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Original Paper

Stochastic Relationship Between the Growth Fraction and Vascularity of Thin Malignant Melanomas

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Tumour vascularity has been proposed as a prognostic indicator for a number of solid tumours. In these respects the situation is unclear for cutaneous malignant melanoma (MM). The small number of MM cases studied to date have indicated controversial preliminary conclusions. Hence, tumour thickness remains currently the most reliable prognostic factor for MM. This variable has been shown in previous work to be correlated with both the S-phase fraction and the growth fraction of the MM cells. We studied the relationship between vascularity and growth fraction of 100 thin (< 1 mm) cutaneous MM using immunohistochemistry (*Ulex europaeus* agglutinin-type 1 (UEA-1) and MIB-1 antibody (Ki-67)) and computerised image analysis. A stochastic relationship was found between intra- and peritumoral vascularity, and between Ki-67 and UEA-1 measures. Approximately 20–40% of PC-MM showing a high Ki-67-positive growth fraction (breakpoint: 30/0.4 mm^2) were characterised by a prominent vascularity at the boundary of the neoplasm. In these cases, vessels often exhibited either heterogeneous lumen sizes or a uniform prominent dilated network. The present study shows that the angiogenic phenotype of MM occurs as a stochastic event during neoplastic progression. Its expression is not mandatory for reaching high values in MM growth fraction. It can be hypothesised that the absence of a link between proliferation and vascularity might account for the unusual outcome of some cutaneous MM, including the dormant growth-stunted type and the metastasising thin MM.

Key words: angiogenesis, growth fraction, melanoma, tumour thickness
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INTRODUCTION

CANCER PROGRESSION and neovascularisation are often linked. Angiogenesis can precede overt neoplasia and is obligatory for tumour progression through certain steps of malignancy. In particular, an increased vascular network is a critical determinant for tumour growth and release of metastatic cells [1–5]. Among such cancers, cutaneous malignant melanoma (MM) exhibits unequivocable angiogenesis [6–20]. However, MM studies have yielded conflicting conclusions about the clinical implications. Some authors regard the extent of MM angiogenesis as a valuable predictive indicator for metastasis [7, 13, 17, 19, 20], while others have not found such a link [9, 15, 16, 18]. Unfortunately, studies published to date on vascularity of thin MM should be interpreted with caution because of the small number of cases studied. In addition, the assessment of angiogenesis may have differed because of the choice of the immunohistochemical method, the definition of countable microvessels and the quantitative methods employed.

Tumour thickness is currently the most reliable prognostic factor for MM. This variable is correlated with both the S-phase fraction [21] and the growth fraction of MM cells [22–28]. In addition, it has been shown in a small number of patients that the MM growth fraction is also closely related to neovascularisation in the peritumoral stroma [8].

The aim of the present study was to re-examine the relationship between vascularity and growth fraction in 100 thin cutaneous MM using immunohistochemistry and computerised image analysis.

MATERIALS AND METHODS

A total of 100 superficial spreading MM measuring from 0.30 to 1 mm in thickness, excised from the limbs of patients

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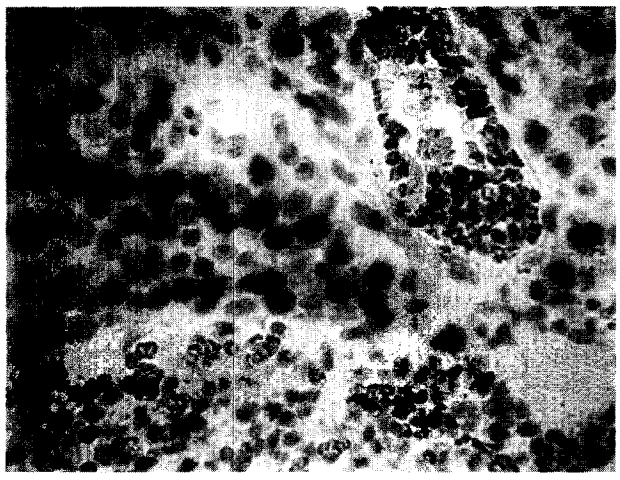

Figure 1. Cluster of Ki-67-positive malignant melanoma cells in the vicinity of a dilated vessel. Magnification × 600.

ranging in age from 24 to 51 years, were used in this study. Cases were excluded if they showed distant metastases at presentation or when there was a second primary malignant neoplasm, either previously or subsequent to the MM excision. Six of the patients developed metastases within the 3–6-year follow-up after excision of the primary neoplasm.

Surgical specimens were fixed in buffered formalin and embedded in paraffin. Representative sections including adjacent apparently normal skin, were processed for immunohistochemical staining using avidin–biotin–peroxidase. Vasculature was highlighted using the lectin *Ulex europaeus* agglutinin-type 1 (UEA-1, 1:80, Dakopatts). The growth fraction of MM cells was assessed through Ki-67 immunoreactivity using the MIB-1 antibody (1:10, Immunotech) after antigen retrieval in a microwave oven set at 750 W. 3-Amino-9-ethylcarbazole was used as chromogen. Positive controls were performed by omitting or substituting reagents of the immunohistochemical procedure.

Quantitative evaluations were made using computerised image analysis (Analysis Olympus). Two main fields of observation were delimited in each section. One field area measuring 0.2 mm in height and 3 mm in length was evaluated at the deepest part of the neoplasm. The contiguous field

Figure 2. Unusual prominent vasculature at the base of a primary cutaneous malignant melanoma. Magnification × 300.

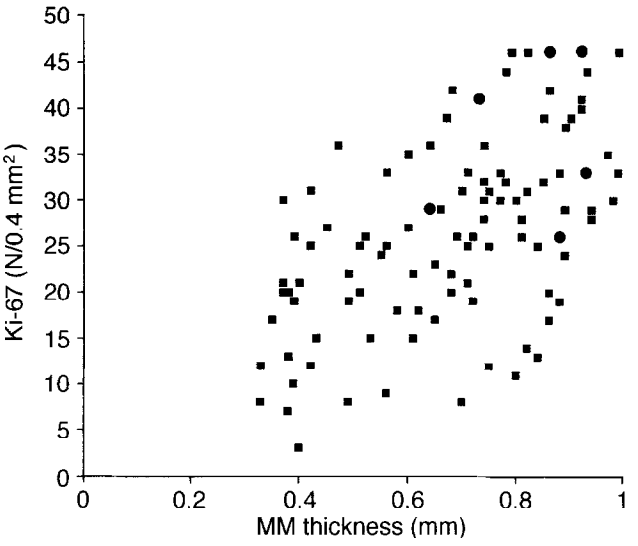

Figure 3. Scatterplot of the malignant melanoma (MM) growth fraction (number of Ki-67-positive cells) versus the thickness of the neoplasm. There was a positive linear relationship ($P < 0.01$) between the two variables. Patients with (●) and without (■) metastasis at follow-up.

of similar size in the peritumoral stroma was also chosen for analysis. The Ki-67-positive MM cells were counted per contiguous 0.04 mm^2 sectors in the intratumoral field. The percentage of the area occupied by vascular profiles was measured in both intra- and peritumoral fields.

Due to skewness of data, the median value and 95% confidence intervals of each analytical variable were calculated for both fields in each MM. The relationships between tumour thickness, numerical density of Ki-67-positive MM cells and extent of vascularity were assessed using regression model analysis. Linear, logarithmic, exponential and power relationships were tested between variables and the coefficient of correlation r was calculated. A P value lower than 0.05 was considered significant.

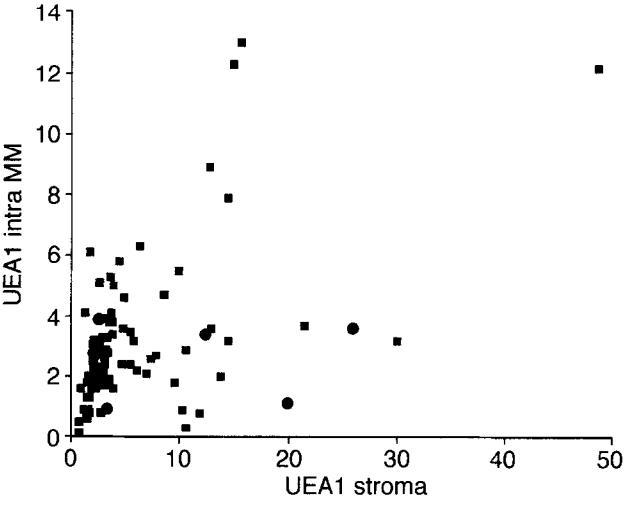

Figure 4. Scatterplot of the stochastic relationship between the intra- and peritumoral vascularity in primary cutaneous malignant melanomas. Values represent the relative area delimited by endothelial cells highlighted by *Ulex europaeus* agglutinin-type 1 (UEA1). Patients with (●) and without (■) metastasis at follow-up.

RESULTS

The primary cutaneous MM varied in their overall proliferative activities and vascular patterns. Most Ki-67-positive MM cells and microvessels revealed by UEA-1 staining were randomly distributed within the neoplasms. In addition, some clusters of proliferating MM cells related or not to increased capillary density were present in the majority of the neoplasms (Figure 1). Compared to the surrounding skin, the peritumoral stroma often exhibited a diffuse and uniform pattern of hypervascularity. In most MM, areas of particularly high vascularity were not clearly identified, thus excluding the identification and further analysis of so-called 'hot spots'. Groups of vessels showing heterogeneous lumen sizes were also focally abutted to the neoplasms. Rare lesions exhibited a rim of prominent vascular network characterised by numerous dilated vessels almost encircling the neoplasm (Figure 2).

A positive linear correlation ($r = 0.55$, $P < 0.01$) was present between MM thickness and the numerical density of Ki-67-positive neoplastic cells (Figure 3). A stochastic relationship was found between intra- and peritumoral vascularity (Figure 4), and between Ki-67 or MM thickness and UEA-1 measures (Figures 5 and 6). Approximately 20–40% of MM exhibiting a high Ki-67-positive growth faction (breakpoint: 30/0.4 mm^2 corresponding to approximately 0.7 mm in thickness; see Figure 3) were characterised by prominent vascularity at the boundary of the neoplasm.

DISCUSSION

Previous studies have differed in the choice of antibody used to highlight microvessels. Inconsistent results given by the immunolabelling of CD31, CD34 and Factor VIII-related antigen (FVIIIRA) have been previously reported [29–31].

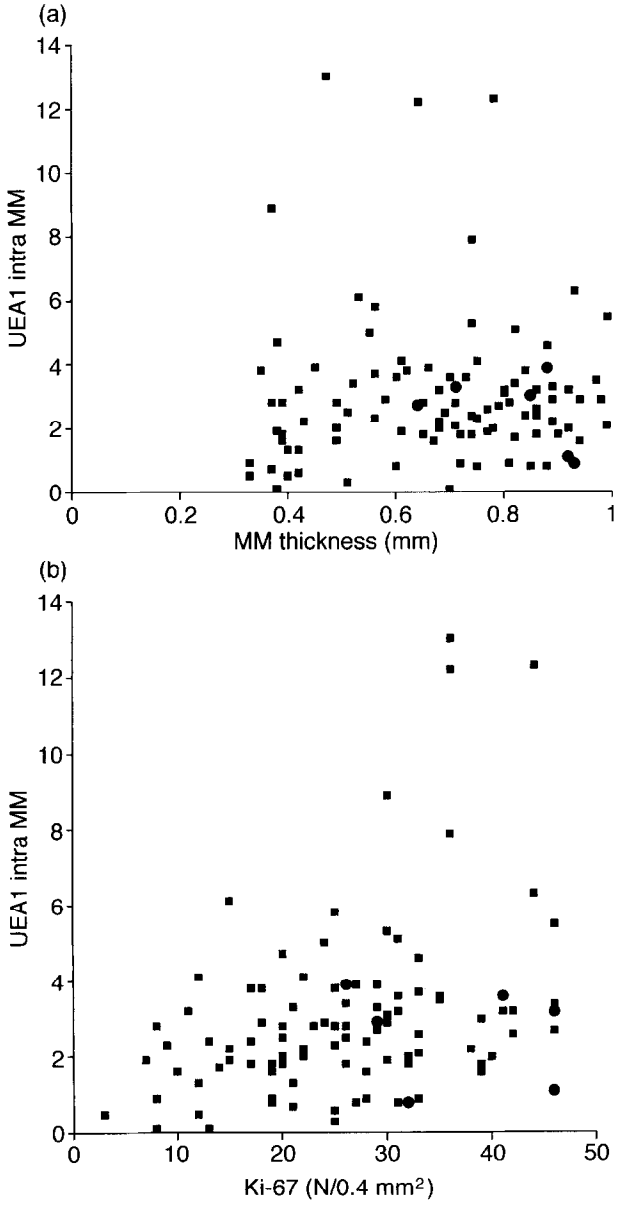

Figure 5. Scatterplot yielding a stochastic correlation between intratumoral vascularity (UEA1 intra-MM) and thickness (a) or growth fraction (b, number of Ki-67-positive cells) of primary cutaneous malignant melanomas (MM). Patients with (●) and without (■) metastasis at follow-up.

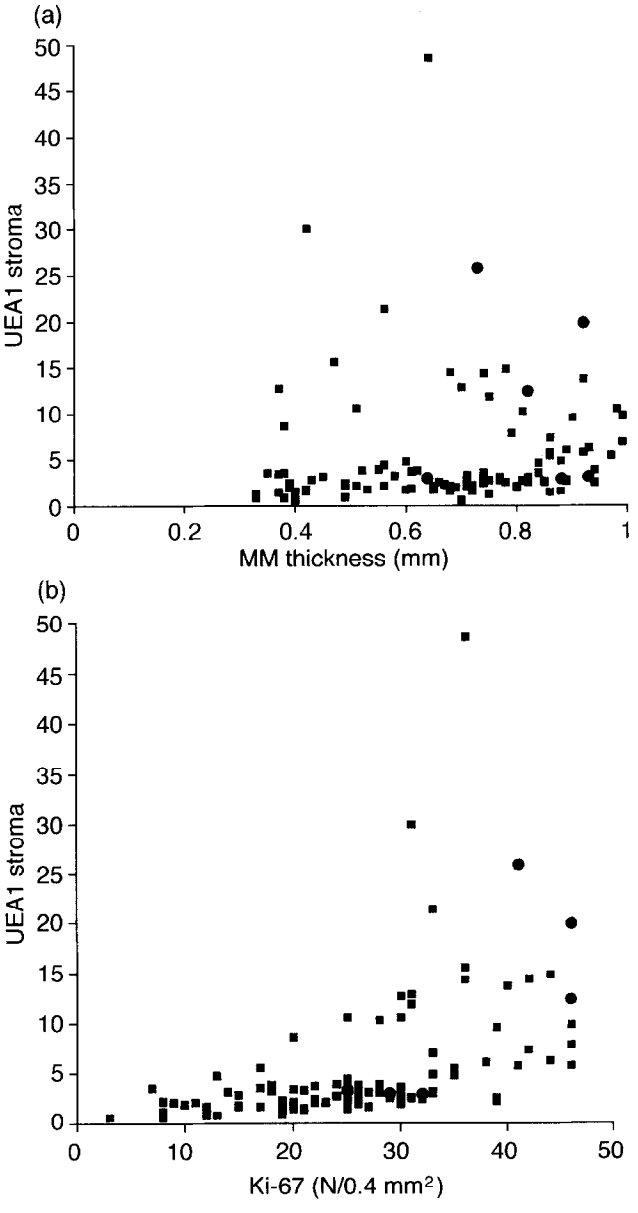

Figure 6. Scatterplot yielding a stochastic correlation between peritumoral vascularity (UEA1 stroma) and thickness (a) or growth fraction (b, number of Ki-67-positive cells) of primary cutaneous malignant melanomas (MM). Patients with (●) and without (■) metastasis at follow-up.

The lectin UEA-1 was chosen for the present work because its reactivity to endothelial cells shows less background staining than antibodies to CD34 [18]. In our hands, UEA-1 is also more sensitive than antibodies to CD31 and FVIIIRA.

A major methodological problem relates to our inability to discriminate between lymphatic vessels and blood vascular structures. Hence, it is not possible using currently available means to identify selectively lymphatic microvessels used for MM metastasic spread. However, the presence of numerous venolymphatic anastomoses allows rapid passage of tumour cells between the two vascular systems and hence renders their distinction less crucial [32].

The intensity of angiogenesis on histological sections can be assessed by microvessel counting or by measuring their density profile area [8]. The issue of reproducibility of microvessel counting has been raised [33]. In addition, such data reflect both the actual number and tortuosity of the vessels. In the present study, we preferred to measure the overall relative area of the peri- and intratumoral vasculature.

There is ample evidence that primary cutaneous MM development in humans is associated with increased vascularity [6–20, 34]. Our observations are in line with these works. The net angiogenesis results from a positive imbalance between stimulator and inhibitor cytokines originating from the malignant cells and accessory cells such as stromal and inflammatory cells [5, 28]. However, the correlation between angiogenesis in the primary neoplasm and clinically recognisable metastatic spread is not so evident because of the unpredictable survival and growth of released MM cells facing the many independent biological steps of the metastatic process [32, 35, 36]. As previously stated, alterations in cell–cell and cell–stroma interactions, degradation of stroma and basement membrane, penetration of vessels, survival in the circulation, extravasation and the ability of tumour cells to grow at their final resting sites are all important in the process of metastasis [36]. Hence, a static count of microvessel density does not necessarily reflect the dynamic process of metastasis. In addition, the increase in vascularity as revealed by immunohistochemistry does not properly reflect the lymphatics which likely represent the major route for MM metastases.

Our data showing the highest values in vascularity outside the margins of MM agree with other studies [6–8, 14]. This may indicate that the specific intratumoral vasculature is a less sensitive target for angiogenic factors than the peritumoral host tissue. The correlation between MM thickness and growth fraction is also a confirmation of previous work [22–28]. The present study shows that the microvascular density often remains constant, even when the MM thickness and growth fraction increase. The apparent angiogenesis steady state in most MM 0.3–1 mm thick probably represents the balance of vasoproliferative stimuli from neoplastic cells and host infiltrate. Such data are in line with the conclusions of previous ones [14] and are at variance with others [8]. In fact, we emphasise the stochastic relationship between vascularity and both the thickness and growth fraction of thin MM. Some lesions exhibit unusual high or low vascularity irrespective of the thickness and growth fraction of the primary tumour. Whether these features would respectively represent a risk factor for metastasis or a clue indicating a growth-stunted neoplasm remains to be determined. The follow-up of these patients ranges from 3 to 6 years and is presently too short to permit conclusions.

It should be emphasised that vascularity is a dynamic process which might fluctuate during MM progression. Data gained by histology are in contrast a snapshot of the situation at the time of excision. Hence, such observations could miss privileged moments when microvasculature is transitorily boosted and/or receptive to a micrometastatic spread.

In summary, the impact of angiogenesis on MM prognosis is a continuing issue as it is for breast cancers [26]. Vascularity appears to have no clear-cut relationship with MM thickness, which is an established prognostic factor, and therefore indirectly indicates that vascularity is not a prognostic factor as well. It would be more profitable to monitor anti-angiogenic therapy than trying to predict metastatic spread through the evaluation of MM vasculature.

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