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Research Article

Vascular structures on dermoscopy and ultrasonography associated with increased intratumoral lymphatic density and expression of Cyclin D1 in acral melanoma- a single center pilot study

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Abstract. Background and objectives: Dermoscopic assessment and histopathological examination have an important role in melanoma diagnosis and management. Studies showed that angiogenesis and lymphangiogenesis play an important role in tumorigenesis, as predictors of tumor growth and metastasis development. The aim of the present study was to correlate dermoscopic structures seen in acral melanoma with ultrasonographic findings regarding tumor vascularization and immunohistochemical parameters evaluating angiogenesis and lymphangiogenesis. Materials and methods: Dermoscopic and ultrasonographic (Doppler US) data from 10 acral melanomas was collected. MVD (microvessel density), LVD (lymphatic vessel density) within and around the tumor, VEGFR2, Ki67 and Cyclin D1 were assessed by immunohistochemistry. All patients in the study gave their informed written consent. Results: In our study, milky red areas/globules and polymorphous vascular pattern on dermoscopic examination was associated with high vascularization on ultrasound examination, stiff appearance on elastography, a tendency to express Cyclin D1 and high intratumoral LVD on immunohistochemistry. The absence of milky red areas/globules on dermoscopy was associated with no vascularization on ultrasound examination and low or absent intratumoral LVD. Conclusion: Our study demonstrates that when combined, dermoscopy, Doppler US, strain elastography and immunohistochemistry can offer a better understanding and management of cutaneous acral melanoma.

Key Words: acral melanoma, ultrasound, dermoscopy, Cyclin D1, D2-40 LVD

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Introduction

Acral melanoma (AM) is a distinct form of melanoma arising in acral sites, primary on palms, soles and nails, accounting for 2-3% of all melanomas (Goydos et al 2016; Bradford et al 2009). It is more frequent in African and Asian population and associated with poor prognosis mostly due to patient late presentation and advanced stages at diagnosis, but misdiagnosis and low public awareness are also contributory (Dawes et al 2016; Desai et al 2017)

Tumor growth and high tumor vascularization along with tumor histology (tumor thickness, presence of ulceration, Clark level of invasion or mitotic rate) are important predictors for the increased risk of metastasis (Srivastavaet al 1989). Doppler US associated with dermoscopy and clinical examination increases diagnostic accuracy and improves melanoma management (Botar Jid et al 2015).

The aim of the present study was to correlate dermoscopic structures seen in acral melanoma with ultrasonographic findings regarding tumor vascularization and immunohistochemical parameters evaluating angiogenesis and lymphangiogenesis.

Materials and methods

The present study was observational, analytic, prospective, transversal, and cohort. Our study included 10 patients diagnosed with primary acral melanoma, admitted within the Department of Dermatology from Emergency County Hospital, Cluj-Napoca between January 2014 and November 2016. The study was approved by the Ethics Committee of the "Iuliu-Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca. Prior to inclusion in the study, all patients signed an informed consent. All the lesions were clinically and dermoscopically evaluated by two experienced dermatologist (LG and RC) using a handheld dermatoscope (Heine Delta 20, Heine Optotechnik Germany or DermLite III DL3 Polarized & Fluid Dermoscope, 3Gen Inc. USA). Specific dermoscopic structures were recorded for each patient. The pigmented pattern included the description of parallel ridge pattern (throughout the lesion or in the periphery) and irregular diffuse pigmentation. The vascular structures assessed were represented by the presence or absence of polymorphous vascular pattern, milky red areas/globules, linear irregular vessels and corkscrew vessels.

Prior to surgery, a high-frequency-ultrasonographic (US) evaluation of tumors was performed by an experienced radiologist, using a Sonotouch and Tablet system from Ultrasonix Medical corporation, Richmond, Canada with an 8-40 Mhz linear transducer. All tumors were evaluated in longitudinal and transverse planes with no exerted compression that could have altered the blood flow. Color and power Doppler were used to assess tumor vascularization (color Doppler: PRF 500-1000 Hz, wall filter 25-50Hz; power Doppler PRF 350-700 Hz, wall filter 22-50Hz). For each tumor the number of vascular pedicles and the aspect of vascularization were assessed (no or low vascularization, <50% - medium vascularization, >50% hypervascularization). Strain elastography was used to appreciate tumor elasticity (soft, medium stiffness, stiff) comparing the tumoral lesion with surrounding normal tissue, assumpting that soft tissues are deformable and stiff tissues are less deformable. Tumor elasticity was qualitatively assessed with the help of strain elastography colours: red coded soft tissues, yellow and green medium stiffness tissues and blue for stiff tissues.

All patients underwent surgical excision and were treated according to the stage proposed by 2009 AJCC Melanoma Staging and Clasification.

The histological slides were reviewed by an experienced pathologist who also performed the immunohistochemistry stains and evaluation. Immunohistochemistry was performed on 4-μm thickness formalin-fixed, paraffin-embedded sections using commercially available markers VEGFR-2 at 1:50 (polyclonal, Abcam) and CD34 at 1:100 (QBend 10, Immunologic) were used to assess MVD, D2-40 at 1:25 (Abcam) for LVD, Cyclin D1 at 1:40 (P2D11F11, Novocastra) and ki67 at 1:600 (MYB1, Immunologic) for tumor cell proliferation. All stains were performed automatically on Ventana, Roche Bench Mark Ultra machine, HIER CC1 with optiview detection kit and ultra view red. All stained slides were viewed and scored on an Olympus BX43 microscope. CD34 MVD was counted within and around the tumor area after scanning the immunostained sections at low magnification (40x) using the Weidner method. [Weidner N Am J Pathol 1995]. Three areas with the greatest number of vessels were selected and the stained vessels were counted. The mean of these areas was used to appreciate the MVD. D2-40 LVD was also appreciated within the tumor and around it using the same method. VEGFR2-was also used to appreciate the tumor vessels. The following scale appreciated the percentage of positive VEGFR2 cells: 0, 0% to 25%; 1+, 26% to 50%; 2+, 51% to 75%; and 3+, greater than 76% to 100%. The staining intensity of VEGFR2 positive cells was noted: 1+, weak staining; 2+, moderate staining; and 3+, strong staining. A total score summing the two of them was noted for each tumor. Cyclin D1 was assessed using the proportion of tumor cells showing a positive reaction (no staining; positive staining in 50% of tumor cells).

The statistical analysis was performed with the help of MedCalc Statistical Software version 17.5.5 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2017). Quantitative data were characterized by median and 25-75 percentiles. Qualitative variables were described with frequency and percent. Differences between groups were assessed by Mann-Whitney test or Chi-square test, whenever appropriate. The correlation between two quantitative variables was verified using the Spearman's rho coefficient. A p value <0.05 was considered statistically significant.

Results

The patient's median age at diagnosis was 62 years (range 50-76). There were four (40%) males and six (60%) females. The mean Breslow index on histopathological examination of tumors was 4.25mm (range 0.7-9.5mm). At the time of initial diagnosis one patient was stage IA, two stage IIA, four stage IIC, one stage IIIC and one stage IV.

Dermoscopic evaluation of the tumors revealed that the majority of tumors, eight (80%) presented a polymorphous vascular pattern, with milky red areas/globules being the most prominent vascular structure. Two (20%) of the tumors presented no or few number of visible vessels, the pigmented component being more prominent. Linear irregular vessels were present in all tumors except one. Corscrew vessels were present in eight (80%) tumors. Irregular diffuse pigmentation was present in all the tumors and parallel ridge pattern was present in 50% of the tumors, especially in the periphery of tumors. None of the analyzed tumors were amelanotic.

On the high-frequency-ultrasound evaluation all tumors presented as hypoechoic masses. Six (60%) had a homogeneous appearance on ultrasonography and four (40%) were inhomogeneous. Two (20%) of the tumors had no vascular structures and presented as soft masses on strain elastography and eight (80%) were hypervascularised, had more than two pedicles and presented a rigid, stiff appearance on strain elastography. Polymorphous vascular pattern on dermoscopy which included milky red areas/glubules was associated with hypervascularised tumors on ultrasonography (Table 1). Milky red areas/ globules on dermoscopy were associated with increased vascularity on ultrasonography and hypervascularised tumors with more than two vascular pedicles on Doppler ultrasound examination. There was a strong correlation ($p=0.02$) between the presence of polymorphous vascular pattern which included milky red areas/ globules with the number of vascular pedicles (>2) and hypervascularization on Doppler US (Table 1).

Table 1. Correlation of dermoscopic structures (polymorphous vascular pattern, milky red areas/globules), immunohistochemical staining for MVD, LVD, Cyclin D1 and vascular pedicles on Doppler US.

Characteristics		No vascular pedicles on Doppler US	Multiple vascular pedicles (>2) on Doppler US	P
Polymorphous vascular pattern	No	2 (100%)	-	0.02
	Yes	-	8 (100%)	
Milky red areas/globules	No	2 (100%)	-	0.02
	Yes	-	8 (100%)	
Intratumoral MVD		8.8 (8.7; -)	9.3 (5.2; 9.8)	0.6
Peritumoral MVD		16.3 (6.2; -)	11.3 (8; 15.1)	1
Intratumoral LVD		0.2 (0; -)	3.2 (0.8; 7.1)	0,05
Peritumoral LVD		2.8 (0.5;)	3.5 (1.8; 5.3)	0.6
VEGFR2		0.2 (0; -)	0.1 (0.01; 0.6)	0.5
Ki67		0.05 (0.05; 0.05)	0.05 (0; 0.1)	1
Cyclin D1		0 (0; 0)	0.22 (0.1; 0.37)	0.06
Breslow index (mm)		2.3 (0.0; -)	4.6 (2.8; 4.9)	0.2

Tumors presenting milky red areas/ globules on dermoscopy had a rigid appearance on elastography. Tumors with no vessels on dermoscopy did not present vessels on ultrasonography and had a soft appearance on strain elastography. We found a strong correlation (p=0.02) between the presence of polymorphous vascular pattern which included milky red areas/globules with the elastographic stiff appearance on strain elastography(Table 1). Mean ultrasonographic depth of tumors was 4.5 mm in concordance with the mean Breslow index on histopathology.

Immunohistochemical analyses of histological slides showed that median MVD within the tumor was 9.12 (6.25; 9.6) and in the peritumoral areas was 11.3 (7.7; 17.1). MVD within the tumor and in the peritumoral area was not associated with the presence of vessels on dermoscopic and ultrasonographic evaluation and did not influence the elastographic stiffness appearance (Table1) Median LVD on immunohistochemical analyses was 1.87 (0.5; 5.5) within the tumor and 3.5 (1.4;5.3) in the peritumoral areas. The absence of polymorphous vascular pattern which included milky red areas/globules on dermoscopy correlated with no or very few LVD within the tumor, and high density of LVD in the peritumoral area (p=0.05)(Table 1).

Milky red areas/globules and polymorphous vascular pattern on dermoscopic examination was associated with high vascularization on ultrasound examination, stiff appearance on elastography, and high intratumoral LVD on immunohistochemistry (Table 1). Positive staining for VEGFR2 and Cyclin D1on immunohistochemistry was noted in seven (70%) of the tumors. VEGFR2 tumor cell positivity did not show any correlation with vascular structures present on dermoscopy and ultrasonography. Cyclin D1 expression on immunohistochemistry tended (p=0.06) to be associated with the presence of a polymorphous vascular pattern which included milky red areas/globules on dermoscopy and with hypervascularised tumors on Doppler ultrasound (Table 1). Rigid tumors on strain elastography also tended (p=0.06) to express Cyclin D1 on immunohistochemistry (Table 1).

Discussion

Acral melanoma is a type of melanoma associated with poor prognosis, late presentation and advanced stages at diagnosis, being more frequent in aged people (Desai et al 2017).The median age of patients in our study was 62 years with a male to female ratio of 1:1.5, in accordance with the published data in the literature. Lallas et al reported a median age of 67.7 years and a male to female ratio of 1:1.6 in 131 patients with acral melanoma and Sondermann et al a median age of 61.6 years in 151 patients with acral melanoma, with higher frequency in males than in females 1.6:1. Patients in our study had more thick tumors at presentation with a median Breslow of 4.25mm than other reports of 2.67 mm (Lallas et al 2015), but Breslow thickness increases in misdiagnosed tumors up to a 3.1mm median (Sondermann et al 2016). In 2018 Mun et al reported a mean Breslow index of 4.0 ± 3.7 mm in invasive melanomas. The majority of cases in our study showed a high Breslow index mostly due to late presentation.

Dermoscopy improves the diagnosis of cutaneous lesions, being able to differentiate based on specific dermoscopic structures (pigmented and vascular structures) benign lesions from malignant ones, and, in case of melanoma, predict the tumor thickness (Argenziano et al 2003; De Giorgi et al 2002). Milky red globules are dermoscopic structures not frequently seen; when present, they highly suggest an invasive melanoma (Braun et al 2009a) and correspond to an area with increased vascularity(Margoob et al 2010; Ungureanu et al 2013). Argenziano et al reported their presence on dermoscopy in only 4.7% of melanomas with vascular structures (Argentiano et al 2004). Reports in the literature regarding linear irregular vessels on dermoscopy in melanomas suggest relative thin melanomas, but they can also be present in benign lesions (Braun et al 2009a; Pizzicheta et al 2004). Corscrew vessels are mainly reported in nodular and desmoplastic melanomas, but also in cutaneous melanoma metastasis (Bono et al 2004). 80% of melanomas in our study presented a polymorphous vascular pattern which included milky

red areas/globules on dermoscopy, vascular pattern and structures associated with a high Breslow index.

High frequency ultrasound is used in cutaneous tumors to estimate tumor thickness and diameter. The mean Breslow index in our study was 4.25mm (range 0.7-9.5 mm) and the mean US thickness was 4.5mm (range 0.5-11.5mm), but the purpose of our study was not thickness measurement. Doppler US assess tumor vascularization, analyzing the distribution of vessels, blood flow amount and vessels velocity. On ultrasonographic evaluation Catalano et al found high tumor vascularization in cutaneous melanomas thicker than 2 mm (Catalano et al 2010; Botar Jid et al 2015). In 2013, Doppler US was reported by Kato M et al to be a useful tool in evaluation of amelanotic melanoma in comparison with other malignant tumors. When comparing dermoscopic vascular structures with Doppler sonographic examination vessels' findings they found no correlation between vessel types seen in dermoscopy and Doppler sonography findings. Our study found a strong correlation between milky red areas/ globules when present in dermoscopy with hipervascularization and a great number of vascular pedicles on Doppler ultrasound.

Tumor angiogenesis and lymphangiogenesis, the generation of new capillary blood vessels and lymphatic vessels from pre-existing vessels showed to be associated with poor prognosis in melanoma (Straume et al 1999; Valencak et al 2004; Dadras et al 2005; Depasquale et al 2005).When assessed for LVD, Straume et al showed an increased peritumoral LVD compared with intratumoral LVD in melanomas with decreased Breslow index. Our study found that tumors with no vessels at dermoscopic and ultrasonographic evaluation showed no, or very few intratumoral LVD. One of the major angiogenic factors is vascular endothelial growth factor (VEGF), who is secreted by tumor and also by host cells (Srivastava et al 2003). VEGF expression was associated with melanoma thickness and progression from radial to vertical growth phase (Erhard et al 1997; Marcoval et al 1997). We did not find an association between the presence of any vascular structures on dermoscopy or on Doppler US with VEGFR2 tumor cell positivity.

There are controversial reports in the literature regarding the biologic role of Cyclin D1 in melanoma progression and metastasis. Some studies showed a correlation with stage, grade and prognosis in breast, colon , lung and head/neck tumors (Yu et al 2001; Oyama et al 1998; Keum et al 1999). In melanoma, Cyclin D1 expression was found in invasive and in situ melanomas on a higher rate than in benign melanocytic lesions (Ramirez et al 2005). In 2020 Kauffman et al reported an increased expression of Cyclin D1 in invasive but still thin (≤1 mm Breslow thickness) melanomas compared to in situ melanomas. In our study Cyclin D1 tended to be associated with highly vascularized tumors on dermoscopy and Doppler US, structures associated with high Breslow index.

Although small, this observational, prospective study is the first to our knowledge that associates dermoscopic vascular structures in acral melanomas with Doppler US intratumoral vascularization and their correlations with lymphatic vessel density on immunohistochemistry. In order to refine and confirm our findings, further studies and larger groups of patients are needed.

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