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Original Paper

High CD44 Surface Expression on Primary Tumours of Malignant Melanoma Correlates with Increased Metastatic Risk and Reduced Survival

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The cell surface glycoprotein CD44 has been implicated in the progression and metastasis of certain human tumours including malignant melanoma (MM). In animal models, certain MM cell lines, expressing high levels of CD44, displayed an augmented capacity for haematogenous metastasis, compared to those with low CD44 levels. To determine whether, *in vivo*, the level of CD44 expressed by primary tumours of MM (PMM) is related to their metastatic potential, CD44 expression on PMM was studied in 92 patients, classified by their metastatic risk based on histological measurement of vertical tumour thickness (VT): *in situ* PMM, low-risk PMM (VT ≤0.7 mm), intermediate risk PMM (VT = 0.71–1.4 mm) and high-risk PMM (VT>1.4 mm). Paraffin-embedded sections were stained immunohistochemically with a panCD44 MAb. The level of CD44 expression on PMM was analysed semiquantitatively with epidermal CD44 staining set as an internal standard. High levels of CD44 were detected in 58.3% of high-risk PMM, 40.6% of intermediate-risk PMM, 36.7% of low-risk PMM and 16.7% of *in situ* PMM. Seventy-four per cent (17/23) of patients who developed and/or died of MM metastasis were CD44 high, and importantly, among these were 5 patients, whose metastatic risk had been estimated low, based on the measurement of VT. Finally, Kaplan–Meier analysis revealed patients whose PMM were CD44 high to have a significantly reduced 5-year survival rate compared to those that were CD44 low ($P < 0.05$). We conclude that in our patient population, a high level expression of CD44 on PMM is associated with increased metastatic risk and reduced survival. © **1997 Elsevier Science Ltd.**

Key words: CD44, melanoma, metastasis, prognostic factors

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INTRODUCTION

CD44 is a broadly distributed cell surface glycoprotein implicated in multiple physiological cellular functions including cell–cell adhesion, cell–substrate interaction and cell activation [1, 2]. CD44 exists in different isoforms which can be generated either by alternative splicing from at least 10 different variant exons (v1–v10) or by differential glycosylation or glycosaminoglycan modification of the mature protein [3–7]. The smallest CD44 isoform, CD44s, is expressed in a variety of tissues, whereas expression of larger CD44 isoforms (CD44v), which are generated by alternative splicing, is restricted mainly to epithelial tissues, including epidermis and cells of the immune system [3–6]. Several studies have shown that CD44 interacts with extracellular matrix (ECM) components, in particular certain isoforms of CD44 are the principal receptors for hyaluronate (HA) [7, 8].

Recently, CD44 isoforms have also been associated with tumour growth and metastasis [9, 10]. A role for CD44 in melanoma growth and metastasis was first suggested by Birch and associates [11], who reported that human malignant melanoma (MM) cells expressing high levels of CD44 formed significantly more lung metastases upon i.v. injections into nude mice than variants of the same cell lines that had low CD44 expression. In another animal model, it was recently shown that high level expression of CD44 isoforms

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significantly enhanced the capacity of mouse melanoma cells to form tumours in nude mice [12]. Herein we demonstrate that, *in vivo*, primary tumours of human melanoma differ in their level of CD44 surface expression, and that this is of relevance for the metastatic risk and survival of melanoma patients.

PATIENTS AND METHODS

Tumour specimen and clinicopathological classification

Paraffin-embedded tumour samples of 92 patients with primary tumours of MM (Table 1) were selected randomly from material collected at the Department of Dermatology, Freiburg, Germany. Subclassification of primary tumours into *in situ* PMM (primary tumours of MM), low metastatic risk PMM, intermediate risk PMM and high risk PMM (Table 1) was based on measurements of the vertical tumour thickness (VT) as determined by H&E histology [13]. All of these patients had been followed-up at regular intervals in our melanoma clinic for at least 5 years after excision of their primary tumours according to standardised procedures [14].

Monoclonal antibodies and staining reagents

MAb Leu44 ($mIgG_1$) specific for the N-terminal portion of CD44, common to all CD44 isoforms, was purchased from Becton Dickinson, Sunnyvale, California, U.S.A. An irrelevant IgG_1 (X63.Ag.8, mouse IgG_1) served as isotype control and was obtained from Dianova, Hamburg, Germany.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tumours were dewaxed, rehydrated and mounted on uncoated slides. To unmask CD44 epitopes, slides were boiled for 5 min in target unmasking fluid (TUF^R, Dianova, Hamburg, Germany), rinsed twice in PBS and stained according to the following protocol: (1) primary MAb (mouse IgG); (2) biotin-conjugated goat-anti-mouse IgG; (3) peroxidase conjugated streptavidin; (4) diaminobenzidine or naphthol–phosphate plus fast red (in case of heavily pigmented tumour specimen) as chromogenic substrates, and finally counterstained with haematoxylin (all reagents from DAKO, Hamburg, Germany). Stained slides were evaluated by four independent dermatopathologists in a blinded manner using a Zeiss Axioskop, equipped with an MC100 camera system. In 87/92 cases, their readings were concordant. The remaining five cases were re-evaluated by all individuals, discussed and a mutual grading was reached. The intensity of CD44 staining on melanoma cells was scored semi-quantitatively on a scale of - to + + + . To correct for staining differences between specimens, the level of CD44 expression on MM cells was compared to that on the overlying epidermis in the same section since keratinocytes are known to express CD44 (2): –: no expression on MM but staining of keratinocytes in the same section; +: MM staining is weaker than in the overlying epidermis; + + : MM staining is identical to that of the overlying epidermis; + + + : surface staining on MM is stronger than on the overlying epidermis. Only those specimens in which >80% of MM cells revealed a reactivity of +, ++ or + + + , were incorporated into the analysis and termed CD44-low (+, ++) or CD44-high (+ + +).

Statistics

The correlation between VT and CD44 levels was calculated using Fisher's exact test. Kaplan–Meier curves were calculated as previously described [15, 16] to estimate the distribution of time to recurrence, defined as time from the excision of primary tumours to the manifestation of metastases using the lifetest procedure of the SAS statistics package. 6 patients who died without metastases were treated as censored. The first of the month was supplied as date of excision or occurrence of metastases for 22 and 14 patients, respectively, because the precise dates could not be ascertained. Statistical significance was calculated using the log-rank test. Two-sided P values were used throughout.

RESULTS

Primary tumours of MM differed markedly in their level of CD44 surface expression as revealed by immunostaining of paraffin-embedded, TUF^R-treated specimens with the panCD44 MAb Leu44. For example, Figure 1a shows a low-risk primary melanoma (VT = 0.7 mm), in which the intensity of CD44 staining on the melanoma cells was much higher than on the surrounding epidermis, a staining pattern that was defined as "CD44-high". In contrast, another low risk PMM (VT = 0.4 mm) displayed a lower level of CD44 on the melanoma cells than the surrounding epidermis, and was classified as "CD44-low" (Figure 1b). Similarly, high-risk melanomas differed with respect to CD44 surface expression. This is exemplified by a high-risk PMM (VT = 1.5 mm) expressing high levels of CD44 on the melanoma cells compared to the epidermis (Figure 1c), while another high-risk PMM (VT = 4 mm) was CD44-low (Figure 1d). To

Table 1. Clinical data of PMM patients

Histological risk group*	n	Clinical classification				Clinical course		
		NMM	SSM	ALM	LMM	Survival	Metastasis	Death
In situ	6	0	6	0	0	6	0	0
Low (VT ⩽ 0.7 mm)	30	0	20	2	8	28	5	2
Intermediate (VT 0.71–1.4 mm)	32	2	23	4	3	30	3	2
High (VT>1.4 mm)	24	10	10	2	2	13	15	11
Total	92	12	59	8	13	77	23	15

* As determined by routine H&E histology.

NMM, nodular malignant melanoma; SSM, superficial spreading melanoma; ALM, acrolentiginous melanoma; LMM, lentigo malignant melanoma.

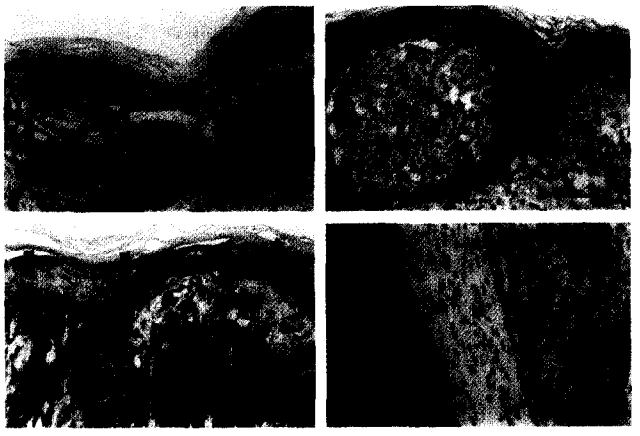

Figure 1. Level of CD44 surface expression on primary tumours of malignant melanoma: (a) CD44-high, low-risk PMM (0.7 mm): the intensity of CD44 staining (brown) on the melanoma cells, arrows, is much higher than on the surrounding keratinocytes, arrowheads (bar = 40 μm). (b) CD44-low, low-risk PMM (0.4 mm): CD44 levels (red) on the melanoma cells, arrows, is much lower than on the surrounding epidermis, arrowheads (bar = 40 μm). Fast red was used as a chromogenic substrate since the tumour was heavily pigmented. (c) CD44-high, high-risk PMM (1.5 mm): high level of CD44 (brown) on the tumour cells, arrows, compared to the epidermis, arrowheads (bar = 40 μm). (d) CD44-low, high-risk PMM (4 mm): low expression of CD44 (brown) on MM cells, arrows, compared to epidermis, arrowheads (bar = 40 μm).

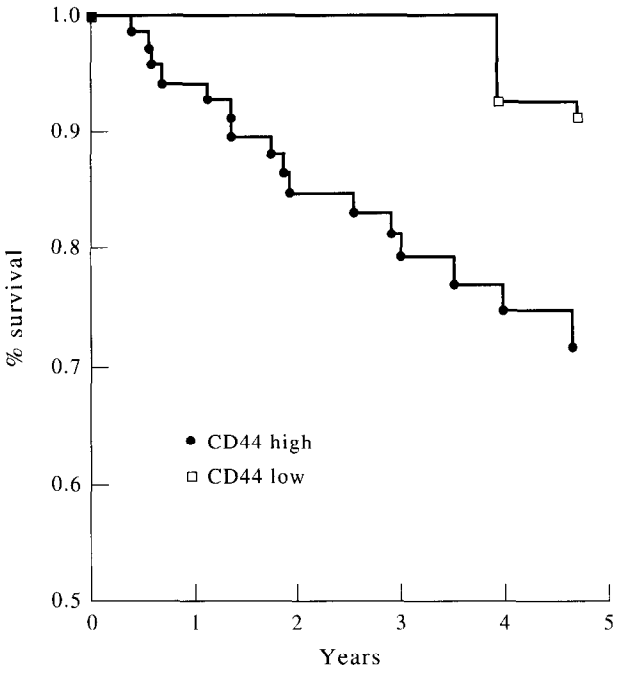

Figure 2. Five-year survival rates in CD44-high and CD44-low patients. Kaplan–Meier curves were calculated for CD44-high (-●-, $n = 54$) and CD44-low patients (-□-, $n = 38$) as detailed in Patients and Methods.

determine whether the intensity of CD44 surface expression correlates with the level of MM invasion, we compared the CD44 staining intensities and the vertical tumour thickness in all PMM: high levels of CD44 were detected in 58.3% of high-risk primary melanomas, 40.6% of intermediate-risk PMM, 36.7% of low-risk PMM and 16.7% of *in situ* PMM (Table 2).

We questioned whether the level of CD44 expression on primary tumours of MM is related to the metastatic risk and/or the risk of death from metastatic melanoma. We analysed CD44 levels on PMM of patients who developed and died of metastasis. Of the 23 patients who progressed to metastatic disease during the 5-year clinical follow-up period, 17 (74%) expressed high levels of CD44 on their primary tumour. Importantly, among these 17 were 5 patients whose metastatic risk had been estimated low, based on the histological measurement of vertical tumour thickness (VT ⩽0.7 mm). The other 6 patients (26%) who developed MM metastasis were CD44-low, with a VT>1.4 mm. Furthermore, of the 15 patients who died of disseminated melanoma, 11 (73%) expressed high levels of CD44 on their primary tumours, among these were 2 patients with a VT ⩽0.7 mm. The other 4 patients (27%) who died of metastatic MM were CD44-low, with a VT>1.4 mm.

These results raised the possibility that the level of CD44 on PMM is related to the clinical course and prognosis of malignant melanoma. To test this hypothesis, we compared the 5-year survival rates of patients with "CD44-high" and "CD44-low" PMM. Kaplan–Meier curves revealed that 5-year survival was reduced significantly ($P < 0.05$, log-rank test) in patients whose primary tumours were CD44-high compared to those who were CD44-low (Figure 2).

Table 2. Comparison of the level of CD44 surface expression and the tumour thickness in PMM

Histological risk group†	CD44 levels*			
	–	+	++	+++
In situ	0‡	0	83.3	16.7
Low	3.3	20.0	40.0	36.7
Intermediate	3.1	25.0	31.3	40.6
High	0	8.3	33.3	58.3

*–: no expression on MM, positive staining on keratinocytes in the same section; +: MM staining is weaker than in the overlying epidermis; ++: MM staining is identical to that of the overlying epidermis; +++: surface staining on MM is stronger than on the overlying epidermis. †As determined by routine H&E histology as detailed in Table 1. ‡Percentage of patients ($n = 92$).

DISCUSSION

In a retrospective study of 92 melanoma patients, we observed their primary tumours to differ substantially in their level of CD44 surface expression. Expression of high CD44 levels on PMM appears to correlate with the vertical tumour thickness of the PMM, although a simultaneous analysis of the two factors in a regression model remains to be carried out in a larger cohort. A significant portion (36.7%) of the low-risk PMM (VT ⩽ 0.7 mm) displayed the "CD44-high" phenotype. This is of importance since, as detailed below, these patients had an increased metastatic risk. We were unable to detect any correlation of CD44 levels with other clinical or histolopathological prognostic criteria such as radial or vertical growth patterns or the presence of regression zones (data not shown).

Patients with high-level CD44 expression on their PMM were at increased risk of developing metastases within the 5-year clinical follow-up period, when compared to the CD44-low patients. At present, we do not know whether high CD44 levels on PMM correlate with haematogenous

spread and/or with a preferential localisation of MM metastases to sites such as the lung. Interestingly, among the 17 CD44-high patients with MM metastases were 5 patients whose metastatic risk had been considered low based on histological measurement of the vertical tumour thickness ($VT \leqslant 0.7$ mm). We realise that the reduced survival rates of the CD44-high population is influenced by the vertical tumour thickness, since many of these patients belonged to the intermediate and high-risk groups, as determined by VT measurements. It, therefore, remains to be determined in a larger validation study, whether the level of CD44 surface expression is an independent prognostic parameter for human malignant melanoma.

We do not know whether the poor prognosis of the CD44-high population is also related to the expression of distinct CD44 isoforms encoded by one or several variant exons [5], since these could not be distinguished by the panCD44 MAb. Recently, Manten-Horst and associates [17] reported that, in a study on a limited number of melanoma patients, CD44 isoforms containing protein sequences encoded by exon v5 are related to disease progression. However, other laboratories including our own have failed to detect a correlation of CD44 variant protein expression and metastatic behaviour in human cutaneous or uveal melanoma [18–20].

The findings presented in this study are compatible with the notion that a high surface expression of panCD44 epitopes on PMM is associated with increased metastatic risk and poor prognosis. This is in accordance with previous *in vitro* studies by Birch and associates [11], who cloned from the same human MM, cell lines expressing either high or low levels of CD44 on their surface. The CD44-high variants displayed enhanced homotypic adhesion, motility and most importantly, formed lung metastases upon i.v. injections into nude mice [11]. A high expression level of CD44 has been shown to correlate with aggressiveness and poor prognosis in other human tumours such as lymphomas or bladder carcinomas [21–24]. However, we note that in contrast to our own findings, Moretti and associates and Manten-Horst and associates did not observe a modulation of CD44 surface expression during melanoma progression [17, 25]. These discrepancies could be due to differences in anti-CD44 MAb employed or in the techniques of tissue fixation, antigen unmasking or scoring of the specimen. Further studies using additional antibodies as well as antibodies against CD44 splice variants should settle this matter. These studies should also address the question of whether or not CD44 expression is an independent prognostic parameter.

At present, we can only speculate on the mechanisms by which a high CD44 surface expression enhances the metastatic potential of melanoma cells. Certain isoforms of CD44, particularly those containing no variably spliced exons, are principal receptors for hyaluronate (HA) [7, 8]. HA has been observed at sites of tumour invasion, and several studies have demonstrated HA to be associated with tumour aggressiveness [24, 26]. Since the dermis is especially rich in HA [7], high surface expression of CD44 may enhance the capacity of MM cells for dermal penetration, intradermal growth and early metastasis. Indeed, the cutaneous growth of the B16 mouse melanoma was directly related to their ability to bind HA via CD44 [12], and the capacity of murine lymphomas to metastasise into peripheral lymph nodes has recently been shown to be CD44- and HA-dependent [27]. Furthermore, the CD44-high, metastatic MM cell variants described by Birch and associates [11] displayed increased HA binding. However, in a later study examining a panel of human and murine MM cell lines, the same investigators linked the HA avidity to the activation state of CD44 rather than to its level of surface expression [20]. It is also possible that CD44-high MM cells possess an increased motility as suggested by *in vitro* studies demonstrating that CD44-high MM have an augmented capacity to migrate on HA-coated surfaces [28].

We conclude that in our patients, a high surface expression of CD44 on primary tumours of malignant melanoma is associated with an increased metastatic risk and a reduced 5-year survival rate. This finding may be of particular prognostic relevance for those melanoma patients whose metastatic risk is considered low based on the measurement of vertical tumour thickness by routine histology.

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