IMMUNOHISTOCHEMICAL AND MOLECULAR CORRELATIONS OF THE VENOUS MICROVASCULARIZATION IN PRIMARY CUTANEOUS MELANOMA

ABSTRACT

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KEY WORDS: melanoma; high endothelial venules; MECA-79; regression; regression; TIL; chemokines; CCL19; CCL21, CCR7.
I. CURRENT STATUS OF THE PROBLEM

The incidence of cutaneous melanoma has increased worldwide in the last decades. Although in early stages it is usually curable by surgical resection, the rates of mortality in metastatic melanoma are still high because of a virtual absence of a truly effective treatment for this malignancy [1,2]. A series of complex interactions and signaling pathways between tumor cells and the immune system are involved in the process of melanoma dissemination to lymph nodes and distant sites [3, 4].

During the immune response under normal circumstances, the traffic of lymphocytes from the blood to the lymph node parenchyma occurs through distinct small collecting venules, called high endothelial venules (HEVs) [5-7]. HEVs are specialized post-capillary venules that are morphologically and functionally distinct from ordinary venules, and can be found in the paracortical and interfollicular areas of the lymph nodes (LN) [8].

In malignant melanoma, the presence of tumor infiltrating lymphocytes (TIL) is associated with an important role in tumor cell killing and regression of this neoplasm. TILs are directly correlated with an effective immune response against tumor cells, constituting a prognostic factor for survival [9].

Martinet et al. [11] described for the first time that HEVs are frequently found in human solid tumors, including cutaneous melanoma, where they may facilitate lymphocyte infiltration into tumors. Martinet et al. [12] also demonstrated that CCL21 and CCL19, among other chemokines, as well as TH1 and naïve T-cell genes, were overexpressed in melanomas with high HEV density. They also found that intratumoral mature dendritic cell and HEV densities were strongly related, and that a higher HEV density correlated with tumor regression, low Clark level of invasion and thin Breslow thickness. HEVs have also been found in melanoma metastases where they are associated with accumulation of lymphocytes in nodular structures which are called tertiary lymphoid organs (TLO) [13]. Tertiary lymphoid organs have significant morphological, cellular and vascular similarities to secondary lymphoid organs, particularly lymph nodes [14]. Certain chemokines, such as CC-chemokine ligand 19 (CCL19) and CC-chemokine ligand 21 (CCL21), are constitutively expressed by stromal cells, dendritic cells within...
lymphoid T zones, and by melanoma tumor cells [15, 16]. CCL19 and CCL21 are translocated through HEV endothelial cells, and interact with chemokine CC receptor 7 (CCR7), thus directing the migration of lymphocytes through the HEVs into the lymph nodes [17-19]. In addition, the expression of CCR7 in melanoma cells has been correlated with tumor cell migration and the development of lymph node metastasis in response to CCL 21 [20, 21].

CHAPTER 4. OBJECTIVES. MATERIALS AND METHODS

4.1 Objectives of the study

The aim of our study was to analyze HEV density as recognized by a specific antibody, MECA-79, in the spectrum of cutaneous malignant melanoma progression: primary melanoma, in transit metastases and distant metastases, and its correlation with clinicopathological parameters as well as with CCL19, CCL21 and CCR7 mRNA expression in the same tumor samples.

4.2 Tumor samples

In this study, we analyzed tumor samples from 85 patients with cutaneous melanoma collected during a 10-year period (2002-2011) at the Department of Pathology, Hospital Clínico Universitario, Valencia.

The cases were selected from consecutive melanoma specimens received at the Department of Pathology with the only condition that the tumor specimens were received fresh and unfixed. Primary and metastatic tumor specimens were manually macrodissected to obtain samples with greater than 90% tumor tissue content. One tumor slice was taken for RNA extraction, which in primary melanomas was the slice immediately adjacent to the thickest area of the tumor. Fresh tumor samples were immediately frozen in liquid nitrogen and stored at -80°C. The samples comprised primary melanomas (n=67), in-transit metastases (n=10) and distant metastases (n=8). Samples from lymph node metastases were not included because high endothelial vessels are a normal component of the lymph nodes. The clinicopathological characteristics of the patients included age, gender, anatomic sites, histological type, ulceration, Clark
level, Breslow thickness, growth phase, mitosis, associated nevi, and the amount of lymphocytic infiltration. Tumor regression was evaluated according to the College of American Pathologists (CAP) 2012 criteria [22]: “replacement of tumor cells by lymphocytic inflammation (definitional), as well as attenuation of the epidermis and non-laminated dermal fibrosis with inflammatory cells, melanophagocytosis, and telangiectasia”. Lymphocytic infiltration was also evaluated following the CAP 2012 criteria [22] (0 = absent: no lymphocytes present, or lymphocytes present but do not infiltrate tumor at all. 1 = non-brisk: lymphocytes infiltrate melanoma only focally or not along the entire base of the vertical growth phase. 2 = brisk: lymphocytes diffusely infiltrate the entire base of the vertical growth phase or the entire invasive component of the melanoma). Clinical follow-up of the patients (lymph node metastases, distant metastases, and disease related mortality) was also evaluated.

**4.2.1 Method of MECA-79 Positive Venules Quantification**

Immunohistochemistry was performed by tissue microarray analysis, using formalin-fixed and paraffin-embedded tissue samples. The immunohistochemical study was performed with HEV-specific mouse monoclonal antibody MECA-79 [24]. Antigen retrieval was performed following the manufacturer’s recommendations. Endogenous peroxidase activity was blocked by incubation in S2023 (Dako) for 10 min. The slides were incubated for 30 min. with primary antibody MECA-79 (final dilution 100 μg/ml) at room temperature. The slides were then counterstained with hematoxylin. Adjacent control sections were incubated with monoclonal anti-sialyl-I antibody at the same concentrations and using the same procedure.

Evaluation of the immunohistochemistry was performed by two persons using an Olympus BX40 light microscope, and LEICA DMD108 digital microscope (Leica Microsystems, Germany). The presence or absence of immunoreactivity and the type of vessel was evaluated. The number of MECA-79-positive vessels per mm2 was recorded for each case.
The absolute number of MECA-79+ vessels present in the tumor area was quantified for each tumor section, and the mean number of positive vessels per mm² calculated for each case.

We found that MECA-79+ vessels could be divided into two different groups: cuboidal and flat.

![Figure 4A](image1.png) ![Figure 4B](image2.png)

**Figure 4A.** The cuboidal aspect of the endothelial cells (C-HEV).

**Figure 4B.** Flat aspect of the endothelial cells (F-HEV).

We defined cuboidal HEVs (C-HEV) when the diameter of the lumen was lower than the height of the endothelium and flat HEVs (F-HEV) when the diameter of the lumen was higher than the height of the endothelium.

**4.2.2 Quantitative RT-PCR**

Total RNA extraction was performed according to the manufacturer’s instructions, using 1 ml TRIzol Reagent (Gibco BRL, Gaithersburg, MD)/50 mg of tissue. RNA purity and quantitation was performed by UV-Vis spectrophotometry from 220 to 750 nm (Nanodrop ND-1000). Relative quantification of messenger RNA (mRNA) of the target genes was performed by Real-Time quantitative reverse transcriptase PCR. Briefly, 0.15 μg of total RNA was converted to single-stranded cDNA using the High-Capacity cDNA Archive Kit (PE Applied Biosystems, Foster City, CA). Then, the PCR reactions were performed using TaqMan Universal PCR Master Mix (PE Applied Biosystems, Foster City, CA) and Assayon- Demand gene expression products, consisting of a mix of unlabeled PCR primers and the TaqMan MGB probes (FAM dye-labeled) for the target genes CCR7 (Hs00171054_m1), CCL19 (Hs00171149_m1),...
CCL21 (Hs00171076_m1), and endogenous reference gene 18S rRNA (Hs99999901_s1) [25]. All reactions were performed in triplicate and the results were automatically analyzed by the 7900 HT Fast real time PCR system and RQ Manager 1.2 software (PE Applied Biosystems Inc., Foster City, CA). For the relative quantification of gene expression, the comparative Ct method was used. Samples which exhibited median Ct values for the endogenous reference gene out of the working range were classified as not suitable for valid normalization and, consequently, excluded from further real-time PCR analysis. The final amount of the target gene, normalized to an endogenous reference gene (ΔCt = Ct target gene – Ct reference gene), was given by the formula: $2^{-\Delta C_t}$ [26], which allowed comparison between the different samples of our study.

4.3 Statistical analysis

The statistical analysis was performed using the SPSS software package (v.17.0). The comparison between groups, as well as correlation between HEV counts and non-continuous clinicopathologic variables, was analyzed by the two-tailed non-parametric Mann–Whitney U (MW) and Kruskal-Wallis tests. For comparison between HEV counts and clinicopathologic or analytical continuous variables Pearson’s correlation was used. A p value of <0.05 was considered statistically significant.

CHAPTER 5. RESULTS

The median age of the patients was 62 years, (59% women and 41% men). The most common location of primary melanoma was the trunk (49%), followed by extremities (36%) and head (15%).

The predominant histologic type was superficial spreading melanoma, SSM (78%), followed by lentigo maligna melanoma (9%) and nodular melanoma (7%). Lymphocytic infiltration was present in 69% of tumors, and partial tumor regression was found in 51% of the cases. MECA-79 positive vessels were detected in 55% (47/85) of melanoma samples.
The density of all MECA-79+ vessels/mm2 and the density of C-HEV were higher in primary melanomas than in melanoma metastases. Areas with the highest C-HEV density also showed a more prominent lymphocytic infiltration. In contrast, F-HEV were not usually surrounded by numerous lymphocytes. We found no significant correlation between MECA-79+ vessels and the histological type, although in contrast, we noted significant differences between HEV density at different anatomic sites (p<0.001), the extremities having the lowest number of vessels.

A positive correlation was found between HEV density and lymphocytic infiltration, the association being stronger with the number of C-HEV (p<0.001). Moreover, we found a positive correlation between HEV density and tumor regression (p<0.005), the correlation being particularly stronger with F-HEV. No significant correlation was found between HEV density and age, gender, Clark level, Breslow thickness, growth phase, mitotic figures, the development of lymph node or distant metastasis and survival.

With regard to intratumoral expression of CCL19 and CCL21 chemokines and their receptor CCR7, 19 out of the 85 cases were excluded because the median Ct values for the endogenous reference gene were outside the working range. Therefore, the potential correlations between relative levels of CCL19, CCL21 and CCR7, and HEV density and type were evaluated in 66 cases. Although there was no correlation between these chemokines and receptor expression and HEV density by Pearson’s correlation test, we found that cases in which C-HEV were present (n=25) had higher levels of CCL19 and CCL21 than cases devoid of C-HEV (n=41) (Mann-Whitney, p=0.007 and 0.016, respectively). Similarly, cases with F-HEV (n=31) had higher levels of CCL19 and CCR7 than cases without F-HEV (n=35) (Mann-Whitney, p= 0.002 and 0.039, respectively). Furthermore, it is interesting that cases in which C-HEV predominated over F-HEV (14/66) had higher levels of CCL19, CCL21, and CCR7 (Mann-Whitney, p=0.007, 0.034, and 0.002, respectively).
CHAPTER 6. DISCUSSION

The presence of tumor-infiltrating lymphocytes (TIL) represents a host immune response against tumor cells. A favorable prognostic impact of TIL has been found in different neoplasms: breast [11], colon [27], lung [28], ovarian carcinomas [29], as well as cutaneous melanoma [10, 30]. Martinet et al. first described the presence of HEVs, which are specialized blood vessels for recruitment and migration of lymphocytes, in cutaneous melanomas [11], and demonstrated that a high density of HEVs is associated with thin melanomas, low levels of invasion and signs of regression; suggesting that HEV density represents a favorable prognostic biomarker for malignant melanoma [12].

Our results support the fact that in cutaneous malignant melanoma, HEVs are present in the majority of primary tumors, and that their presence and density can predict the efficiency of the host response against tumor cells. In regard to melanoma metastases, the presence of HEVs was first described by Cipponi et al. [13]. According to our findings, the HEV density in melanoma metastases is very low, as is the number of TIL, compared with primary tumors.

Based on our observations, we propose that two types of HEVs can be found in cutaneous melanomas: MECA-79-positive HEVs lined by cuboidal endothelium (C-HEV) and MECA-79-positive vessels covered by flat endothelium (F-HEV). Accordingly, we found a strong correlation between the density of C-HEV and the presence and degree of lymphocytic infiltration. Topographically, the highest density of lymphocytes was located in areas with a high density of C-HEV, a fact that provides support to the important role of these blood vessels in the recruitment of TIL in primary melanomas. Interestingly, we found that the density of MECA-79-positive vessels covered by F-HEV in primary melanomas showed the highest correlation with established tumor regression. We may explain the presence of F-HEV in areas with a late phase of regression by the fact that at this time lymphocytes are no longer needed and, therefore, the endothelial cells in these vessels lose their characteristic cuboidal appearance which is important for the functional state which permits entry of lymphocytes into the tumor.
Based on our results, the evaluation of HEV density, and particularly the presence of C-HEV in primary melanomas may serve as a reflection of an active stage of immune response mediated by high levels of CCL19 and CCL21 among other cytokine mediators. These findings might be applied to patients with cutaneous melanoma treated with Ipilimumab, a drug which blocks cytotoxic T-lymphocyte-associated antigen 4 and, as a consequence, potentiates an antitumor T-cell response [31]. Since the primary tumors will already have been excised in all these patients before treatment, we believe that the presence and type of HEV could be evaluated both in the primary tumor as well as in the recurrent or metastatic tumors, biopsied during or immediately after treatment, in order to find differences, particularly in the presence of C-HEV, which may provide evidence of a potentiation of the immune response by the treatment. With regard to HEV density, we have shown that it is lower in untreated metastatic tumors than in primary tumors. Therefore, further studies should be performed in order to demonstrate if HEV type and/or density are modified in melanomas treated with Ipilimumab.

**CONCLUSIONS**

In this study we analyzed tumor samples from 85 patients diagnosed with cutaneous melanoma samples collected over a 10 years period (2002-2011) at the Department of Pathology, University Clinic Hospital of Valencia.

Cases were selected from consecutive specimens of melanoma received from the Department of Pathology, the tumor specimens received were fresh and unfixed. Samples of primary and metastatic tumors were manually sectioned to obtain samples containing more than 90 % of tumor tissue.

1) The age average of the patients included in the study was 62 years old (59 % women and 41 % men) with a preponderance of females.

2) The predominant histological type was superficial spreading melanoma in a proportion of 78 %, followed by malignant lentigo, nodular and acral melanoma.

3) We studied the presence of regression in different histopathological types and we noted the presence in: 52 % of cases with superficial spreading melanoma, 67 %
of those diagnosed with lentigo malignant and 25 % for those with acral melanoma.

4) Analyzing the number of MECA -79 positive vessels and gender of patients included in the study, we observed a higher density of MECA -79 positive venules of male patients.

5) The most numerous venules MECA -79 were detected positive for the cases that presented lentigo malignant melanoma. As we found no statistically significant correlation between venules MECA -79 positive and histological type, in contrast we found significant differences between HEV density and different anatomical sites (p < 0.001) and the extremities having the lowest number vessels.

6) The regression was noted in 51 % of cases included in the study.

7) We observed a higher density of MECA -79 positive venules in primary melanomas that presented areas of regression. Moreover, we found a positive correlation between HEV density and tumor regression (p < 0.005), but the correlation was particularly strong for F-HEV type of venules.

8) We found a positive correlation between HEV density and lymphocyte infiltration, the association being stronger C-HEV density (p < 0.001).

9) Our results support the idea that in primary cutaneous malignant melanoma can be detected high endothelial venules and density of this type of vessels can predict the efficiency of host response against tumor cells. We found that the density of MECA -79 positive vessels type F-HEV in the primary melanomas showed the strongest correlation with tumor regression. We may explain the presence of F-HEV in areas with a late phase of regression by the fact that at this time lymphocytes are no longer needed and, therefore, the endothelial cells in these vessels lose their characteristic cuboidal appearance which is important for the functional state.

10) As a final conclusion, identification and quantification of HEV may be of great interest to providing information on biological and prognostic immune response to the tumo, thus providing a starting point for developing new therapeutic strategie. Our findings, the strong association between the density of F-HEV and late regression phase of primary melanoma has not been previously reported.
Based on our results, we support the idea that determining the density of F- HEV venules associated with higher levels of expression of chemokines CCL19, CCL21 and CCR7 receptor, may serve as useful indicators of tumor regression in melanoma.
SELECTIVE REFERENCES


