analysis, the presence of ulceration was correlated with a non-brisk infiltrate, whereas in multivariate analysis, lymph node invasion and a non-brisk infiltrate were associated with a higher risk of death.*

Conclusions: *TILs density grade represents an independent prognostic factor for the OS. Therefore, we conclude that an accurate prognosis may be provided by TILs status in patients with pT3 malignant melanoma.*

Key words: *brisk, lymph nodes, melanoma, survival, tumor infiltrating lymphocytes*

Introduction

Malignant melanoma is the major cause of mortality among skin cancers, being the most lethal skin malignancy. It is considered to be the second most frequent neoplasm in youth in United States and first most frequent worldwide, with increasing incidence and mortality rates estimated that more than 55,000 cancer deaths were related to melanoma, aproximatively 0.7% of the total cancer-related deaths [1-4]. Numerous public health campaigns and efforts were undertaken in the last decade regarding the establishment of a rapid diagnostic and an efficient treatment for these patients, the long-term prognosis being significantly improved by introducing modern therapies, using

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mitogen-activated protein kinase kinase (MEK) and BRAF inhibitors, as well as anti-programmed cell death protein 1 (PD-1) and anti-programmed cell death-1 ligand 1 (PD-L1) agents.

Mortality in melanoma is, as for many other cancers, related to visceral metastatic spread, which involves a series of complex interactions between the tumor and the host's immune response [5]. Melanomas have been shown to be genetically and phenotypically heterogeneous tumors, harboring different genetic alterations in three main oncogenes: BRAF, NRAS and c-KIT [6,7]. In addition, melanoma is considered a highly immunogenic tumor, very responsive to immunologic manipulation, easily stimulating immune reactions in the host organism [8-10]; therefore, the role of immune response has been intensively studied in recent years in the search of developing potential biomarkers with prognostic significance in cancer immunotherapy [11-15].

Tumor infiltrating lymphocytes (TILs) are considered as local histopathological reflection of the host's immune response against cancer cells [16-19], that infiltrate tumors and disrupt tumor cells [20,21]. Many studies suggest the presence of TILs predicts a lower rate of lymph node metastasis and it is correlated with improved OS [22-27]. Moreover, TILs are a heterogeneous group comprising not only effector T cells, but also of tolerogenic or T regulatory (Treg) cells, functionally exhausted T cells, natural killer (NK) cells, macrophages, dendritic cells, myeloid-derived suppressor cells and other immune cell types, being linked with improved prognosis in several types of cancers, not only in malignant melanoma [18,28-32]. Currently, prevention and early diagnosis are the most effective strategies for decreasing the occurrence of this tumor and improving its prognosis [33]. However, these immune cells are producing a wide spectrum of cytokines, chemokines, growth factors and interleukins, and experimental evidence has shown the capacity of the immune system to be exploited in the fight against melanoma [34-38]. Therefore, enhancing the immune response was intensively investigated, ellaborating several targeted agents and immune checkpoint inhibitors (monoclonal antibodies targeting programmed death 1 receptor and its ligand - PD1 and PD-L1 and cytotoxic T-lymphocyte associated protein 4 - CTLA 4), which determined an important progress regarding the treatment of metastatic melanoma, with improved prognosis [39-41].

In this study, we analyzed patients with a pathological pT3 stage cutaneous malignant melanoma that underwent surgical and oncological treatment (chemotherapy or immunotherapy) at the Institute of Oncology "Prof. Dr. I. Chiricuta", in order to determine whether TILs status (brisk, non-brisk) can be considered an independent prognostic factor for lymph node status and OS.

Methods

Study population

We analyzed 1842 patients with histologically confirmed malignant melanoma, that were admitted at the Institute of Oncology "Prof. Dr. I. Chiricuta" from Cluj-Napoca, a comprehensive cancer center. We retrospectively consulted the Institutional databases over a period of 15 years, from January 2000-December 2015 and 114 patients were finally included in the analysis. The disease was staged according to the latest American Joint Committee on Cancer (AJCC-8th edition-2018) staging system [42]. The patients were considered eligible for the study based on the following inclusion criteria: (i) histologically confirmed malignant melanoma; (ii) patients that have undergone surgery exclusively at our Cancer Center; (iii) patients with stage II and III malignant melanoma (pathological T3 staging, with or without lymph node involvement - Breslow thickness 2.01 - 4.00 mm); (iv) patients that were not previously diagnosed with another malignancy; (v) patients with adequate follow-up. The patient treatment and follow-up were done according to the institutional melanoma guidelines, in line with EU recommendations (ESMO). Patients that were treated in other cancer centers or those that did not follow the standard protocols, were excluded from the study. Lymph node status was evaluated by surgery, either by direct lymph node dissection (LND) or by sentinel lymph node biopsy (SLNB), and completed with lymph node dissection if positive within 2 months maximum from diagnosis. All study participants provided informed consent on admission and all clinical information for each patient was coded; finally, the study was approved by the institutional ethical committee.

Pathology

Patient age and sex were recorded from the clinical observation papers and the following features of the primary tumor were extracted from the histopathology reports: histologic subtype, tumor thickness (Breslow index), Clark level of invasion, mitotic rate (per mm^2), presence or absence of ulceration, TIL density grade, microsatellitosis, presence or absence of regression, lymph node status. All pathology reports for the patients included in the study were reviewed by another pathologist, and there was 100% concordance with the databases.

A three-tier TILs density grading schema was used to assess TILs density: absent/non-brisk, brisk; absent was considered as an absence of TILs or, if TILs were present, they were not opposed to tumor cells. The other two groups provided lymphocytes at the site of the tumors, non-brisk being defined as a focal TILs infiltrate and brisk as TILs that involved the entire base of the vertical growth phase of the tumor or showing diffuse

permeation of the vertical growth phase [23]. Information about survival and deaths from melanoma or from other causes were obtained from the National Registries of Population Records and National Cancer Registries.

Statistics

Clinicopathological parameters and TILs grade were used for descriptive and inferential statistics. OS was calculated from the date of histological confirmation of melanoma to death or for censored data the date of the last follow-up.

Statistical analyses were done using IBM SPSS v20.0 software and included chi square test, Fisher's test, Mann-Whitney U test, as well as univariate and multivariate analysis, using Cox proportional hazard models. Survival estimations were determined using Kaplan Meier method and differences were evaluated by log rank test. A statistically significant p value was set at ≤0.05.

Results

A total number of 114 patients were enrolled in this study. The median patient age was 65 years, with a higher tendency to affect women (57% vs 43%). All clinicopathological characteristics are presented in Table 1.

Sixty-eight patients presented a brisk infiltrate whereas 46 had a non-brisk or absent infiltrate. On univariate analysis, melanoma patients with non-brisk infiltrate were more likely to present with ulceration, which was statistically significant (p=0.021), whereas age, sex, mitotic rate, perineural invasion, angiolymphatic invasion, regression, microsatellitosis or lymph node invasion were not correlated with the presence or absence of TILs.

On multivariate analysis, the presence of microsatellitosis, with a HR of 3.6 (95% CI, 1.345-9.740) was statistically significant correlated with a higher risk of death (p=0.01). Moreover, lymph node invasion, with a HR of 2.2 (95% CI, 1.074-4.538) and a non-brisk or absent infiltrate, with a HR of 2.6 (95% CI, 0.928-7.656), were also significantly associated with a lower rate of OS (p=0.031 respectively, p =0.043). In addition, other parameters such as age over 65 years with a HR of 1.245 (95% CI, 0.603-2.569), the presence of angiolymphatic invasion with a HR of 1.738 (95% CI, 0.577-5.237), the presence of perineural invasion with a HR of 2.143 (95% CI, 0.256-17.931), a Breslow score over 3 mm and a Clark scale over 4 were also correlated with a higher risk of death,

Table 1. Patients characteristics stratified according to brisk and non-brisk infiltrate

Characteristics	*Total (100%)*	*Brisk (26.3%)*	*Non-brisk (73.7%)*	*p value*
Median age, years (18-92)	66 (57.75-76)	66.5 (59.5-76.25)	65.5 (56.25-76)	0.65
Sex				
male	48	15	33	0.3
female	66	15	51	
Median Breslow score (2-4)	3(2.5-3.76)	3 (2.5-3.85)	3 (2.5-3.71)	0.82
Median Clark score (2-5)	4 (3-4)	4 (2.5-4)	4(3-4)	0.41
Median mitotic rate (1-32)	5 (3-9)	5 (3-7.25)	5 (3-10)	0.52
Ulceration				
absent	33	4	29	0.021
present	81	26	55	
Perineural invasion				
absent	109	29	80	1
present	5	1	4	
Angiolymphatic invasion				
absent	95	24	71	0.56
present	19	6	13	
Regression				
absent	87	23	64	0.95
present	28	7	20	
Microsatellitosis				
absent	101	28	73	0.34
present	13	2	11	
Lymph node invasion				
N0	68	17	51	0.69
N+	46	13	33	

Table 2. Multivariate Cox regression analysis

Parameters	*Hazard ratio*	*95% CI*		*p value*
		Lower	*Upper*	
Microsatellitosis (1=present, 0=absent)	3.619	1.345	9.740	0.011
TIL (1=non-brisk, 0=brisk)	2.665	0.928	7.656	0.043
Age (1=over 65 years, 0=under 65 years)	1.245	0.603	2.569	0.550
Lymph nodes (1=N+, 0=N0)	2.208	1.074	4.538	0.031
Angiolymphatic invasion (1=present, 0=absent)	1.738	0.577	5.237	0.326
Perineural invasion (1=present, 0=absent)	2.143	0.256	17.931	0.482
Breslow score (1=more than 3, 0=3 or less)	1.070	0.524	2.187	0.850
Clark score (1=4 or more, 0=less than 4)	1.043	0.642	1.697	0.864
Sex (1=female, 0=male)	0.635	0.294	1.372	0.248
Ulceration (1=present, 0=absent)	0.578	0.238	1.403	0.226

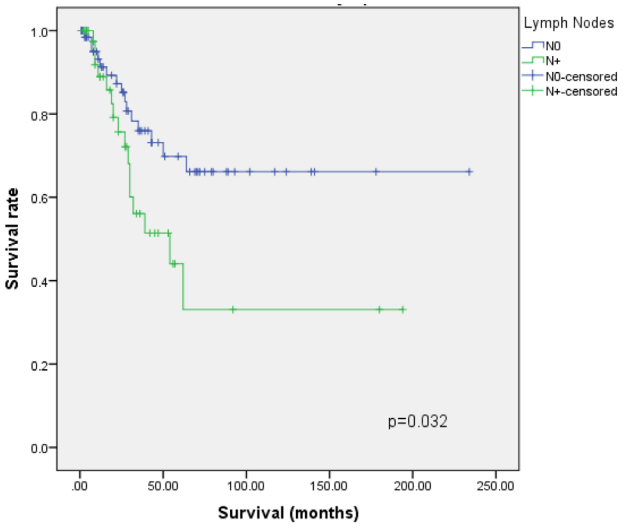

Figure 1. Overall survival in melanoma patients according to lymph node status (N0 vs N+).

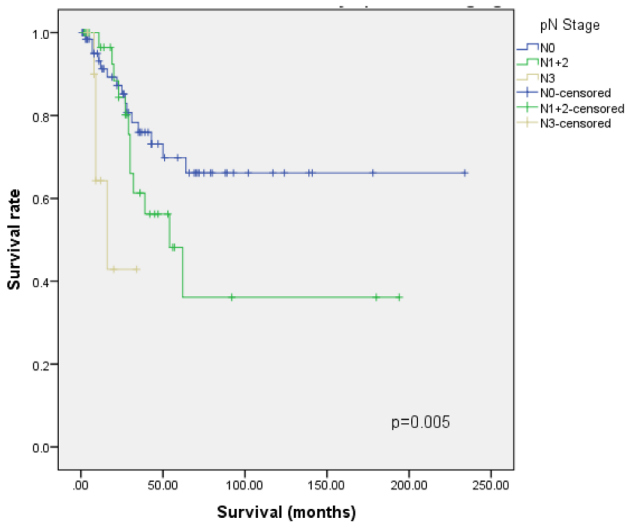

Figure 2. Overall survival in melanoma patients according to lymph node status (N0 vs N1+2 vs N3).

but without being statistically significant. Multivariate analysis parameters are presented in Table 2.

The median survival of the patients was 142.87 months (95% CI:117.74 - 168.33). Therefore, we compared the survival rates of the patients according to the lymph node status and according to the brisk or non-brisk infiltrate. The median OS of the patients with no lymph nodes metastasis (N0) was 164.78 months (95% CI:135.139-194.42), in comparison to patients with positive lymph node metastasis (N+) (86.177 months, 95% CI:49.482-122.871), with a p value of 0.032. Furthermore, we also determined the OS according to N stage (N0, N1+2, N3), which was statistically significant (p=0.005). Therefore, the median survival of N1+2 patients was 93.594 months (95%

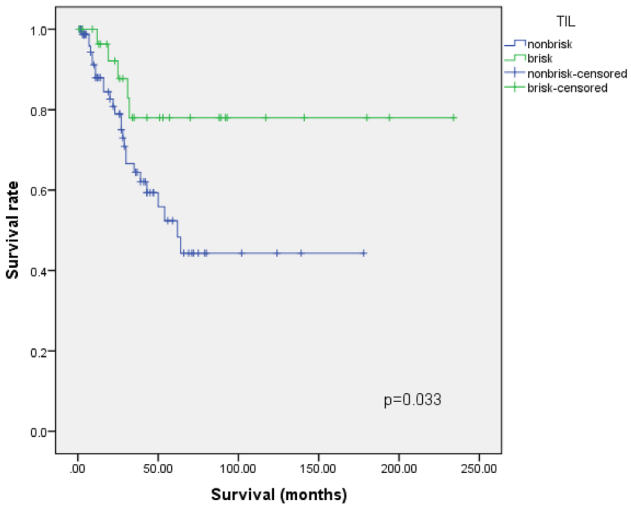

Figure 3. Overall survival in melanoma patients according to TILs status.

CI:53.893-133.295), as opposed to 21.114 months (95% CI:12.272-29.957) for N3 patients, results that are shown in Figures 1 and 2.

Subsequently, a brisk infiltrate was correlated with improved OS (187.87 months, 95% CI:151.86-223.87), in comparison to 96.69 months (95% CI:73.88-119.5) for patients with non-brisk or absent TILs (Figure 3) (p=0.03).

Discussion

The results of the current study show that TILs density grade is an independent prognostic factor for patients with pT3 melanoma, a brisk infiltrate being correlated with improved OS. These findings validate the existing literature data regarding malignant melanoma and highlight the important role of immune system in the pathophysiology of this disease.

More than a century ago, in 1863, Robert Virchow described the involvement of leukocytes in malignant tumors for the first time. Initially, it was thought to be associated with human cancer, along with mononuclear cells, with these cells occurring at sites of chronic inflammation. Moreover, a study on animal models suggested that lymphocytes might have a protumorigenic role in inflammatory processes [5,43]. However, later studies demonstrated that the presence of TILs correlates with improved survival in patients with different cancers, such as malignant melanoma, colorectal, non small lung and breast cancer [30-32,44,45].

Over time, numerous studies were conducted regarding the most important prognostic factors, in order to formulate the most accurate prognosis; the attention was concentrated especially on the antitumor immunology and lymph node status, along with tumor thickness and ulceration status. Controversy existed since then, as the clinical behaviour of melanoma is unpredictable. Three decades ago, Clark et al formulated a classification of TILs of 3 groups for quantifying the presence of lymphocytes at the site of a tumor, which is widely used nowadays: absent, brisk and non-brisk [46-48]. In the same study, the authors concluded that mitotic rate, TILs, anatomic site of primary melanoma, sex and histologic regression represent independent predictors of 8-year survival, respectively; a brisk infiltrate was associated with a significantly better prognosis compared to non-brisk or absent [20,49]. Clemente et al reported similar findings in a study on 285 melanoma patients, concluding that only tumor thickness and presence of TILs were statistically signficant regarding the prognosis [50]. Other studies, however, have reported no correlation between TIls and prognosis on OS [51-53].

The importance of TILs was therefore intensively studied and subsequently confirmed by results from other research papers. More recent studies [26-30], that included larger cohorts of patients, started from the premise that the presence of TILs is correlated with a negative lymph node metastasis. Therefore, Taylor et al highlighted that the absence of TILs along with an increased tumor thickness and the presence of ulceration are independent predictors of SLN metastasis [27]. However, in their studys TILs grade was not associated with OS, in contrast to our study. Burton et al reported in a study on 515 patients that on multivariate analysis TIL response represented a significant predictor of SLN metastasis but was not a significant independent factor predicting disease-free survival (DFS) or OS [29].

In 2012, in a study conducted at the Melanoma Institute Australia (MIA) by Azimi et al, the authors redefined TIL grade, based upon TILs density (mild, moderate, marked) and distribution (focal, multifocal and diffuse), in a 4-tier system for grading [0 to 3]. They demonstrated that in tumors with absent TILs, SNL positivity was 27.8%, in comparison with 5.6% in tumors with dense TILs. Moreover, OS was 100% in patients with dense infiltration. Therefore, they concluded that TIL grade may be considered as an independent predictor of lymph node status, as well as of recurrence-free survival and melanoma-specific survival, being the first study to highlight this association between TILs and all three outcomes in malignant melanoma patients [26].

However, the presence of a dense lymphocytic tumor infiltrate showed no survival improvement for melanomas in radial growth phase for early-stage melanomas [54]. Moreover, in a study on 750 patients, Tas et al concluded that TIL grade may be used as a significant predictor for negativity of nodal involvement, but was not correlated with an improved OS [55].

In the present study, the presence of TILs correlated with a lower rate of lymph nodes involvement and an improved OS, being able to consider TIL grade as an independent predictor of prognosis. These findings concur with the existing data in the literature, and highlight the behaviour of pT3 stage malignant melanoma, which is usually unpredictable and difficult to manage, and offers the posibility to stratify patients in order to best benefit from the treatment. However, contrary to other studies and to what we expected, we could not establish a correlation between TIL density grade and other predictors of lymph nodes metastasis, such as Breslow index, Clark scale, mitotic rate or ulceration. Microsatelitosis on the other hand, in a multivariate analysis, showed statistically significance re-

garding OS with a HR of 3.6 (95% CI, 1.3 to 9.7). To sum up, TIL density grade represents an important parameter that should be taken into consideration in all patients with malignant melanoma, regardless of stage. Prospective studies are needed in the future to validate these findings.

In the last years, the role of lymphocytes in tumors has been documented and it is shown to be a critical component in disease progression and prognosis. The heterogeneity of lymphocytes has been described in several studies, as these cells realize an effective immune surveillance against malignant melanoma. The existence of regulatory T cells (Tregs), that suppress the antitumor immunity, has led to more research on the functions of lymphocytes and therefore specific targets have been discovered, resulting to the immune checkpoint inhibitors, with excellent results in patients with metastatic disease, both in melanoma and in other types of cancer. This recent progress emphasizes the close relationship between the evolution of malignant melanoma, host immune response and tumor microenvironment, and highlights that TILs might be used both as therapeutic targets and for predicting and optimizing the response to melanoma immunotherapy [18,27,32,39].

The limitations of this study concur with the existing retrospective studies in the literature with possible errors when including patients in the database. However, in order to prevent this from happening, all the histopathology reports were revised by a different pathologist.

Tumor thickness remains the strongest predictor of lymph node metastasis and OS and by this histological characteristic, the entire therapeutic management is guided. Therefore, we consider that, due to the heterogenity of melanoma, it is imperative to define and introduce into the guidelines other prognostic factors, such as TILs and microsatellitosis, in order to stratify the patients and manage to guide a better therapeutic approach, with the purpose of improving the quality of life and the OS of these patients.

In conclusion, we analyzed patients with pT3 cutaneous malignant melanoma and highlighted the importance of TILs regarding the pathophysiology of this disease. In order to formulate an accurate prognosis, we consider appropriate to utilize TILs as predictors for lymph nodes status and OS, which should be revised in order to be included in the AJCC staging system. Therefore, in the future, by managing to better understand the mechanisms controlling TILs in relation to tumor progression, we might be able to predict a more precise prognosis, as well as using TILs as potential biomarkers in order to predict the responses to immunotherapy, and, more likely to develop important new immunotherapeutic strategies for treating patients with malignant melanoma.

Conflict of interests

The authors declare no conflict of interests.

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