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Cancer–testis antigen expression in primary cutaneous melanoma has independent prognostic value comparable to that of Breslow thickness, ulceration and mitotic rate ☆

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ABSTRACT

To determine the effect of Cancer–Testis Antigen (CTAg) expression on the natural history of primary cutaneous melanoma we compared its impact on prognosis with that of known prognostic factors and its relationship with other clinicopathologic characteristics.

The immunohistochemical expression of three CTAgs (MAGE-A1, MAGE-A4 and NY-ESO-1) in 348 cases of stage I and stage II primary cutaneous melanoma was analysed and correlated with clinicopathologic characteristics, relapse free survival (RFS) and overall survival (OS). A Cox proportional hazards regression model was used to analyse factors which independently predicted RFS.

All three CTAgs were significantly co-expressed with each other ($P < 0.001$). The median RFS for patients with CTAg-negative tumours and CTAg-positive tumours was 72 months and 45 months, respectively, ($P = 0.008$). Univariate analysis demonstrated that the impact of CTAg expression on RFS was comparable in magnitude to that of Breslow thickness, ulceration and tumour mitotic rate. Multivariate Cox regression analysis indicated that CTAg expression was a powerful independent predictor of RFS (risk ratio (RR) = 1.715, 95% confidence interval (CI) = 0.430–0.902, $P = 0.010$). In contrast, CTAg expression was demonstrated to have no prognostic impact on overall survival.

This study demonstrates CTAg expression in primary cutaneous melanoma is a strong independent predictor of RFS and it is comparable to other known important prognostic factors. CTAg expression has no relationship with overall survival, suggesting anti-melanoma immunity directed towards CTAg expression may contribute to the natural history of the disease. In view of these results, further investigation of the function of CTAgs and their potential use in therapeutic targeting is warranted.

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1. Introduction

Cutaneous melanoma is a major and increasing public health issue worldwide and its incidence continues to rise in individuals of European origin.[1] In the United States it is predicted to be the fifth and sixth most common cancer in men and women, respectively, in 2009.[2] Wide local excision of the primary tumour is currently the standard treatment for cutaneous melanoma[3] and is successful particularly for patients with thin tumours.[4] In many melanoma treatment centres, sentinel lymph node biopsy is offered to patients with melanomas >1 mm thick or to those with melanomas <1 mm thick if other adverse prognostic factors are present. Surgery is also considered the mainstay for treating melanoma that has metastasised to regional lymph nodes.[5] For patients with widespread metastatic disease, the outcome is poor,[4] single agent or combination chemotherapy has little efficacy[6] and at present treatment is directed at palliation of symptoms rather than curing the tumour.[7]

With such limited therapeutic options for advanced metastatic disease, interest in vaccine development is high. Antigens that are relatively specific for cancer, known as cancer–testis antigens (CTAgs), have been demonstrated to serve as targets through their presentation on HLA molecules for immune recognition by cytotoxic T lymphocytes (CTLs).[8] CTAgs are expressed by many different tumours as well as by spermatogonia and trophoblast but not by other normal somatic cells.[9] Previous work involving CTAgs has focused on assessment of the expression patterns of these antigens in cancers[10–12] as well as the development, use and immune response monitoring of cancer vaccines in the clinical setting.[13] However, very little is currently known about the correlation of CTAg expression with clinical data such as known prognostic factors and survival in melanoma patients. Most previous studies are disadvantaged by the small number of tumours studied and/or the limited numbers of antigens tested.[12,14]

This study assessed CTAg expression in a large series of primary cutaneous melanomas and correlated the expression of these antigens with other clinico-pathologic features, relapse free survival (RFS) and overall survival (OS).

2. Materials and methods

2.1. Patients and clinical specimens

Archival formalin-fixed, paraffin-embedded tissue blocks of stage I or stage II primary cutaneous melanomas from patients treated at the Austin Health Melanoma Clinic (Melbourne, Australia) or the Melanoma Institute Australia (MIA) (Sydney, Australia) were retrieved. Sections were cut and stained with hematoxylin–eosin to confirm the diagnosis of primary melanoma and then immunohistochemistry was performed as described below. All protocols were approved by the Austin Health Human Research Ethics Committee.

2.2. Antibodies

Monoclonal antibodies to MAGE-A1[15] and NY-ESO-1[16] were produced by the Biological Production Facility at the Ludwig Institute for Cancer Research (Melbourne, Australia). MA454 (MAGE-A1) was diluted 1:50 for staining. E978 (NY-ESO-1)[17] was used at 3 μg/ml. The monoclonal antibody supernatant 57B (Dr. G. Spagnoli, Surgical Research Centre, Basel, Switzerland) was diluted 1:100 for staining. Monoclonal antibody 57B was raised against MAGE-A3. 57B was originally reported to detect only the MAGE-A3 protein[15] however it was later observed to recognise other CTAgs.[17] In paraffin-embedded tissue sections, 57B has been shown to recognise MAGE-A4 predominantly regardless of the expression of other MAGE genes[18] and therefore for this study it was reported as such.

2.3. Immunohistochemistry

Four-micron thick sections of formalin-fixed paraffin-embedded tissue were cut and then dried at 37 °C overnight. Slides were dewaxed in xylene and rehydrated through alcohols. Water bath antigen retrieval was performed for 30 min using EDTA buffer pH 8.0 (NeoMarkers, Fremont, CA) for MA454 and E978 or citrate buffer pH 6.0 (NeoMarkers, Fremont, CA) for 57B staining. Antibody binding and counterstaining was performed as previously described.[16] The chromogen used was 3-amino-9-ethyl-carbazole (Sigma–Aldrich, St. Louise, MO) and the application of Crystal Mount (Biomeda, Foster City, CA) preceded dehydration and mounting in DePeX (BDH 36125). Known antigen-positive tumours were used as positive controls. Negative substitution controls included replacing the primary antibody with the antibody diluent solution and an IgG matched control antibody.

2.4. Interpretation

Tumour cell CTAg expression was estimated microscopically by two pathologists (D.M. and S.D.) and Ludwig Institute for Cancer Research clinical investigators (I.D.D. and J.S.C.). Slides were scored by eye as a percentage of tumour cells staining for each antigen and assigned to one of two groups: positive (any cells staining) and negative (no staining).

2.5. Human sera

Human sera were obtained from 313 patients treated at the Austin Health Melanoma Clinic (Melbourne, Australia) under informed consent and approved by the Austin Health Human Research Ethics Committee. Sera was assigned to one of four groups, AJCC classified stage I, II, III or IV. All sera were collected prior to surgery for the relevant stage of disease.

2.6. ELISA to detect serum NY-ESO-1 autoantibodies

Maxisorp 96-well plates (Nunc, Roskilde, Denmark) were coated with 0.05 μg/well recombinant NY-ESO-1 (Dr. Roger Murphy, Ludwig Institute for Cancer Research, Melbourne, Australia), in phosphate buffered saline (PBS) and incubated

overnight at 4 °C. Plates were washed three times with 0.2% Tween-20 in PBS (the same for all subsequent washes) and blocked for 1 h at room temperature (5% powered skin milk in PBS with 0.05% azide). Plates were washed and incubated with patient and control serum diluted 1/1000 in blocking buffer at room temperature for 2 h. Plates were washed and incubated with goat anti-human IgG alkaline phosphatase-conjugated antibody (Chemicon International, Temecula, CA) at 1:1000 in blocking buffer for 1 h at room temperature. Plates were developed with the AttoPhos AP Fluorescent substrate system (Promega, Madison, USA) for 30 min in the dark at room temperature, the reaction was stopped by the addition of [3N] NaOH and colour development was measured at $A_{450\ nm}$.

2.7. Statistical analysis

CTAg expression and clinicopathologic variables were analysed by the Pearson's Chi-square test. The threshold for statistical significance was a *P*-value less than 0.05. Survival was reported as either the number of months from initial diagnosis to first relapse or the number of months from initial diagnosis to death. Survival curves were generated using the Kaplan–Meier method and survival distributions were compared with log-rank statistics. Patients who were lost to follow-up over 5 years were treated as censored. The independent prognostic value of variables was analysed using the multivariate Cox proportional hazards model. This multivariate analysis was performed using parameters that were statistically significant in the univariate analysis. CTAg expression status and other parameters with potential prognostic significance were tested by univariate analysis. Multivariate analysis was subsequently performed to identify the most powerful independent predictors of survival. Statistical analyses were conducted using GraphPad Prism 4.03 (La Jolla, CA, USA) and MedCalc (Mariakerke, Belgium) statistical software.

3. Results

3.1. Patients

In total, 348 primary stage I and II cutaneous melanomas were collected and typed for CTAg expression. These included 271 melanoma patients treated at the Austin Health Melanoma Clinic between 1994 and 2008, and 77 patients treated at the

Table 1 – Relationship between clinicopathologic parameters and CTAg expression in cutaneous primary melanoma.

Parameter	Subgroup	Total *n*=	MAGE-A1 positive			MAGE-A4 positive			NY-ESO-1 positive		
			n=	%	*P* value	*n*=	%	*P* value	*n*=	%	*P* value
Age at diagnosis	<56 years	165	42	(26)	Not significant	20	(12)	0.038	61	(35)	Not significant
	>55 years	156	54	(35)		38	(24)		59	(40)	
Patient gender	Male	193	54	(28)	Not significant	27	(14)	Not significant	69	(35)	Not significant
	Female	128	42	(33)		31	(24)		51	(40)	
AJCC clinical staging	I	125	30	(24)	Not significant	18	(14)	Not significant	38	(30)	Not significant
	II	196	66	(34)		40	(20)		82	(42)	
Tumour site	Trunk	122	35	(29)	Not significant	17	(14)	Not significant	42	(33)	Not significant
	Extremity	22	4	(19)		1	(5)		8	(38)	
	Limb	118	36	(31)		27	(23)		50	(43)	
	Head and Neck	59	21	(36)		13	(22)		20	(34)	
Tumour histology	Nodular	105	34	(32)	Not significant	22	(21)	Not significant	40	(37)	Not significant
	Superficial Spreading	172	51	(30)		30	(17)		67	(39)	
	Other[a]	44	11	(25)		6	(14)		13	(34)	
Tumour ulceration	Absent	205	51	(25)	Not significant	33	(16)	Not significant	70	(34)	Not significant
	Present	116	45	(39)		25	(22)		50	(43)	
Tumour thickness	<1.0 mm	54	13	(24)	Not significant	7	(13)	Not significant	16	(29)	Not significant
	1.1-4.0 mm	207	58	(28)		34	(16)		80	(38)	
	>4.0 mm	60	25	(42)		17	(28)		24	(41)	
Tumour infiltrating Lymphocytes (TILs)	Brisk	11	3	(27)	Not significant	3	(27)	Not significant	4	(36)	Not significant
	Non-brisk	74	29	(39)		15	(20)		29	(39)	
	Absent	109	31	(28)		18	(17)		44	(39)	
Tumour mitotic rate	0 mm^2	49	13	(27)	Not significant	5	(10)	Not significant	14	(29)	Not significant
	$\geqslant 1\ mm^2$	272	83	(31)		53	(19)		106	(39)	
MAGE-A1 expression	Negative	225	0	(0)	<0.001	13	(6)	<0.001	62	(27)	<0.001
	Positive	96	96	(100)		45	(47)		58	(61)	
MAGE-A4 expression	Negative	263	51	(19)	<0.001	0	(0)	<0.001	83	(31)	<0.001
	Positive	58	45	(78)		58	(100)		37	(65)	
NY-ESO-1 expression	Negative	201	38	(19)	<0.001	21	(11)	<0.001	0	(0)	<0.001
	Positive	120	58	(48)		37	(31)		120	(100)	
Any CT antigen	Negative	156	0	(0)	<0.001	0	(0)	<0.001	0	(0)	<0.001
	Positive	165	96	(58)		58	(37)		120	(73)	

[a] Other: lentigo maligna, acral lentiginous, mixed.

MIA between 1992 and 2004. Of these, 250 Austin tumours and 71 MIA tumours had complete clinical data and were subsequently included in the clinicopathologic/CTAg analysis (Table 1). Only stage II tumours were analysed for RFS (Fig. 2 and Table 2) and OS (Fig. 4). An additional 21 Austin and 6 MIA stage II tumours were also included in Figs. 2 and 4 and Table 2 as they had sufficient clinical data for univariate survival analysis but not enough for clinicopathologic/CTAg analysis. For both the Austin and MIA cohorts, 36% of the melanomas were stage I and 64% were stage II.

Of the 223 stage II patients used in the univariate survival analysis, the median follow-up RFS times for Austin and MIA patients were 20 months (range 1–166) and 40 months (range 2–112), respectively. The overall number of patients from both cohorts who relapsed was 105 (47%) of which 94 (54%) were from the Austin Health group and 11 (23%) were from the MIA group. To detect any result bias due to different collection procedures we analysed both tumour cohorts independently as well as collectively. With regards to CTAg expression the results from both cohorts were almost identical (see below). In addition with respect to RFS we also internally validated our tumour cohorts by examining known prognostic factors.

3.2. Immunohistochemistry

MAGE-A1, MAGE-A4 and NY-ESO-1 expression was assayed by immunohistochemical staining using antibodies MA454, 57B and E978, respectively. Representative examples of staining with each antibody are illustrated in Fig. 1. The distribution of expression of each antigen varied from region to region within individual tumours.

3.3. Association of CTAg expression quantitation with stage of disease

The intensity of expression and percentage of cells positive for MAGE-A1, MAGE-A4 and NY-ESO-1 for each stage of melanoma within the sample cohort has been previously reported by our group.[11] This study clearly demonstrated that CTAg expression was acquired and expression increased with advancing melanoma stage.

Table 2 – Univariate survival analysis for relapse free survival (RFS) in stage II melanoma patients.

Parameter	Subgroup	Median RFS (months)	Total *n*=	(%)	Risk ratio (RR)	95% confidence limits (95% CI)	*P* value
MAGE-A1 expression	Positive	45	73	(33)	0.626	0.375–0.911	0.018
	Negative	70	150	(67)			
MAGE-A4 expression	Positive	62	44	(20)	0.861	0.501–1.445	ns
	Negative	51	179	(80)			
NY-ESO-1 expression	Positive	45	89	(40)	0.664	0.430–0.962	0.032
	Negative	70	134	(60)			
MAGE-A1 and/or MAGE-A4 expression	Positive	45	80	(36)	0.667	0.412–0.975	0.038
	Negative	67	143	(64)			
MAGE-A1 and/or NY-ESO-1 expression	Positive	45	120	(54)	0.604	0.401–0.878	0.009
	Negative	72	103	(46)			
MAGE-A4 and/or NY-ESO-1 expression	Positive	45	107	(48)	0.620	0.405–0.893	0.012
	Negative	72	116	(52)			
MAGE-A1, MAGE-A4 and/or NY-ESO-1 expression	Positive	45	123	(55)	0.600	0.398–0.872	0.008
	Negative	72	100	(45)			
Tumour ulceration	Present	40	132	(59)	0.497	0.346–0.754	<0.001
	Absent	87	91	(41)			
Tumour thickness	<4.01 mm	72	154	(69)	0.482	0.257–0.649	<0.001
	>4.00 mm	29	69	(31)			
AJCC clinical staging	IIA	86	105	(47)	Not applicable	Not applicable	<0.001
	IIB	48	72	(32)			
	IIC	28	46	(21)			
Tumour infiltrating lymphocytes	Brisk	27	7	(5)	Not applicable	Not applicable	ns
	Non-brisk	42	52	(38)			
	Absent	30	78	(57)			
Tumour mitotic rate	0 mm^2	90	21	(9)	0.426	0.294–0.952	0.034
	$\geqslant 1$ mm^2	49	202	(91)			
Patient age at diagnosis	>56 years	48	94	(42)	1.404	0.962–2.113	ns
	>55 years	62	129	(58)			
Patient gender	Male	59	145	(65)	0.932	0.617–1.400	ns
	Female	49	78	(35)			

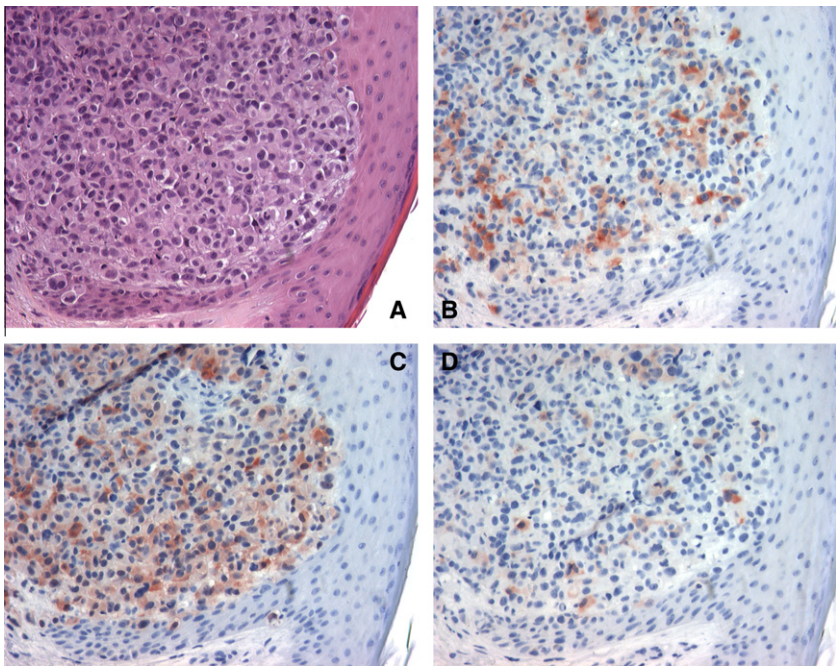

Fig. 1 – Immunohistochemistry staining of primary melanoma from the same field of the one tumour. (A) H & E stain, (B) MAGE-A1, (C) MAGE-A4 and (D) NY-ESO-1.

3.4. *Association of CTAg expression with clinicopathologic characteristics*

Analyses were performed to identify any association between CTAg expression and the following clinicopathologic parameters: patient age at diagnosis, patient gender, AJCC clinical stage, tumour anatomic site, melanoma histologic subtype, ulceration, Breslow thickness, tumour infiltrating lymphocytes (TILs) and tumour mitotic rate (TMR) (Table 1). CTAg tumour expression for MAGE-A1, MAGE-A4 and NY-ESO-1 was associated with increasing age at diagnosis, female gender, increasing AJCC stage, the presence of ulceration, increasing tumour thickness and higher TMR. The CTAg expression association with any of these clinical characteristics however, was not statistically significant except that between MAGE-A4 and increasing age at diagnosis ($P = 0.038$). There was no association between CTAg expression and anatomic tumour site, melanoma subtype, TILS or TMR. Any combination of co-expression of the three CTAgs tested occurred frequently and was statistically significant ($P < 0.001$).

3.5. *Association of CTAg expression with relapse free survival: univariate analysis*

Univariate survival analysis and CTAg expression was performed on 233 stage II primary cutaneous melanoma patients and was analysed by the Kaplan–Meier method (Fig. 2 and Table 2). MAGE-A1 (Fig. 2E) or NY-ESO-1 (Fig. 2G) tumour expression conferred significant reduced patient RFS (risk ratio (RR) = 0.626, 95% confidence interval (CI) = 0.375–0.911, $P = 0.018$ and RR = 0.664, 95% CI = 0.430–0.962, $P = 0.032$, respectively). The median RFS for patients with MAGE-A1 or NY-ESO-1 positive tumours was 45 months and for MAGE-A1 or NY-ESO-1 negative tumours it increased to 70 months. MAGE-A4 expression (Fig. 2F) did not confer a statistically significantly worse RFS, however prior to 50 months follow-up, MAGE-A4-positive tumours had reduced RFS time than MAGE-A4-negative tumours. Expression of any combination of different CTAgs was also statistically significant ranging from $P = 0.038$ for MAGE-A1 and/or MAGE-A4 to $P = 0.009$ for MAGE-A1 and/or NY-ESO-1. Expression of any CTAg (MAGE-A1, MAGE-A4 or NY-ESO-1) (Fig. 2H) conferred the worst RFS (RR = 0.600, 95% CI = 0.398–0.872, $P = 0.008$). The median RFS for any positive antigen expression was 45 months and for tumours negative for all three CT antigens the median RFS was 72 months.

To validate the tumour cohort we also performed univariate survival analysis of known prognostic factors. As expected, tumour thickness, ulceration, and mitotic rate were statistically significant prognostic factors ($P < 0.001$, <0.001 and 0.034, respectively). Patients with ulcerated tumours had a median RFS of 40 months in comparison to 87 months for those with non-ulcerated tumours ($P < 0.001$). Patients with thicker tumours (>4.00 mm) relapsed faster than ones with thinner tumours (<4.01 mm) with median RFS of 29 months and 72 months, respectively ($P < 0.001$). Tumours with a TMR greater than or equal to 1 mm^2 had a median RFS of 49 months compared to tumours with no mitotic figures in whom RFS was 90 months ($P = 0.034$) (Fig. 2A–C).

Comparison of AJCC staging with CTAg expression demonstrated a reduction in the median RFS from stage IIA, IIB to stage IIC (86 months, 48 months and 28 months, respectively $P < 0.001$). Patients with stage IIA tumours on average relapsed at 86 months which was significantly reduced to 48 months and 28 months in patients with stage IIB and stage IIC primary tumours, respectively ($p < 0.001$) (Fig. 2D).

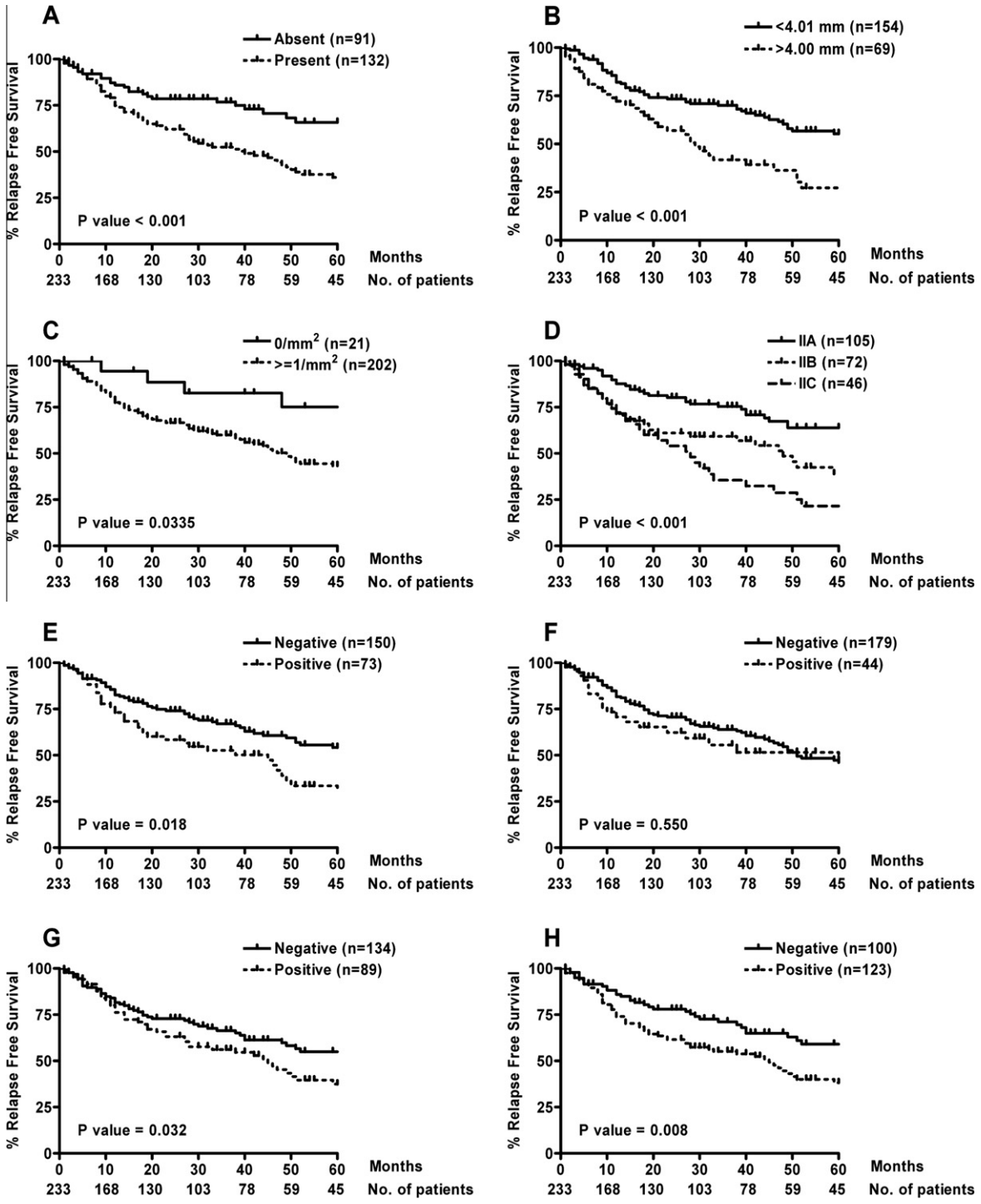

Fig. 2 – Univariate analysis of relapse free survival (RFS) in stage II primary cutaneous melanoma according to CT antigen expression. Distributions were estimated using the Kaplan–Meier method. Tick marks represent the time of last follow-up for patients who remained alive. (A) Tumour ulceration, (B) tumour thickness, (C) mitotic rate, (D) tumour AJCC staging, (E) MAGE-A1, (F) MAGE-A4, (G) NY-ESO-1 and (H) Any CT antigen.

Younger patients at diagnosis tended to have a reduced median RFS compared to older patients, however this was not statistically significant. Only 137 tumour samples reported TILs; in these cases, the presence of TILs showed no correlation with survival. There was also no correlation between patient gender and RFS (Table 2).

3.6. *Association of CTAg expression with relapse free survival: multivariate analysis*

The independent prognostic value of clinicopathologic variables were analysed using the multivariate Cox proportional hazard model to assess the effects of CTAg expression on

Table 3 – Multivariate survival analysis of significant variable for relapse free survival (RFS) in stage II melanoma patients.

Parameter	Subgroups	Risk ratio (RR)	95% Confidence limits (95% CI)	P value
Any CT antigen	Positive or negative	1.715	1.140–2.580	0.010
Tumour ulceration	Present or Absent	1.798	1.166–2.773	0.008
Tumour thickness	<4.01 mm or >4.00 mm	1.865	1.237–2.813	0.003
Tumour mitotic rate	0 mm^2 or $\geqslant$1 mm^2	1.911	0.821–4.444	0.135

RFS whilst controlling for confounding clinical covariates. This multivariate analysis was performed using parameters that yielded a P value <0.05 by univariate analysis. Tumour immunoreactivity with any of the three CTAgs tested was found to be an independent predictor of patient RFS (RR = 1.715, 95% CI = 1.140–2.580, $P = 0.010$). Ulceration and tumour thickness were also significant independent predictors of RFS ($P = 0.008$ and $P = 0.003$, respectively). TMR was not demonstrated to be an independent prognostic factor ($P = 0.135$) which is probably a reflection of the small patient group in the 0 mm^2 group. The significance level of the overall model fit was statistically significant, $P < 0.001$ (Table 3).

3.7. *Association of CTAg expression with overall survival: univariate analysis*

Overall survival analysis was performed on 233 stage II primary cutaneous melanoma patients and was analysed by the Kaplan–Meier method (Fig. 4).

MAGE-A1 (Fig. 4A), MAGE-A4 (Fig. 4B), NY-ESO-1 (Fig. 4C) or any CTAg (Fig. 4D) tumour expression was demonstrated to have no relationship with overall patient survival. ($P = 0.611$, $P = 0.566$, $P = 0.677$ and $P = 0.453$, respectively). Tumour thickness and AJCC staging were both prognostic factors ($P = 0.005$ and $P = 0.030$, respectively) but not ulceration ($P = 0.370$) or mitotic rate ($P = 0.084$) (data not shown).

3.8. *Association of spontaneous NY-ESO-1 antibodies in patient serum with AJCC staging*

Sera were assayed for the presence of NY-ESO-1 antibodies from 313 melanoma patients (Fig. 3). NY-ESO-1 antibodies were not detected in patients with stage I or stage II disease but 6% of stage III patients and 16% of stage IV patients were NY-ESO-1 antibody positive. A relationship between the absence or presence of anti-NY-ESO-1 antibodies and prognostic factors or survival in AJCC stage II or IV patients could not be demonstrated, (data not shown). 27 of the 31 patients who had detectable levels of anti-NY-ESO-1 antibodies, had tumour tissue examined for the presence of NY-ESO-1 proteins and all found to be positive.

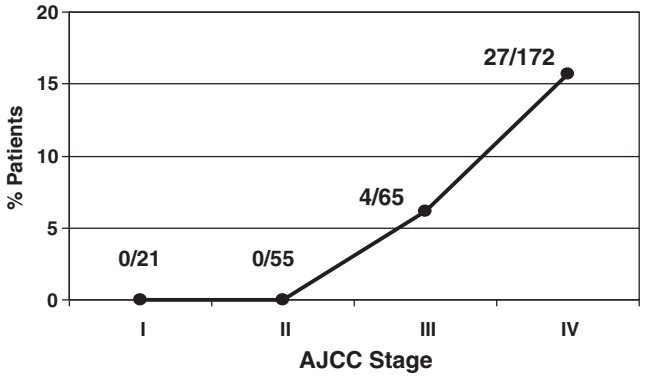

Fig. 3 – Enzyme linked immuno-sorbent assay (ELISA) for the detection of serum anti-NY-ESO-1 antibodies in melanoma patients according to AJCC staging. Number of positive patients for each stage are indicated.

4. Discussion

The molecular analysis of melanoma is proving to be increasingly important for understanding subclassification and for determining optimal treatment. The CTAgs have proven to be of particular interest because they are targets for immune recognition and therefore immunotherapy.[13] Little is known about their function and influence on the behaviour of melanoma, so we have performed the first detailed study that relates the expression of these key molecules to clinical outcomes.

Although there was no tumour selection on the basis of previous patient treatment or any other demographic factors, the Austin Health patients overall tended to relapse earlier than the MIA patients. Unlike the MIA patients, many Austin patients were referred to the Melanoma Clinic after disease recurrence and their primary tumours collected retrospectively. Our main findings however were that results from both melanoma treatment centres were concordant, reflecting the selection of similar patient populations by each group.

We therefore pooled all patient data in the analysis. In our current study NY-ESO-1 expression was found in 30% of stage I and 40% of stage II melanomas. This is somewhat lower than we previously reported[15] due to improved laboratory techniques and differences in patient selection, including the exclusion of non-cutaneous melanoma in the current series.

Our results show that CTAg expression in cutaneous melanoma has independent prognostic value comparable to that of Breslow thickness and ulceration. Most previous studies have also reported that CTAg expression is associated with poorer event free survival and/or overall survival in a variety cancers such as melanoma,[11,12,14] neuroblastoma,[19] non-small cell lung cancer,[20–22] ovarian cancer,[23] breast cancer,[24] multiple myeloma,[25] colorectal cancer,[26] bladder cancer[27] vulvar cancer,[28] gastrointestinal stromal tumours,[29] squamous cell carcinoma[30] and stomach cancer.[31] However some studies have reported no association with survival in primary mucosal melanoma of the head and neck[32] or that CTAg expression is associated with good prognosis in neuroblastoma,[33] hepatocellular carcinoma,[34] urothelial carcinoma,[35]

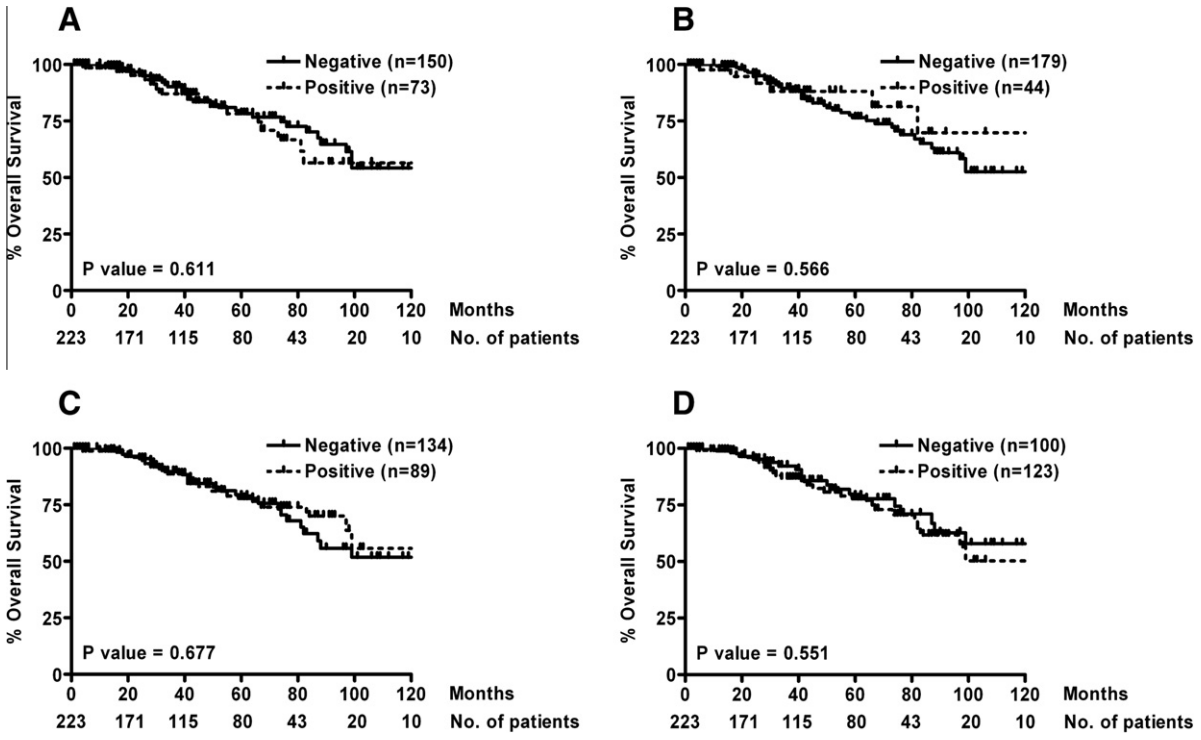

Fig. 4 – Univariate analysis of overall survival in stage II primary cutaneous melanoma according to CT antigen expression. Distributions were estimated using the Kaplan–Meier method. Tick marks represent the time of last follow-up for patients who remained alive. (A) MAGE-A1, (B) MAGE-A4, (C) NY-ESO-1 and (D) any CT antigen.

breast cancer[36] and oesophageal cancer.[37] Apparent discrepancies between these studies probably reflect the small cohort of patients studied. The median number of patients analysed in these studies was only 65. Other possibilities are the inclusion of different tumour types, heterogenous expression of different CTAgs and varying laboratory methods of detection and interpretation.

The tantalising observation made here is that unlike relapse free survival, CTAg expression had no relationship with overall patient survival. We have no clear explanation for this; however as seen in Fig. 3, immunity against NY-ESO-1 became detectable as disease stage increased. No antibody was detectable in primary stage I or stage II tumour bearing subjects. In contrast patients with stage III or stage IV disease had readily detectable antibody in their serum which increased with advancing disease suggesting that the antibody response to NY-ESO-1 is dependent on the persistence and level of antigenic stimulation. We and others have shown that antibody responses against NY-ESO-1 are correlated in parallel with emerging T-cell immunity.[13,38,39]

Sixteen percentage of stage IV patients had detectable antibody, which is similar to the magnitude of the separation of the survival curves (approximately 20%) seen in Fig. 2. We therefore hypothesise that evolving immunity may modify tumour progression in these patients. If so, this provides indirect evidence that immune responses against CTAgs have the capacity to influence the natural history of melanoma. This further provides a rationale for the use of CTAgs as immunogens for the treatment of cancer, particularly early in the course of the disease. Since spontaneous immunity developed late in the course of the disease, the timing of vaccine intervention against CTAgs may be important and we propose that clinical trials designed to assess the impact of vaccination against CTAgs should target patients with earlier stage disease. Inducing earlier immune responses in the course of the disease may enable this impact on clinical outcomes to take effect before disease progression can potentially pre-empt the development of CTAg expression which tends to increase with advancing disease stage.

To assess the impact of vaccination against CTAgs, a mouse model has been previously reported by our group in which NY-ESO-1 transfected B16 melanoma cells were transplanted into C57BL/6 mice.[40] In this study, vaccination with NY-ESO-1 ISCOMATRIX™ was found to induce effective anti-cancer immunity in a protective model and established that achieving anti-cancer immunity at a sufficiently early stage of the disease could modify the natural history of melanoma progression. This has been evaluated prospectively in a randomised clinical trial undertaken by the Ludwig Institute for Cancer Research LUD 03009. This study has completed accrual and is currently under analysis. The results of this study will be reported in due course.

In summary, we have clearly demonstrated that CTAg expression in primary melanoma is an important factor that is associated with time to first disease relapse. The magnitude of impact is equivalent to that seen for ulceration and tumour thickness which are currently accepted critical prognostic factors in melanoma. The lack of impact on overall survival points to the potentially important role of emerging immunity and highlights the need to consider tumour stage and

evolving immunity when designing immunotherapeutic strategies aimed at modifying the natural course of disease.

5. Conflict of interest statement

None declared.

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