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REVIEW ARTICLE

Tumor infiltrating lymphocytes as a prognostic factor in malignant melanoma. Review of the literature

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Summary

Malignant melanoma represents the major cause of mortality among skin cancers, with increasing incidence and mortality rates worldwide. Despite the numerous public health campaigns and the efforts undertaken in the last decade regarding the establishment of a rapid diagnostic and an efficient treatment for these patients, the long-term prognosis has not been significantly improved. Thus, numerous studies were conducted in order to establish a more accurate prognosis, tumor infiltrating lymphocytes (TILs) being considered in many studies as independent prognostic factors of lymph node metastasis and overall survival in patients with melanoma. Moreover, immunotherapy has been intensively studied and evolved in recent times, and represents a promising treatment option for patients with advanced stage (metastatic) malignant melanoma.

In this review article, we provided a literature overview on the histological classification, the history and the essential role of TILs, as well as the implications of regulatory T (Treg) cells and FOX P3 transcription factor in malignant melanoma.

Key words: *FOXP3, immune response, lymphatic metastasis, malignant melanoma, prognostic factors, tumor infiltrating lymphocytes, Treg*

Introduction

Malignant melanoma represents the major cause of mortality among skin cancers, with increasing incidence and mortality rates worldwide. Despite the numerous public health campaigns and the efforts undertaken in the last decade regarding the establishment of a rapid diagnostic and an efficient treatment for these patients, the long-term prognosis has not been significantly improved.

The incidence of melanoma is increasing, with 232,000 new cases (1.6% of new cancers) in 2012, affecting far more frequently the white population, both males and females, with the highest incidence in Australia and New Zealand (40.3 and 30.5 per 100,000, respectively). Moreover, it was estimated that more than 55,000 cancer deaths were related to melanoma, aproximatively 0.7% of the total cancer-related deaths [1]. In 2010, in the North-Western part of Romania, aproximatively 80 new cases of malignant melanoma were reported, both in males and females, with a sex ratio of 1.01, while in 2011, the incidence was 78 for males and 92 for females, with a sex ratio of 0.85.

As for many other cancers, mortality is mainly related to metastatic spread to sites distant from the primary tumor, involving complex interactions between the tumor and the patient immune system [2]. Therefore, despite the fact that

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the pathological prognostic factors remain the most important, recent studies [3-9] focused on the prognostic value of the immune response of the host to the tumor and specifically on the prognostic value of TILs.

Virchow was the first to describe, in 1863, the involvement of leukocytes in malignant tumors, which are now referred to as TILs, and it was thought that these cells occur at the sites of chronic inflammation. Moreover, a study on animal models suggested that lymphocytes might have a protumorigenic role in inflammatory processes [10].

However, further studies demonstrated a correlation between the presence of TILs and a favorable prognostic outcome and improved survival in patients with malignant melanoma, ovarian and colorectal cancers [11,12], being considered as the result of immune response of the host against cancer cells.

Tumor infiltrating lymphocytes in melanoma and their prognostic value

Clark et al. described in 1989 a classification of TILs which is used even now, for quantifying the presence of lymphocytes at the site of a tumor [13,14]. They divided the infiltrate into three groups: absent, nonbrisk and brisk. Absent was considered as an absence of TILs, or if TILs were present, they were not apposed to tumor cells. The other two groups provided lymphocytes at the site of the tumors, nonbrisk being defined as a focal TILs infiltrate and brisk as TILs that involve the entire base of the vertical growth phase of the tumor or showing diffuse permeation of the vertical growth phase. Subsequently, Clemente et al. [3] formulated a study in which they described in detail the two main patterns of brisk infiltrate : diffuse and peripheral patterns that have the same prognostic significance.

Moreover, Azimi et al. from the Melanoma Institute Australia, conducted a study on 1865 patients and formulated a new TILs grade, based on TILs density (mild, moderate, marked) and TILs distribution (focal, multifocal and diffuse), defined as : grade 0 – absent TILs, grade 1 – mild or moderate focal, or mild multifocal lymphocyte infiltrate, grade 2 – marked focal or moderate or marked multifocal, grade 3 – moderate or marked diffuse infiltrate of the lymphocytes [15].

Among patients with malignant melanoma, the formulation of an accurate prognostic system is essential, regarding the need for further investigations, the postoperative management, the overall survival and the assignment of risk in patients entering clinical trials. According to the latest studies, the most important prognostic factor is considered to be the thickness of the tumor, but it was well demonstrated that mitotic rate, ulcerative state, TILs infiltrate, anatomic site, and also patient characteristics, such as age, gender, should be taken into consideration [16-21].

Moreover, in the 7th edition of the American Joint Committee on Cancer (AJCC) , the presence or absence of mitoses have been introduced as staging criteria for tumors [22], as studies demonstrated that the mitotic rate can provide precise stratification of the recurrence risk and is a strong independent predictor of metastatic potential , being more powerful than ulceration [23,24].

Numerous studies were directed on the effects of TILs as prognostic factors. Day et al. [25] in 1981 and Clark et al. in 1989 [4], in a study of 264 patients reported that patients with a moderate to marked infiltrate had a significantly better prognosis in comparison with those with absent TILs, as a brisk infiltrate was associated with 88% 8-year survival compared with 75% and 59% 8-year survival for patients with nonbrisk or absent infiltrate. Subsequently, other studies conducted by various researchers validated this theory [26-28].

Moreover, Clemente et al. in a study of 285 patients obtained a 5-year survival of 77% in patients with infiltration present, compared to 37% in those with absent infiltrate, in this study both tumor thickness and TILs being considered independent prognostic indicators [3]. Thomas and colleagues found, in a study on 3330 primary invasive melanomas, that absent TILs were associated with a higher AJCC tumor stage, in comparison with nonbrisk and brisk infiltrate, which correlated with a lower tumor stage. Moreover, they observed that melanoma-related death rate was 30% in patients with absent TILs and 80% when nonbrisk and brisk infiltrate was present [29].

However, other authors demonstrated no correlation between the value of TILs and the patient survival [30]. These results may be related to the fact that thin and with radial growth phase melanomas (little is known about the role of TILs as a prognostic indicator for these melanomas) were included, and that the TILs and the lymphocytes in the vicinity of the tumor may be misclassified.

Malignant melanoma can metastasize by two principal pathways: hematogeneous and lymphatic than by locoregional direct invasion. The

Table 1. Literature studies analysing the association of TILs in primary melanomas with overall survival and lymph node status.

Study	*Year*	*Number of patients*	*Conclusions*
Day et al. (19)	1981	107	This study emphasizes the importance of anatomic sites of the tumor when formulating prognosis for patients with malignant melanoma.
Clark et al. (20)	1989	264	Presence of TILs was associated with a significantly better prognosis in comparison with the absence of TILs, as a brisk infiltrate was associated with better 8-year survival compared to nonbrisk or absent infiltrate.
Morton et al. (27)	1992	237	The development of intraoperative identification of the sentinel lymph node, the lymph node nearest the site of the primary melanoma, on the direct drainage pathway was introduced in 1992. Thus, this technique identifies, with a high degree of accuracy, patients with early stage melanoma who have nodal metastases and are likely to benefit from radical lymphadenectomy.
Clemente et al. (8)	1996	285	A multivariate analysis comparing thickness, mitotic rate and presence of TILs showed that only thickness and presence of TILs were significant and independent positive histologic prognostic factors.
Kruper et al. (37)	2006	327	Risk for SLN positivity can be identified by incorporating biologically based variables such as VGP, TILs, and mitotic rate along with thickness into a prognostic model, which, if validated, can be used in order to select patients to undergo or be spared SLN biopsy.
Taylor et al. (38)	2007	887	The absence of TILs, increased Breslow thickness, presence of ulceration and male sex are predictors of SLN metastasis in patients with primary cutaneous melanoma.
Mandala et al. (39)	2009	1251	The absence of TILs predicts SLN metastasis, which in a multivariate analysis represented the most significant predictor of overall and disease free survival.
Burton et al. (40)	2011	515	In multivariate analysis TIL response was not a significant independent factor predicting DFS or OS, but however represented a significant predictor of SLN metastasis.
Azimi et al. (41)	2012	1865	TIL grade represents an independent predictor of SLN status and survival among patients with melanoma, a pronounced TIL infiltrate having an excellent prognosis.
Thomas et al. (24)	2013	3330	A higher TIL grade in patients with primary melanoma is associated with better survival. Thus, TIL grade deserves prospective investigation in order to determine whether it should be included or not in future AJCC staging revisions.
Egger et al. (28)	2016	1998	Histopathological characteristics (Breslow thickness, ulceration) and patient characteristics (age, anatomic localisation) are important independent factors predicting survival and reccurence. These factors can be used to stratify prognosis among patients with tumour-negative SLN and to formulate long-term follow-up strategies.

most common route is the lymphatic spread to a regional lymph node basin that drains the area of the primary tumor [31]. Therefore, using sentinel lymph node biopsy (SLNB), which is a minimally invasive procedure that can determine the status of the locoregional lymph nodes, numerous studies were formulated in order to correlate the status of the SLNB with TILs. The technique was first introduced and described by Morton et al. in 1992, and it was extensively debated and improved since then [32,33].

Nowadays, it is recommended to be done for all patients with a Breslow thickness of ≥ 1 mm, and for selected cases < 1 mm, in order to reduce the patient morbidity by avoiding a complete lymphadenectomy for staging and prognostic purposes [33-35]. Moreover, it was suggested that the SLNB in patients with clinically negative lymph nodes will detect metastases in aproximatively 15-22% of the cases [36-38]. The status of SLN is essential for staging and for the formulation of a prognostic model, having an important impact

in guiding the management and the subsequent postoperative steps in patient care [39,40]. Also, it is assumed that the inflammation and the immunologic activity influence the size of lymph nodes, as it may reflect the immunogenicity of malignant melanoma or even indicate an ineffective response by the immune system of the host [41,42].

Kruper et al. [43] in a study of 682 patients and Taylor et al. [5] in a study of 887 patients, demonstrated that large tumor thickness, high mitotic rate and absence of TILs were independent predictors of SLN metastasis, but were not associated with overall survival. Similar studies have been reported subsequently [6,7], and a large study was conducted by Azimi et al. investigators from Melanoma Institute Australia (MIA), in 2012 on 1865 patients, the largest study involving TILs [8]. They succeeded to show a significantly inverse association between TIL grades and the SLN status. Therefore, they concluded that TIL grade may be considered an independent predictor of SLN status, as well as of melanoma-specific survival and recurrence free survival, in patients with primary cutaneous melanoma ≥ 0.75 mm in thickness, being the first study to demonstrate an association of the lymphocyte infiltrate and all three outcomes.

Regulatory T cells (Tregs) and FOXP3 transcription factor involvement in malignant melanoma

Development and progression of malignant melanoma implies numerous mechanisms in order to escape the antitumor immune responses. One of these mechanisms involves the activation of Treg cells, which are defined as immunosupressors of the activity of other T cells through undefined pathways, being identified by the transcription factor forkhead box P3 (FOXP3) [3]. TILs are expressed by the majority of CD3+ T cells, which include both CD4+ and CD8+ subsets. The CD8+ subset are cytotoxic lymphocytes that comprise CD3+ and CD3- cells, which can induce apoptosis and therefore result in killing the tumor cells and induce regression of melanoma, being correlated with a better prognosis [44-47]. On the other hand, CD4+ cells are grouped in two subsets, the CD4 + CD25- T cells (T helper cells), which enhance the activity of CD8+ cells against the tumor cells, and correlate with improved survival, and CD4+CD25+ T cells (Tregs) that downregulate the activity of immune response, inducing immunosuppression [48,49].

The transcription factor FOX P3 regulates the differentiation and the immunosuppressive function of Tregs, and recently it has been reported to be also expressed in normal tissues, like normal breast, prostate and ovarian epithelium [50-52]. On the other hand, FOX P3 increased expression has been demonstrated in colorectal cancer, pancreatic adenocarcinoma [53], hepatocellular carcinoma [54], bladder cancer [55], thyroid carcinoma [56], leukemia [57], cervical cancer [58] and malignant melanoma [59-61], with a different prognostic significance, as in colorectal adenocarcinoma it is correlated with a good prognosis [60], compared to hepatocellular carcinoma, where it is associated with a poor prognosis [62], a discrepancy that is explained by the fact that FOXP3+T cells include both Tregs and non-Tregs [61]. Thus, numerous studies [59-63] were elaborated in order to demonstrate a possible association between the expression the FOXP3 and different prognostic factors.

Several studies were conducted, especially on patients with malignant melanoma, Treg cells being considered a major impediment for immunotherapy.

Starting from this, Ebert et al. conducted a study and reported that FOXP3 expression can be found in both metastatisc melanoma tissue samples and cell lines derived from the tumor [59].

Moreover, Niu et al. demonstrated that the expression of FOXP3 in melanoma cells managed to inhibit the proliferation of anti-CD3/anti-CD28 activated T cells and to secrete immunosuppressive factors such as TGF-β [61]. Also, Quaglino et al. managed to establish a correlation between FOXP3 expression in primary malignant melanoma and the development of visceral metastases [60]. Ebert and colleagues, in a study that included 146 stage III and IV melanomas from Australia, obtained a FOXP3 expression only in 12% (18 cases) of the cases by immunohistochemistry, and in less than 1% of the cells in these tumors [59].

In addition, Miracco and colleagues conducted a retrospective study on 66 selected patients who underwent surgery for vertical growth phase primary melanoma, between 1990-2000, with a 5-year follow up, and analysed the correlation between CD4+CD25+ FOXP3 Treg cells by immunohistochemistry and tumor relapse. They concluded that the percentage of Tregs inside the tumor mass and at its periphery and among TILs was significantly higher in cases that recurred than in those that did not [63].

In addition to the activation of Treg cells, with their immunosuppressive activity, there are

numerous known mechanisms for melanoma immune evasion like loss of the melanoma differentiation antigen (MDA) [64], a reduced MHC class I expression, through which melanoma target cells are guarded from cytotoxic T lymphocytes mediated lysis [65] or evading natural killer (NK) cells-mediated destruction, by increasing the inhibitory NKG2D ligands by the melanoma cells [66] . Moreover, deletion of immune effector populations, clonal anergy induced tolerance, as well as tumor induced immune tolerance and T cell exhaustion are other immune evasion mechanisms demonstrated in the physiopathology of malignant melanoma [67] .

Conclusions

In this article, we presented a literature overview on the histological classification, and the history and the essential role of TILs in malignant melanoma. Due to the increasing mortality among the patients suffering from this pathology, numerous studies have been conducted in order to establish a more accurate prognosis, TILs being considered in many studies as independent prognostic factors in melanoma. Moreover, immunotherapy has been intensively studied and evolved in recent times, and represents a promising treatment option for patients with advanced stage (metastatic) malignant melanoma ; thus, TILs might be used both as therapeutic targets and for predicting and optimizing the response to melanoma immunotherapy.

However, additional research and studies are needed to optimize the patient response to cancer immunotherapy, as the physiopathology of malignant melanoma comprises numerous immune evasion mechanisms, which are difficult to antagonize as yet.

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Conflict of interests

The authors declare no confict of interests.

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