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Association of High Molecular Weight Melanoma-associated Antigen Expression in Primary Acral Lentiginous Melanoma Lesions with Poor Prognosis[1]

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ABSTRACT

In a recent study we detected marked differences in the antigenic profile of acral lentiginous melanoma (ALM) and nodular melanoma lesions. Furthermore, we showed that the human high molecular weight melanoma-associated antigen (HMW-MAA) is expressed with a significantly higher frequency in metastatic than in primary ALM lesions. Because of the potential role of HMW-MAA in the metastatic process of melanoma cells, in the present investigation we tested whether HMW-MAA represents a useful prognostic marker in ALM. Primary ALM lesions removed from 32 patients were stained with anti-HMW-MAA monoclonal antibody (mAb) in an immunoperoxidase reaction. The results were correlated with the expression of other markers defined by mAb, with clinical parameters of the disease, and with histopathological characteristics of the lesions. Only 9 of the 32 primary ALM lesions tested were stained by anti-HMW-MAA mAb. Expression of HMW-MAA was the only variable associated with patients' survival and disease-free survival. Both were significantly shorter in patients with HMW-MAA expression in their primary lesions. These results suggest that HMW-MAA may represent a novel prognostic marker in ALM, since phenotyping of primary ALM lesions with anti-HMW-MAA mAb may provide information about the prognosis of the disease which cannot be obtained with known prognostic parameters.

INTRODUCTION

Until 1975, the clinicopathological classification of cutaneous malignant melanoma included three main types: superficial spreading melanoma, lentigo maligna melanoma, and NM.[3] In 1975, another type, designated ALM, was introduced by Clark *et al.* (1) to describe a variant of melanoma characterized by palmar and plantar distribution. ALM differs from NM in a number of characteristics, which include incidence in different ethnic groups, clinical course of the disease, and histopathological characteristics of lesions, in addition to the site of anatomic localization (2–4). Recently, we found that primary and metastatic ALM lesions differ from their NM counterparts in the expression of melanoma-associated antigens defined by mouse mAb (5). Furthermore, at variance with previous investigations in which no difference in the distribution of HMW-MAA was detected in primary and metastatic NM lesions (6), we unexpectedly found that the HMW-MAA is expressed with a significantly lower frequency in primary than in metastatic ALM lesions (5). This finding in conjunction with the potential role of HMW-MAA in the metastatic process of melanoma cells (7–9) suggests that HMW-MAA may play an important role in the biology of ALM cells and in the clinical course of the disease.

To determine the prognostic significance of HMW-MAA expression in ALM, in the present investigation we correlated the expression of HMW-MAA in primary lesions from 32 patients with ALM with clinical parameters and histopathological characteristics of the lesions. For comparison purposes, we also tested the expression in primary ALM lesions of other markers which have been described as progression markers in melanoma, *i.e.*, HLA-DR antigens (10) and transferrin receptor (11), and a marker which does not correlate with the clinical course of the disease, *i.e.*, T4 tyrosinase (5).

MATERIALS AND METHODS

Melanoma Lesions. Primary ALM lesions were obtained from racially Japanese patients who underwent surgery in the Departments of Dermatology at Kumamoto University Medical School, Kumamoto, Japan, and at Sapporo Medical College, Sapporo, Japan. Four lesions were removed from nail beds, two from palms, and 26 from soles. The diagnosis of ALM was based on the anatomic site of lesions and on clinical and histopathological characteristics. Tissues were processed within 15 min following surgical removal. Each tumor tissue was divided into two parts. One half was fixed in 10% buffered formaldehyde and processed for routine histopathology. The other half of the specimen was snap frozen in liquid nitrogen and stored at −80°C until use. Four-μm-thick cryostat sections were dried and fixed in absolute acetone for 1 min. Under these fixation conditions, cryostat sections could be stored for at least 3 months at −20°C without loss of reactivity with anti-melanoma-associated antigen mAb.

mAb and Conventional Antisera. mAb 225.28, TP41.2, and TP61.5 to distinct determinants of HMW-MAA, anti-HLA-DR mAb CL413, anti-transferrin receptor mAb PAL-M1, and anti-T4 tyrosinase mAb TMH-1 were developed and characterized as described previously (6, 11–14). All of the mAb, but mAb TMH-1, are of mouse origin. mAb TMH-1 is of rat origin. The reactivity of the mAb preparations used in the present investigation was monitored by testing with known positive tissue substrates.

The Vectastain ABC Kit and biotinylated anti-rat IgG xenoantibodies were purchased from Vector Laboratories, Burlingame, CA.

Histopathological Analysis of Lesions. The presence of epithelioid and spindle-type melanoma cells, the degree of lymphocyte infiltrate, and the melanin content in melanoma cells were evaluated using hematoxylin-eosin staining. The percentage of melanoma cells with epithelioid and spindle characteristics was determined by evaluating the number of nests of cells with spindle characteristics in one whole section of each lesion. The degree of lymphocyte infiltrate was scored as − when no lymphocyte was detected, ± when the lymphocyte infiltrate was scarce, + when the lymphocyte infiltrate was unifocal, ++ when the lymphocyte infiltrate was multifocal, and +++ when the lymphocyte infiltrate was extensive. Melanin content was scored as − when melanin was not detected, ± when the content was scarce, + when the content was low, and ++ when the content was high.

Indirect Immunoperoxidase Staining. This assay was performed utilizing the Vectastain ABC Kit according to the instructions of the manufacturer. The procedure has been described in detail elsewhere (5). A lesion was classified as negative when no staining of melanoma cells was detected. The percentage of stained melanoma cells in each section and the staining intensity were estimated independently by two investigators. Variations in the percentage of stained cells enumerated by the two investigators were 10% or less. The average percentage was calculated and used as a result; each value has been rounded off to the nearest multiple of 10. The staining intensity was graded − when no staining was detected, ± when the staining was faint or barely

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[3] The abbreviations used are: NM, nodular melanoma; ALM, acral lentiginous melanoma; HMW-MAA, high molecular weight melanoma-associated antigen; MAA, melanoma-associated antigen; mAb, monoclonal antibody(ies); PBS, phosphate-buffered saline.

detectable, + when the staining was homogeneous, and ++ when the staining was strong and homogeneous. Cells graded ± were included in the positive cells in the calculation of the percentage of stained cells.

Statistical Analysis. Fisher's exact test was used to assess the association of an individual factor with negativity or positivity of staining with anti-HMW-MAA mAb 225.28. In both survival and disease-free survival analyses, the traditional Kaplan-Meier actuarial method and the log rank test were not used because of the small number of patients in the study. Instead, a much more sensitive fully parametric survival method was applied to the data. Specifically, since both the survival and disease-free survival times were empirically verified to closely follow an exponential distribution, the exponential survival regression method was used in both the univariate and multivariate analyses of the survival times. The method of maximum likelihood was used to estimate the exponential parameters, and likelihood ratio tests were used to assess both univariate and multivariate significance of prognostic factors (15).

RESULTS

Primary ALM lesions were obtained from 18 male and 14 female patients with the average age of 64 and 74 years (range, 38–91 and 40–87 years), respectively. Five patients had stage 1 melanoma, 7 stage 2, 16 stage 3, and 4 stage 4. The classification of the patients according to the tumor-node-metastasis system (16) is shown in Table 1. The median survival of the 32 patients was 49.3 months (range, 25.7–94.7 months). The median survival of the 28 disease-free patients was 39.6 months (range, 20.6–76.1 months).

The mean thickness of the 32 primary lesions tested was 4.3 mm (range, 0.3–12.0 mm). The level of thickness was II in 2 lesions, III in 7, IV in 14, and V in 9. The lymphocyte infiltrate was – in 5 lesions, ± in 11, + in 14, and ++ in 2. The melanin content was – in 2 lesions, ± in 2, + in 24, and ++ in 4. Spindle cells were detected in 13 lesions; the percentage was 36.2 ± 19.2 (mean ± SD) with a range of 10–50.

The 32 primary ALM lesions were stained in the immunoperoxidase reaction with anti-HMW-MAA mAb 225.28, anti-HLA-DR mAb CL413, anti-transferrin receptor mAb PAL-M1, and anti-T4 tyrosinase mAb TMH-1. Representative staining patterns are shown in Fig. 1. The following points are noteworthy. (*a*) Only 9 lesions were stained by anti-HMW-MAA mAb 225.28l; in 7 lesions the percentage of stained melanoma cells was at least 80. The percentage of stained melanoma cells was 75.6 ± 18.8 (mean ± SD) in the stained lesions. This mean and SD are higher and lower, respectively, than those reported in the previous publication (5), since in the latter these parameters were calculated utilizing also the lesions not stained by anti-HMW-MAA mAb. (*b*) Previous studies have shown a differential expression of distinct determinants of HMW-MAA in melanoma lesions (17, 18). To exclude the possibility that the different staining patterns obtained with the 32 ALM lesions tested reflected the differential expression of the determinant recognized by anti-HMW-MAA

Table 1 *Clinical characteristics of the ALM patients investigated*

Stage	TNM[a] classification	No.	Disease-free survival	Survival
I	$pT_1N_0M_0$	2[b]	41, 2[c]	41, 2
	$pT_2N_0M_0$	3	17, 17, 16	17, 17, 16
II	$pT_{3a}N_0M_0$	6	31, 48, 36, 24, 6, 3	31, 48, 36, 24, 6, 16
	$pT_{3b}N_0M_0$	1	20	21
III	$pT_{4a}N_0M_0$	9	32, 17, 22, 5, 3, 11, 2, 8, 8	32, 32, 22, 5, 8, 11, 3, 18, 15
	$pT_{4b}N_0M_0$	1	21	21
	$pT_1N_1M_0$	1	7	33
	$pT_{3a}N_1M_0$	1	32	32
	$pT_{4a}N_1M_0$	3	60, 12, 7	60, 12, 16
	$pT_{4a}N_{2a}M_0$	1	6	6
IV	$pT_{3b}N_1M_1$	1		14
	$pT_{3b}N4_{M_1}$	1		2
	$pT_{4a}N_1M_1$	2		12, 11

[a] T, tumor; N, node; M, metastasis.
[b] Number of patients.
[c] Number of months.

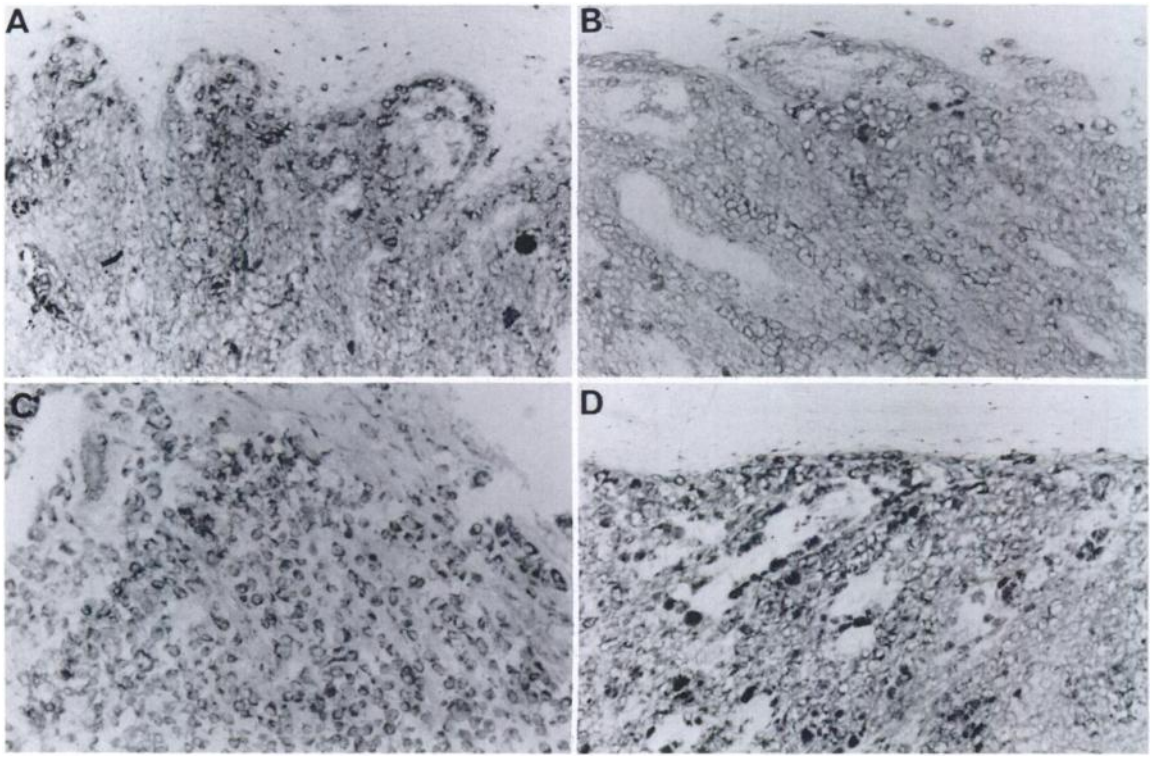

Fig. 1. Immunoperoxidase staining of 4-μm cryostat sections of an ALM primary lesion with anti-HMW-MAA mAb 225.28 (*A*), anti-HLA-DR mAb CL413 (*B*), anti-transferrin receptor mAb PAL-M1 (*C*), and anti-T4 tyrosinase mAb TMH-1 (*D*). × 100.

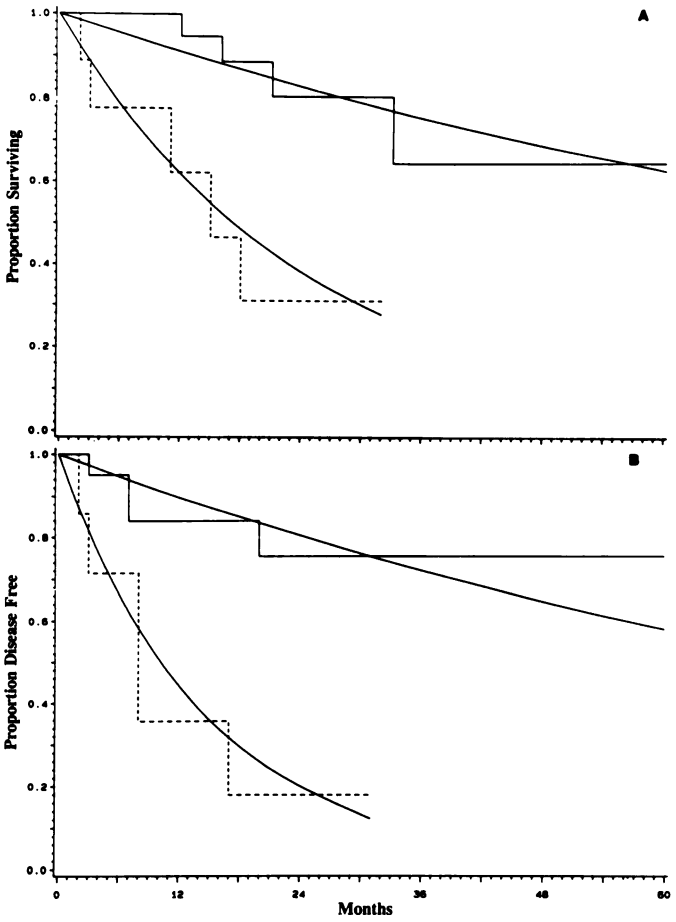

Fig. 2. Association between HMW-MAA expression in primary ALM lesions and patients' survival and disease-free survival. Survival of the 23 patients without detectable HMW-MAA expression in their primary lesions was significantly ($P = 0.02$) longer than that of the 9 patients with HMW-MAA expression in their primary lesions (*A*). Disease-free survival of the 22 patients without detectable HMW-MAA expression in their primary lesions was significantly ($P = 0.003$) longer than that of the 6 patients with HMW-MAA expression in their primary lesions (*B*).

Table 2 *Effect of HMW-MAA expression in primary ALM lesions on the estimated survival and disease-free survival*

	Survival		Disease-free survival	
Time (yr)	HMW-MAA −	HMW-MAA +	HMW-MAA −	HMW-MAA +
1	91.1 (78.6, 96.6)[a]	61.9 (35.0, 83.0)	89.7 (75.5, 96.1)	44.4 (18.2, 74.2)
2	83.0 (62.5, 93.5)	38.3 (13.7, 70.8)	80.4 (57.9, 92.4)	19.8 (4.0, 59.1)

[a] Percentage of patients with 95% confidence interval in parentheses.

mAb 225.28 and not of HMW-MAA molecules, ALM lesions were stained also with anti-HMW-MAA mAb TP41.2 and TP61.5, which recognize distinct and spatially distant determinants from that defined by mAb 225.28 (12, 13). The staining pattern obtained with the three anti-HMW-MAA mAb was very similar. Furthermore, incubation of ALM lesions with a pool of the three anti-HMW-MAA mAb enhanced the intensity of staining but did not change the percentage of stained melanoma cells in each lesion. (*c*) Twenty-three ALM lesions were stained by anti-HLA-DR mAb CL413. The percentage of stained melanoma cells was 45.6 ± 24.1 (mean ± SD) in the stained lesions. (*d*) Nineteen lesions were stained by anti-transferrin receptor mAb PAL-M1. The percentage of stained melanoma cells was 56.8 ± 18.3 (mean ± SD) in the stained lesions. (*e*) Thirty lesions were stained by anti-T4 tyrosinase mAb TMH-1. The percentage of stained melanoma cells was 63.0 ± 31.4 (mean ± SD) in the stained lesions.

HMW-MAA expression in ALM lesions was significantly associated only with patients' age and with disease stage. HMW-MAA was detected in only 3 (17%) of the 18 patients younger than 70 years, but it was detected in 6 (43%) of the 14 patients 70 years and older ($P = 0.02$ by Fisher's exact test). Furthermore, HMW-MAA was detected in only 1 (8%) of the 12 patients with melanoma in stage 1 or 2, but it was detected in 8 (40%) of the 20 patients with melanoma in stage 3 or 4 ($P = 0.006$ by Fisher's exact test).

Both the univariate and multivariate exponential survival analyses showed that patients' sex and age, stage of disease, thickness of lesion, percentage of epithelial type cells and lymphocyte infiltrate in lesions, and HLA-DR antigen and transferrin receptor expression were not associated with either survival or disease-free survival. In contrast, HMW-MAA expression was significantly associated with survival, measured as time between surgical removal of primary lesion and death ($P = 0.02$ by likelihood ratio test), and with disease-free survival, measured as time between surgical removal of primary lesion and detection of metastasis or local recurrence ($P = 0.003$ by likelihood ratio test). Median survival and median disease-free survival were not reached in the patients without detectable HMW-MAA expression in their primary lesions but were only 17.3 months (95% confidence interval from 7.2–41.7 months) and 10.3 months (95% confidence interval from 4.3–24.7 months), respectively, in the patients with HMW-MAA expression in their primary lesions (Fig. 2). In Table 2, comparisons of the estimated percentages and 95% confidence interval of surviving patients and of disease-free patients at 1 and 2 years from the diagnosis of the disease in the groups of patients with and without HMW-MAA expression in their primary lesions are given.

DISCUSSION

Immunohistochemical staining with mAb has detected HMW-MAA in about 30% of primary ALM lesions and has shown that HMW-MAA expression in primary ALM lesions is significantly correlated with clinical parameters of the disease. HMW-MAA expression is directly correlated with the stage of the disease and inversely correlated with patients' survival and disease-free survival. This conclusion has to be interpreted with caution, since the practical difficulties of obtaining frozen primary ALM lesions from patients with known clinical courses of the disease have restricted our investigations to a limited number of patients. Furthermore the lack of reactivity with formalin-fixed, paraffin-embedded tissues of the anti-HMW-MAA mAb available to us has not allowed us to perform retrospective studies utilizing collections of fixed ALM lesions stored in departments of dermatology and pathology. In spite of these limitations, we are reporting our data, since the described usefulness of HMW-MAA as a prognostic marker in ALM is expected to prompt other investigators to assess the validity of our conclusions, thus facilitating the analysis of a large number of patients with ALM.

If not fortuitous, the association between HMW-MAA expression in primary ALM lesions and poor clinical course of disease may reflect the aggressive characteristics of undifferentiated or poorly differentiated melanoma cells which express HMW-MAA. The latter possibility is supported by the classification of HMW-MAA as an early differentiation marker in cells of the melanocyte lineage (19) and by its expression by melanocytes in fetal skin but not by melanocytes in adult skin (20). An alternative, but not exclusive, mechanism is suggested by the following *in vivo* and *in vitro* lines of evidence which argue in favor of a role of HMW-MAA in the metastatic process of melanoma cells. HMW-MAA has a significantly higher expression in metastatic tissue than in primary ALM lesions (5). Furthermore, anti-HMW-MAA antibodies markedly reduce the functional properties of

melanoma cells in *in vitro* assays which are believed to correlate with their *in vivo* metastasizing potential (8, 9). Whatever the mechanism(s) underlying the association of HMW-MAA expression in primary ALM lesions with patients' disease-free survival and survival, the present results suggest that phenotyping of primary ALM lesions with anti-HMW-MAA mAb may provide information about the prognosis of the disease, which cannot be obtained utilizing known prognostic parameters. Therefore, HMW-MAA may represent a novel prognostic marker in ALM.

The all or none staining pattern of ALM lesions with anti-HMW-MAA mAb subdivides them into two subsets. Whether the two subsets are derived from melanocytes at different stages of differentiation with differential ability to express HMW-MAA or whether during the malignant transformation one type of melanoma cell loses its ability to synthesize, transport, and/or express HMW-MAA remains to be determined. This information may suggest therapeutic approaches to ALM which aim at inhibiting HMW-MAA expression by melanoma cells.

HLA-DR antigen and transferrin receptor expression in primary ALM lesions is not correlated with the prognosis of the disease. This finding is at variance with the previously reported correlation between HLA-DR antigen (10) and transferrin receptor (11) expression in NM and poor prognosis of the disease which had been mainly found in patients with NM. If not caused by technical reasons such as specificity of mAb, sensitivity of immunohistochemical techniques used, and/or number of patients investigated, these conflicting results may reflect the different role played by HLA-DR antigens and by transferrin receptor in the biology of ALM and NM cells.

The surprising finding of the lack of correlation in both univariate and multivariate analyses between prognosis and two well-known risk factors, *i.e.*, lesion thickness and stage of disease, deserves comment. In the univariate analysis, the lack of prognostic significance of lesion thickness may reflect the preponderance of patients with lesions in the highest thickness range, where survival is less strongly associated with increase in lesion thickness (21). As far as disease stage is concerned, the unusual finding of 17 patients with lesions in the highest thickness range with negative lymph nodes biases the prognostic significance of lymph node involvement in favor of no difference. In the multivariate analysis, the lack of effect of lesion thickness and disease stage may reflect the major role played by HMW-MAA in the prognosis of the disease. If studies by other investigators of a large number of patients with ALM confirm our findings, the routine application of HMW-MAA in the immunopathological characterization of ALM lesions will greatly benefit from the development of anti-HMW-MAA antibodies which react with formalin-fixed, paraffin-embedded tissues.

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