Tumor Antigen Expression in Melanoma Varies According to Antigen and Stage

Catherine Barrow,1 Judy Browning,1 Duncan MacGregor,1,2 Ian D. Davis,1 Sue Sturrock,2 Achim A. Jungbluth,3 and Jonathan Cebon1

Abstract

Purpose: Melanoma cells express antigens that can induce T-cell and antibody responses. Obtaining a detailed understanding of antigen expression in primary and metastatic melanoma is essential if these molecules are to be useful targets for immunotherapy of melanoma.

Experimental Design: Malignant melanomas (n = 586) from 426 patients were typed for antigen expression. Multiple samples were available from 86 individuals, enabling analysis of antigen expression patterns over time. Paraffin-embedded samples were tested by immunohistochemistry for the presence of the differentiation antigens: gp100, Melan-A, tyrosinase, and the “cancer/testis” antigens MAGE-A1, MAGE-A4, and NY-ESO-1.

Results: Samples were primary tumors (n = 251), lymph node metastases (n = 174), s.c. metastases (n = 71), and distant metastases (n = 90). The differentiation antigens were strongly expressed in 93% to 95% of tumors regardless of stage. In contrast, the frequency of cancer/testis antigen expression in primary tumors for MAGE-A1, MAGE-A4, and NY-ESO-1 was lower (20%, 9%, and 45%, respectively). MAGE-A1 and MAGE-A4 were acquired with advancing disease (to 51% and 44% in distant metastases, respectively) but not NY-ESO-1, which remained positive in 45%. MAGE-A1 expression was twice as prevalent in ulcerated primaries as in nonulcerated primaries (30% versus 15%; P = 0.006) and in thicker as opposed to thin melanomas (26% versus 10%; P = 0.1).

Conclusions: This large series describes patterns of antigen expression in melanoma and their evolution over time. This will help inform decisions about selection of patients and target antigens for melanoma immunotherapy clinical trials.

Imaging, Diagnosis, Prognosis

Malignant melanoma is increasing in incidence worldwide, and although early primary melanomas can be cured surgically, this cancer can be rapidly fatal when metastases have developed. Regrettably, therapeutic options for metastatic disease are limited. Nonetheless, it has proven to be a disease capable of inducing immune responses and thus has long been considered a model tumor for the study of cancer immunotherapy.

This immunogenicity is often mediated by the recognition of tumor antigens expressed by melanoma cells, which are recognized by CTLs through presentation on HLA molecules (1). The most relevant antigens on melanomas can be broadly classified into two classes: differentiation antigens that are only expressed by normal and malignant cells derived from melanocytes but not other cell types (2); and “cancer/testis” antigens that are expressed in a wide variety of tumor types and germ cells of normal adult testis, but expression in nongametogenic tissues is restricted to placenta (3, 4). These antigens all represent potential targets for cancer immunotherapy (5–10); thus, an understanding of their expression patterns is essential if effective immunotherapies are to be developed against malignant melanoma (11). It is also essential to understand whether there is any temporal change in the expression pattern of any of these antigens; that is, whether their presence changes over the course of time.

Most previous studies of antigens in melanoma have focused on either differentiation antigens (12–15) or cancer/testis antigens (16–20) separately; however, multiple targets may be chosen for the development of polyvalent vaccines, and these may include molecules from both classes. Thus, knowledge about the patterns of expression of each in individual tumors is essential. In addition, prior studies have been disadvantaged by small sample numbers (14, 15) and limited numbers of antigens tested (12, 14, 15, 21). Finally, very limited information is available about the changes that occur in the expression of each antigen during the progression of melanoma in individual patients. Thus, there is a need to study both classes of antigens in adequate numbers of tumors and to

Authors’ Affiliations: 1Ludwig Institute for Cancer Research, Melbourne, Australia; 2Department of Anatomical Pathology, Austin Health, Heidelberg, Victoria, Australia; and 3Ludwig Institute for Cancer Research, New York, New York

Received 7/18/05; revised 9/23/05; accepted 9/29/05.

Grant support: Ludwig Institute for Cancer Research.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Jonathan Cebon, Ludwig Institute for Cancer Research, Austin Health, Heidelberg, Victoria 3084, Australia. Phone: 61-3-9496-5462; Fax: 61-3-9457-6698; E-mail: Jonathan.Cebon@ludwig.edu.au.

©2006 American Association for Cancer Research.

doi:10.1158/1078-0432.CCR-05-1544
follow antigen expression patterns in individuals longitudinally through the course of their disease.

In the present study, we have assessed antigen expression in a series of 586 primary and metastatic melanoma samples. These were assayed using standardized immunohistochemistry, which was done in a single laboratory. All samples were derived sequentially from an Australian melanoma clinic where patients were screened for suitability as candidates for clinical trials with vaccines. Three differentiation antigens and three cancer/testis antigens were evaluated. The present analysis characterizes in detail the relationship between antigen expression and tumor stage as well as location of disease and whether there is a relationship between antigen expression and known prognostic variables, such as Breslow thickness and presence of ulceration in primary tumors. We also analyze the expression of one antigen in relation to the others and the temporal evolution of antigen expression in individual patients whose tumors were resected on more than one occasion.

Materials and Methods

**Patient population.** All samples came from patients attending the melanoma clinic at Austin Health (Melbourne, Australia). For patients referred from other institutions, paraffin blocks or unstained slides were collected from various pathology laboratories elsewhere in Australia.

**Tissue acquisition and banking.** Formalin-fixed paraffin sections were obtained from primary and metastatic tumors of patients with malignant melanoma. Sections of each tumor were routinely stained with H&E to confirm the diagnosis. All patients provided written informed consent for antigen typing in accordance with protocols approved by the Austin Health Human Research Ethics Committee.

**Antibodies.** Monoclonal antibodies (mAbs) to NY-ESO-1 (22, 23), Melan-A (16), tyrosinase (24), and MAGE-A1 (25) were generated by our group previously and used at a concentration of 3 μg/ml for mAb E978 (NY-ESO-1; ref. 22), 2.67 μg/ml for mAb A103 (Melan-A; ref. 16), 5.59 μg/ml for mAb T311 (tyrosinase; ref. 24), and 1:50 dilution for mAb MA4454 (MAGE-A1; ref. 25). The mAb supernatant 57B, which recognizes MAGE-A4, was kindly supplied by Dr. G. Spagnoli (Surgical Research Centre, Basel, Switzerland) and used at a 1:100 dilution (26).
Anti-gp100 mAb (HMB45) was purchased from DakoCytomation (Carpinteria, CA) and used at a 1:20 dilution (27).

**Immunohistochemistry and antigen retrieval.** Formalin-fixed paraffin sections were prepared and dried overnight at 37°C. Following dewaxing in xylene and rehydration through alcohols, microwave antigen retrieval was done for 10 minutes using EDTA buffer (pH 8; NeoMarkers, Fremont, CA) for NY-ESO-1 and MAGE-A1 and citrate buffer (pH 6; NeoMarkers) for gp100, Melan-A, tyrosinase, and MAGE-A4.

Immunohistochemistry was done using the Dako Envision+ kit (DakoCytomation) or Vectastain Elite Universal Avidin-Biotin Complex kit (Vector Laboratories, Burlingame, CA). All sections were submitted to 3% H2O2/PBS for 10 minutes to block endogenous peroxidase. Endogenous biotin activity was quenched by sequential application of egg white and skim milk according to published methods for the Vectastain Elite Universal Avidin-Biotin Complex kit (28, 29). All incubations were done at room temperature using the Shandon Sequenza immunostainer. The chromogen was 3-amino-9-ethyl-carbazole (Sigma-Aldrich, St. Louis, MO), and slides were counterstained with Mayer’s hematoxylin (Amber Scientific, Belmont WA, Australia). Application of Crystal Mount (Biomed, Foster City, CA) preceded dehydration and mounting in DePeX (BDH 36125).

Specimens of known antigen-positive tumors were used as a positive control, and negative substitution controls, where the antibody diluent solution (10% FCS in PBS, pH 7.6) replaced the primary antibody, were included with every immunohistochemical test.

**Interpretation.** All the samples were prepared using the same methods and were analyzed in the Department of Anatomical Pathology at Austin Health by a single pathologist (D.M.). Consensus was reached on staining distribution following review of all sections by subsequent case conference by D.M. in conjunction with the Ludwig Institute clinical investigators (C.B., I.D.D., and J.C.).

To assess change in antigen expression with melanoma progression, samples were grouped into the following four categories: primary melanomas (n = 251), lymph node metastases (n = 174), s.c. metastases (n = 71), distant metastases (all other sites; chiefly liver, gut, lung, and brain; n = 90). The extent of staining was scored as percentage of malignant cells testing positive for the presence of each antigen and assigned to one of six groups: negative, <5%, 6% to 25%, 26% to 50%, 51% to 75%, >75%.

**Statistical methods/analysis.** Statistical analyses were done by the University of Melbourne Statistical Consulting Centre. The extent of staining was analyzed in relation to tumor (primary, lymph node metastasis, s.c. metastasis, and distant metastasis), in patients for whom more than one sample was available, presence or absence of ulceration of the primary, and according to Breslow thickness of the primary. The cumulative percentage of cells staining for each antigen was compared using ordinal logistic regression and expressed as a cumulative percentage graph or as an odds ratio with 95% confidence intervals. An odds ratio of >1 or <1, with 95% confidence intervals not crossing unity, indicated a significant increase or decrease in staining relative to the comparator.

**Results**

**Patients.** Unselected melanoma tumor samples (n = 586) were obtained sequentially from 426 individuals with malignant melanoma who attended the Austin Health melanoma clinic in Melbourne between 1997 and 2003 inclusive. There was no selection based on stage, previous therapy, or any other demographic factors. There was no deliberate selection of metastatic tumors for analysis, although these studies were essentially limited to patients for whom surgical resection was a clinically appropriate diagnostic or therapeutic intervention. Such patients generally had oligo-metastatic disease.

For all primary tumors, which were resected with a wide margin, normal melanocytes stained positively for the three differentiation antigens but not for the cancer/testis antigens. In tumors with patchy antigen distribution, there was no consistent correlation in staining among the three cancer/testis antigens. There were sometimes islands of cells, which clearly stained for several cancer/testis antigens simultaneously. In other tumors, or in other parts of individual heterogeneously staining tumors, no such correlation was seen. Representative examples of immunohistochemical staining with antibodies against gp100, Melan-A, MAGE-A1, MAGE-A4, NY-ESO-1, and S100 are shown in Fig. 1. The figure shows that the distribution of each antigen varied from being absent (patient 1, NY-ESO-1; Fig. 1C) to a few cells as shown for MAGE-A4 (Fig. 1J), to more uniform expression, such as MAGE-A1 (Fig. 1F). Acquisition of NY-ESO-1 staining is shown for patient 1 (Fig. 1G), where <25% of cells were scored positive on review of the whole section.

**Patterns of antigen staining.** Each tumor was stained for the presence of all six antigens, and Fig. 2 shows the extent of staining for each within each of the four categories comprising primary, lymph node, s.c. metastases, and distant metastases. The three differentiation antigens were expressed in the majority of tumor samples (91-98%) regardless of location. These antigens also tended to be widely expressed within
individual tumors (i.e., >50% of malignant cells staining positive) in 55% to 90% of cases. There was no significant difference between levels of antigen expression in primary tumors or metastases, either in terms of frequency of positive tumors, or number of positive cells per tumor. In contrast, the cancer/testis antigens were more likely to be absent particularly in primary tumors. The prevalence for MAGE-A1, MAGE-A4, and NY-ESO-1 was 20%, 9%, and 45%, respectively. There was a clear pattern of cancer/testis antigen expression acquisition with progressive tumor stage for MAGE-A1, which increased from 20% to 51% ($P < 0.001$) and MAGE-A4, which increased from 9% to 44% ($P < 0.0001$). This, however, was not seen for NY-ESO-1, which remained positive in the range of 43% to 53% irrespective of the stage of the disease. The prevalence of cancer/testis antigen expression at any time, regardless of tumor stage, was 37% for MAGE-A1 and 46% for NY-ESO-1; MAGE-A4 was the least prevalent of the antigens being present in only 29% of the 586 tumors examined. Present, the proportion of cells that stained for any of the cancer/testis antigens was substantially less than that for the differentiation antigens. Indeed, only a small proportion of the primary tumors (3-28%) contained malignant cells, which were positive for cancer/testis antigens in >50% of cells (Fig. 2). A slightly higher percentage of metastatic tumors showed expression in >50% of the tumor. In tumors with patchy antigen distribution, there was no consistent correlation in staining among the three cancer/testis antigens. There were sometimes islands of cells, which clearly stained for several cancer/testis antigens simultaneously. In other tumors, or in other parts of individual heterogeneously staining tumors, no such correlation was seen.

Table 1 shows the number of tumors that expressed multiple antigens. For primary melanomas, all three differentiation antigens tended to be present simultaneously: 94% of these stained for all three differentiation antigens, whereas only 1.6% expressed two antigens and 1.2% expressed one. This was quite different for the three cancer/testis antigens that showed simultaneous expression in only a minority of primary tumors (5.2%), whereas 11.2% primaries expressed two cancer/testis antigens and 37% primary melanomas were positive for at least one cancer/testis antigen. If only one cancer/testis antigen was expressed, it was most likely to be NY-ESO-1 (75 of 251, 30%), in contrast to MAGE-A1 (13 of 251, 5.2%) and MAGE-A4 (4 of 251, 1.6%).

For the differentiation antigens, these patterns were largely maintained when metastases were studied (lymph node, s.c. metastases, and distant metastases combined; $n = 335$). Eighty-nine percent of samples expressed all three differentiation antigens compared with 5.1% for two antigens and 3% for one. The pattern was different for the cancer/testis antigens, however, because expression tended to be more prevalent when compared with primary tumor samples. Of the 335 metastases, 29 (23.6%) of samples expressed all three cancer/testis antigens compared with 76 (22.7%) for two antigens and 73 (21.8%) for one only.

The samples were grouped into those testing positive and negative for each antigen and then studied to determine how frequently the remaining antigens were expressed in each. For example, of the 545 gp100-positive tumors, Melan-A was simultaneously present in 540 of 545 (99%), tyrosinase in 534 of 545 (98%), MAGE-A1 in 191 of 545 (35%), MAGE-A4 in 158 of 545 (29%), and NY-ESO-1 in 256 of 454 (47%). Conversely, for the 41 of 586 (7%) tumors where gp100 was absent, Melan-A was present in 17 of 41 (41%), tyrosinase in 19 of 41 (46%), MAGE-A1 in 15 of 41 (36%), MAGE-A4 in 14 of 41.

### Table 1. Differentiation antigens and cancer/testis antigens: distribution among primary tumors and metastases

| Samples                      | No. antigens expressed | 3 | 2 | 1 | 0 |
|------------------------------|------------------------|---|---|---|---|
| Primary tumors ($n = 251$)   |                        |   |   |   |   |
| Differentiation antigens     |                        | 237 (94%) | 4 (1.6%) | 3 (1.2%) | 7 (2.8%) |
| Cancer/testis antigens       |                        | 13 (5.2%) | 28 (11.2%) | 93 (37%) | 118 (47%) |
| Metastases ($n = 335$)       |                        | 223 (89%) | 17 (5.1%) | 10 (3%) | 10 (3%) |
| Differentiation antigens     |                        | 79 (23.6%) | 76 (22.7%) | 73 (21.8%) | 107 (32%) |
| Cancer/testis antigens       |                        |                        |                        |                        |                        |

### Table 2. Alternative antigen expression in antigen-positive and antigen-negative tumors

| Antigen | gp100 ($n = 596$) | Tyrosinase ($n = 596$) | NY-ESO-1 ($n = 596$) | MAGE-1 ($n = 596$) | MAGE-4 ($n = 596$) |
|---------|------------------|------------------------|---------------------|-------------------|-------------------|
| Melan-A |                  |                        |                     |                   |                   |
| gp100   |                  |                        |                     |                   |                   |
| Tyrosinase |             |                        |                     |                   |                   |
| NY-ESO-1 |                 |                        |                     |                   |                   |
| MAGE-1  |                 |                        |                     |                   |                   |
| MAGE-4  |                 |                        |                     |                   |                   |

NOTE: All tumors have been designated as positive or negative for each of the six antigens (columns). The presence of each of the remaining five antigens was determined (rows), and the numbers and percentages are shown.
(33%), and NY-ESO-1 in 15 of 41 (36%). Similar analyses were also done for each of the remaining antigens (Table 2) and after stratifying according to the site of the sample (primary, lymph node, s.c. metastases, and distant metastases). The following conclusions were drawn:

- The expression of differentiation antigens was generally high and synchronous. Consequently, in those tumors where one of these antigens was absent, it was very likely that none of the other differentiation antigens was present. The percentages for the presence of a particular differentiation antigen in a specific tumor, which was negative for another differentiation antigen, ranged from 18% to 46% and was highest for tyrosinase in gp100-negative tumors and Melan-A in tyrosinase-negative lesions.

- When one cancer/testis antigen was absent, the other cancer/testis antigens were present in 10% to 37%, levels which were comparable with those seen in tumors where the cancer/testis antigens were present. Similarly, the absence of a cancer/testis antigen did not seem to correlate with any reduction of differentiation antigen expression (antigen remained positive in 94-96% of cases).

Site of distant metastasis. Of the 90 distant metastases studied, 58 were from brain (18), lung (28), and small bowel (12). The remaining 32 samples consisted of metastases resected from liver, adrenal, ovarian, thyroid, spleen, and other miscellaneous sites. Antigen staining for these metastases is shown in Fig. 3. All three differentiation antigens were highly prevalent regardless of the metastatic site. Tyrosinase was widely expressed in 100% of bowel metastases. Cancer/testis antigens were expressed in <50% of these metastatic tumors with very few differences between each of the sites. The one exception to this was NY-ESO-1, which was seen more commonly in the brain (62%), although in most of these samples (>80%), staining was only seen in a minority (<50%) of cells.

Relationship between antigen expression and other prognostic factors. In view of the increased expression of MAGE-1 and MAGE-4 in association with disease progression, we evaluated the relationship between antigen expression and two key prognostic factors in primary melanomas: ulceration and tumor thickness. Of the 251 primary melanomas, 242 (96%) were assessable for ulceration. The majority were nonulcerated (72% versus 28% ulcerated). Figure 4 shows that expression of the differentiation antigens did not differ with ulceration status. In contrast, MAGE-A1 was expressed at a significantly higher level in ulcerated tumors (30% versus 15%; \(P < 0.006\)) compared with nonulcerated primaries. There was also a trend to an increased prevalence of MAGE-A4 expression in ulcerated compared with nonulcerated primaries (14% versus 7%), but this was not significant (\(P = 0.1\)). NY-ESO-1 prevalence did not change appreciably with ulceration.

Figure 5 shows the relationship between antigen expression and the thickness of primary melanomas. Of the 251 tumors, 234 (93%) were assessable for Breslow thickness and further
subdivided into three groups representing different ranges of thickness: <1 mm (n = 51), 1.1 to 4.0 mm (n = 127), and >4 mm (n = 56). More than half (54%) were between 1.1 and 4 mm thick. MAGE-A1 and MAGE-A4 expression tended to be more frequent in thicker as opposed to thin tumors, although it did not reach significance (P = 0.1 and 0.5, respectively). NY-ESO-1 antigen was most prevalent in those tumors within the 1.1 to 4.0 mm range. This increased prevalence of NY-ESO-1 in tumors of intermediate thickness as opposed to thin tumors was significant (P = 0.002); however, there was no consistent trend, because expression of NY-ESO-1 was lower in tumors exceeding 4 mm (Fig. 5). In contrast, there was a consistent trend for loss of differentiation antigen expression, as primary tumors became thicker. In thin melanomas, there was 100% expression for all three differentiation antigens, decreasing to 90% to 95% expression for tumors >4 mm. For gp100, this was statistically significant (P = 0.003) but not for the other two differentiation antigens Melan-A (P = 0.2) or tyrosinase (P = 0.6).

**Evolution of antigen expression with disease progression.** To evaluate the evolution of antigen expression in individual patients during the course of their disease, we studied 86 individuals who had resections on more than one occasion. Of the 586 tumor samples, 246 (42%) came from these 86 individuals: 43 (50%) contributed two samples to the analysis, 23 contributed three samples, 12 contributed four samples, six contributed five samples, one contributed six, and a further individual contributed seven tumor samples at separate time points. Thirteen individuals did not have primary lesions available for assessment. Table 3 summarizes the patterns of tumor antigen expression in these individuals over time.

In some cases, the numbers of cells in a tumor, which expressed individual antigens, were seen to increase or decrease. In other cases, expression was acquired when it had previously been absent or was lost after having previously been present. In yet other cases, levels fluctuated. The differentiation antigens were always expressed on at least one occasion, and although there was a trend for antigen loss in some tumors, this was not universal because between 9% and 17% of these tumors had an increase in antigen expression. MAGE-A4 was the most commonly acquired antigen (40% of individuals), and once acquired, was very rarely lost. In contrast NY-ESO-1 was most commonly lost (in 31% of individuals) over time. However, despite this, it was present at some point in a series of sequential samples in 76% of cases.

**Discussion**

The exact knowledge of the expression of melanocyte differentiation antigens and cancer/testis antigens has important implications in the field of diagnostic surgical pathology and in the immunotherapy of cancer. First, melanocyte differentiation markers are widely used as tool for the diagnosis of melanocytic lesions. Furthermore, vaccines to cancer/testis antigens and differentiation antigens are now being developed for the treatment of melanoma, and a detailed understanding of antigen expression is critical to understand the constraints associated with targeting these molecules. This will not only make it possible to optimize patient and antigen selection but also to evaluate the relationship between antigen expression and clinical outcomes. This is the largest published series of patients and melanoma samples evaluating six frequently

| Table 3. Patterns of tumor antigen expression over time in 86 individuals with multiple samples |
|-----------------------------------------------|
| **Tumor Antigen** | **Lost** | **Reduced** | **Acquired** | **Increased** | **No change** | **Fluctuated** | **Absent** | **Total** |
| gp100 | 6 (7%) | 18 (21%) | 2 (2%) | 15 (17%) | 29 (34%) | 16 (19%) | 0 (0%) | 86 (100%) |
| Melan-A | 4 (5%) | 17 (20%) | 1 (1%) | 8 (9%) | 42 (49%) | 14 (16%) | 0 (0%) | 86 (100%) |
| Tyrosinase | 5 (6%) | 6 (7%) | 1 (1%) | 13 (15%) | 46 (53%) | 15 (17%) | 0 (0%) | 86 (100%) |
| MAGE-A1 | 3 (3%) | 1 (1%) | 21 (24%) | 11 (13%) | 2 (2%) | 12 (14%) | 36 (42%) | 86 (100%) |
| MAGE-A4 | 0 (0%) | 2 (2%) | 28 (33%) | 6 (7%) | 4 (5%) | 8 (9%) | 38 (44%) | 86 (100%) |
| NY-ESO-1 | 22 (26%) | 4 (5%) | 14 (16%) | 5 (6%) | 5 (6%) | 15 (17%) | 21 (24%) | 86 (100%) |

NOTE: Lost, antigen that was initially present (usually within primary tumor) and was absent in subsequent sample(s). Reduced, antigen that was initially present and was present but in fewer cells within subsequent sample(s). Acquired, antigen that was absent in the initial sample and was present in subsequent sample(s). Increased, antigen in the initial sample was present in more cells in subsequent sample(s). Fluctuated, levels increased and decreased over the time course. Absent, antigen was never present.
selected target antigens: Melan-A, gp100, tyrosinase, NY-ESO-1, MAGE-A1, and MAGE-A4.

In summary we found:

- All three differentiation antigens were not only highly prevalent but also widely expressed in all melanoma samples, regardless of stage or location of disease. This supports the findings of other authors who found that expression for each was consistently in the order of ≥90% tumors (15, 24, 30–34).
- The cancer/testis antigens MAGE-A1 and MAGE-A4 were less prevalent and, when present, expressed in fewer tumor cells than were the differentiation antigens. This was particularly apparent in primary tumors, but the same pattern was also seen in secondary deposits regardless of site.
- There was a clear pattern of acquisition of the MAGE antigens with progressive stage that was not seen with any of the differentiation antigens or NY-ESO-1.
- Expression of MAGE-A1 and MAGE-A4 tended to be more common in thicker and in ulcerated melanomas. It is not clear whether the higher levels of expression of these antigens related to the tumors being more inherently biologically aggressive, or simply that thicker and/or ulcerated melanomas are of a higher T stage than others, and expression increased as a function of stage.
- NY-ESO-1 expression did not follow the same pattern as MAGE-A1 and MAGE-A4. It failed to increase with progression and stage, was lost most commonly in patients with serial biopsies, and most interestingly, tended to be more common in brain metastases. All of these observations would be consistent with the observation that NY-ESO-1 quite frequently elicits spontaneous immune responses and may therefore be the antigen most susceptible to “immunoediting.” Immunoediting is the concept that the immune system may not only protect against the development of cancers but can also sculpt the tumor phenotype, resulting in the selection of tumor variants that can evade immune defences (35, 36). Such “sculpting” may explain each of these observations: loss of antigen more commonly, failure to acquire antigen with disease progression, and persistence of antigen in the immunologically privileged central nervous system.

To our knowledge, ours is the largest series reported in terms of the numbers and types of melanoma samples analyzed (i.e., primaries and metastases) as well as the numbers and the combination of antigens analyzed. Our study also comprises a subseries of sequential melanoma samples acquired from single individuals and thus provides a unique insight into the evolution of antigen expression over time in a series of patients with repeated tumor resections. A high degree of reporting consistency was maintained because all samples were stained at a single center, reported by a single pathologist, and reviewed by a panel of investigators.

Gajjar et al. (37) found MAGE-A1 protein expression in melanoma metastases in only 27% of cases. Our findings are more in keeping with those of Brasseur et al. (38), who reported MAGE-A1 in 48% of metastases and 16% of primary melanomas, although that study was based on reverse transcription-PCR analysis. Previous studies did not find a difference in expression of MAGE-A4 between primary and metastatic melanomas, and no correlation with stage or thickness could be established (39), although a correlation could be seen with the presence of an inflammatory infiltrate. However, the study consisted of fewer cases, and tumor and metastatic lesion were not derived from the same patients (39). Likewise, few studies have looked at patterns of NY-ESO-1 expression in melanoma, although one smaller study did find NY-ESO-1 mRNA expression to be increased in metastases compared with primary samples (32). Two other studies found NY-ESO-1 expression in ~36% (23) and 31.6% of melanomas (40), respectively. However, both studies have analyzed smaller sample numbers and did not distinguish between primary tumors and metastases.

The observation that antigens may be lost or acquired is important because it highlights the possibility for both the loss and gain of immune susceptibility during the course of the disease. The mechanisms that underlie such changes are not well characterized; however, in the case of Melan-A, they can include the secretion of soluble factors by the tumor (41). Nonetheless, in the absence of an adequate immune response, there may be little change in the tumor antigen phenotype because there may be no selective pressure applied to the evolving tumor. In contrast, a tumor that develops in the face of an active immune response against one or more antigens can evolve to escape the control of the immune system. The consequent sculpting of the immunogenic phenotype of the tumor may facilitate tumor progression (35, 36).

In contrast, the acquisition of antigen MAGE-A1 and MAGE-A4 expression with tumor progression suggests that these antigens are less susceptible to immunoediting. Furthermore, because levels were higher in thicker compared with thin melanomas, and ulcerated compared with nonulcerated lesions, it is possible that these antigens could be prognostic indicators in their own right.

NY-ESO-1 deserves special mention because it may be a more useful target for immunotherapy than seems to be the case based on the one-time expression assessment. Unlike the differentiation antigens, NY-ESO-1 is not well tolerated. Thus, it is immunogenic and frequently induces spontaneous immune responses (3). Although only present in 46% of tumors analyzed, expression of this antigen varied over the course of the disease and, when studied in sequential samples from individuals, was present on at least one occasion in 76% of patients. This larger number represents the proportion of patients whose tumors could potentially be targeted by an immune response against NY-ESO-1 following successful vaccination. The implications of this should be analyzed carefully in future clinical trials, because it may well be possible that induction of an anti-NY-ESO-1 immune response early in the course of the disease may prevent the development of metastases that express this antigen.

In conclusion, this large series details the patterns of expression for six key antigens in melanoma. It provides the basis for selection of patients and antigens for use in immunotherapy clinical trials.

**Acknowledgments**

We thank Drs. Lloyd Old and Eric Hoffman and Prof. Munro Neville of the Ludwig Institute for Cancer Research for their invaluable advice and guidance.
References

1. Boon T, Old LJ. Cancer tumor antigens. Curr Opin Immunol 1997;9:681 – 3.
2. Kawakami Y, Rosenberg SA. Human tumor antigens recognized by T-cells. Immunol Res 1997;16:313 – 39.
3. Chen YT, Old LJ. Cancer-testis antigens: targets for cancer immunotherapy. Cancer J Sci Am 1999;5:16 – 7.
4. Scanlan MJ, Simpson AJ, Old LJ. The cancer/testis genes: review, standardization, and commentary. Cancer Immun 2004;4:1.
5. Davis ID, Chen W, Jackson H, et al. Recombinant NY-ESO-1 protein with ISCOMATRIX adjuvant induces broad integrated antibody and CD4+ and CD8+ T cell responses in humans. Proc Natl Acad Sci U S A 2004;101:10697 – 702.
6. Gnjatic S, Eger J, Chen W, et al. CD8(+) T cell responses against a dominant cryptic HLA-A2 epitope after NY-ESO-1 peptide immunization of cancer patients. Proc Natl Acad Sci U S A 2002;99:11813 – 8.
7. Jager E, Gnjatic S, Nagata Y, et al. Induction of primary NY-ESO-1 immunity: CD8+ T lymphocyte and antibody responses in peptide-vaccinated patients with NY-ESO-1+ cancers [In Process Citation]. Proc Natl Acad Sci U S A 2000;97:12198 – 203.
8. Scott AM, Cebon J. Clinical promise of tumour immunology. Lancet 1997;349:9819 – 22.
9. Davis ID, Jefford M, Parente P, Cebon J. Rational approaches to human cancer immunotherapy. J Leukoc Biol 2003;73:3 – 29.
10. Rosenberg SA. Shedding light on immunotherapy for cancer. N Engl J Med 2004;350:1461 – 3.
11. Gattoni-Celli S, Cole DJ. Melanoma-associated tumor antigens and their clinical relevance to immunotherapy. Semin Oncol 1996;23:754 – 8.
12. Marincola FM, Hijiya YM, Fetsch P, et al. Analysis of expression of the melanoma-associated antigens MART-1 and gp100 in metastatic melanoma cell lines and in situ lesions. J Immunother Emphasis Tumor Immunol 1996;19:192 – 205.
13. de Vries TJ, Fourkour A, Wobbes T, et al. Heterogeneous expression of immunotherapy candidate proteins gp100, MART-1, and tyrosinase in human melanoma cell lines and in human malignant lesions. Cancer Res 1997;57:3223 – 9.
14. Corringer JP, Abadi A, Fetsch P, et al. Comparative analysis of the in vivo expression of tyrosinase, MART-1/Melan-A, gp100 in metastatic melanoma lesions: implications for immunotherapy. J Immunother 1998;21:27 – 31.
15. Kageshita T, Kawakami Y, Hira S, Ono T. Differential expression of MART-1 in primary and metastatic melanoma lesions. J Immunother 1997;20:460 – 5.
16. Chen YT, Stockert E, Jungbluth A, et al. Serological analysis of Melan-A (MART-1), a melanocyte-specific protein homogeneously expressed in human melanomas. Proc Natl Acad Sci U S A 1996;93:5915 – 9.
17. Van den Eynde B, Peeters O, De Backer O, Gaugler B, Lucas S, Boon T. A new family of genes coding for an antigen recognized by autologous cytolytic T lymphocytes on a human melanoma. J Exp Med 1995;182:689 – 98.
18. Couli PG, Van den Eynde BJ, van der Bruggen P, Van Pel A, Boon T. Antigens recognized by T-lymphocytes on human tumours. Biochem Soc Trans 1997;25:544 – 8.
19. Sahin U, Tureci O, Chen YT, et al. Expression of multiple cancer/testis (CT) antigens in breast cancer and melanoma: basis for polyvalent CT vaccine strategies. Int J Cancer 1998;78:387 – 9.
20. Treci O, Chen YT, Sahin U, et al. Expression of SSX genes in human tumors. Int J Cancer 1998;77:19 – 23.
21. Hoffbauer GF, Schafer C, Noppen C, et al. MAGE-3 immunoreactivity in formalin-fixed, paraffin-embedded primary and metastatic melanoma: frequency and distribution. Am J Pathol 1997;151:1549 – 53.
22. Vaughan HA, Svobodova S, Macgregor D, et al. Immunohistochemical and molecular analysis of human melanomas for expression of the human cancer-testis antigens NY-ESO-1 and LAGE-1. Clin Cancer Res 2004;10:8396 – 404.
23. Jungbluth AA, Chen YT, Stockert E, et al. Immunohistochemical analysis of NY-ESO-1 antigen expression in normal and malignant human tissues. Int J Cancer 2001;98:856 – 60.
24. Jungbluth AA, Iversen K, Coplan KA, et al. T311-an anti-tyrosinase monoclonal antibody for the detection of melanocytic lesions in paraffin embedded tissues. Pathol Pract Res 2000;196:235 – 42.
25. Chen YT, Stockert E, Chen Y, et al. Identification of the MAGE-1 gene product by monoclonal and polyclonal antibodies. Proc Natl Acad Sci U S A 1994;91:1004 – 8.
26. Landry C, Brasseur F, Spagnoli GC, et al. Monoclonal antibody 57B stains tumor tissues that express gene MAGE-A4. Int J Cancer 2000;86:835 – 41.
27. Bacchi CE, Bonetti F, Pea M, Martignoni G, Gown AM. HMB-45. A review. Appl Immunohistochem Mol Morphol 1996;4:73 – 85.
28. Miller RT, Kubier P. Blocking of endogenous avdin-binding activity in immunohistochemistry. The use of egg whites. Appl Immunohistochem Mol Morphol 1997;5:63 – 6.
29. Miller RT, Kubier P, Reynolds B, Henry T, Turnbow H. Blocking of endogenous avidin-binding activity in immunohistochemistry. The use of skim milk as an economical and effective substitute for commercial biotin solutions. Appt Immunohistochem Mol Morphol 1999;7:63 – 5.
30. de Vries TJ, Smeets M, de Graaf R, et al. Expression of gp100, MART-1, tyrosinase, and S100 in paraffin-embedded primary melanomas and locoregional, lymph node, and visceral metastases: implications for diagnosis and immunotherapy. A study conducted by the EORTC Melanoma Cooperative Group. J Pathol 2001;193:13 – 20.
31. Dalerba P, Ricci A, Russo V. High homogeneity of MAGE, BAGE, GAGE, tyrosinase and Melan-A/MART-1 gene expression in clusters of multiple simultaneous metastases of human melanoma: implications for protocol design of therapeutic antigen-specific vaccination strategies. Int J Cancer 1998;77:200 – 4.
32. Goydos JS, Patel M, Shih W. NY-ESO-1 and Ctpp1 expression may correlate with stage of progression in melanoma. J Surg Res 2001;98:76 – 80.
33. Jungbluth AA, Busam KJ, Gerald WL, et al. A013: an anti-melan-a monoclonal antibody for the detection of malignant melanoma in paraffin-embedded tissues [see comments]. Am J Surg Pathol 1998;22:595 – 602.
34. Busam KJ, Iversen K, Coplan KA, et al. Immunoreactivity for A013, an antibody to melan-a (MART-1), in adrenocortical and other steroid tumors. Am J Surg Pathol 1998;22:57 – 63.
35. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoeediting: from immunosurveillance to tumor escape. Nat Immunol 2002;3:991 – 8.
36. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immuno surveillance and immunoeediting. Immunity 2004;21:137 – 48.
37. Gajjar NA, Cochran AJ, Binder SW. Is MAGE-1 expression in metastatic malignant melanomas really helpful? Am J Surg Pathol 2004;28:883 – 8.
38. Brasseur F, Rinaldi D, Lihnard D, et al. Expression of MAGE genes in primary and metastatic cutaneous melanoma. Int J Cancer 1995;63:375 – 80.
39. Busam KJ, Iversen K, Berwick M, Spagnoli GC, Old LJ, Jungbluth AA. Immunoreactivity with the anti-MAGE antibody 57B in malignant melanoma: frequency of expression and correlation with prognostic parameters. Mod Pathol 2000;13:459 – 65.
40. Bolli M, Schulz-Thater E, Zajac P, et al. Expression of MAGE-1 in non-melanotic malignant melanoma: implications for vaccine development. Cancer Immunol Immunother 2003;52:169 – 76.
41. Bacchi CE, Bonetti F, Pea M, Martignoni G, Gown AM. HMB-45. A review. Appl Immunohistochem Mol Morphol 1996;4:73 – 85.
42. Miller RT, Kubier P. Blocking of endogenous avidin-binding activity in immunohistochemistry. The use of egg whites. Appl Immunohistochem Mol Morphol 1997;5:63 – 6.
43. Miller RT, Kubier P, Reynolds B, Henry T, Turnbow H. Blocking of endogenous avidin-binding activity in immunohistochemistry. The use of skim milk as an economical and effective substitute for commercial biotin solutions. Appl Immunohistochem Mol Morphol 1999;7:63 – 5.
Tumor Antigen Expression in Melanoma Varies According to Antigen and Stage

Catherine Barrow, Judy Browning, Duncan MacGregor, et al.

_Clin Cancer Res_ 2006;12:764-771.

Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/12/3/764

Cited articles
This article cites 34 articles, 10 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/12/3/764.full#ref-list-1

Citing articles
This article has been cited by 23 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/12/3/764.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/12/3/764.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.