Immunohistochemical and Molecular Analysis of Human Melanomas for Expression of the Human Cancer-Testis Antigens NY-ESO-1 and LAGE-1

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ABSTRACT

目的: NY-ESO-1和LAGE-1是同源的癌症-testis抗原,它们在不同的癌症中表达。这是典型肿瘤中评估患者适合度的临床试验所必需的。这项研究评估了用于区分这些两个同源但不同抗原的分型策略。这些结果表明，两种抗原的PCR方法不能提供关于微小异质性方面的信息。在LAGE-1基因中，同源性基因在非正常细胞中的表达特异性通过抑制寡核苷酸引物的结合，由此显示了qRT-PCR作为分型方法的精确性。

结论: NY-ESO-1分型通过抑制与分子方法和抗原的结合而产生新的NY-ESO-1表达。因为没有mAb结合LAGE并且没有同表达LAGE和NY-ESO-1之间的协调表达。

INTRODUCTION

为了优化癌症疫苗的开发，了解那些因素是导致成功或失败的一个疫苗是重要的。这包括了解抗原分布和工具的优缺点。NY-ESO-1是一个癌症-testis抗原，表达在70%的常见癌症中，包括黑色素瘤(34%；引文1)，膀胱癌(32-80%；引文2,3)，肺(21%；引文3)，以及某些罕见的癌症，如软骨肉瘤(80%；引文4)。初始免疫组织化学研究使用单克隆抗体(mAb) ES121显示了在所有癌症细胞中均匀的表达。表达可以范围从相对较少的细胞到罕见的细胞如软骨肉瘤。

更多最近，一个同源的抗原称为LAGE-1已经被描述了(6, 7)，因为有同源性 hilary@ludwig.edu.au.

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restricted antigenic peptides have been identified from LAGE-1 and alternative putative proteins. CTLs, specific for these epitopes, have been found (8, 9).

When evaluating patients as candidates for vaccine therapy, it is important to characterize accurately NY-ESO-1 expression in their tumors and to distinguish between reactivity for NY-ESO-1 and LAGE-1. This is particularly so if immune responses to nonshared epitopes are found to be clinically important. To establish the optimal methods for typing, comparisons of three independent assays for NY-ESO-1 and LAGE-1 were done: (a) immunohistochemical typing with two mAbs that bind NY-ESO-1 in paraffin-embedded tissue; (b) reverse transcription-PCR (RT-PCR); and (c) quantitative real-time (qRT-PCR) for each antigen. Here, we report the comprehensive analysis of these antigens in 120 malignant melanomas with these methods.

**MATERIALS AND METHODS**

**Tumor Samples and Cell Lines.** Tumor samples were collected from patients with melanoma with their informed consent and either snap frozen in liquid nitrogen and stored at −70°C for RNA extraction, or formalin-fixed and paraffin-embedded tissue sections were cut for immunohistochemistry. The melanoma cell line SK-MEL-37 was provided by the Ludwig Institute for Cancer Research (LICR), New York Branch. Protocols were approved by the Human Research Ethics Committee, Austin Health, Melbourne, Australia.

**Antibodies.** Two anti–NY-ESO-1 mAbs, E978 and ES121, were compared for reactivity by immunohistochemistry. The generation of these reagents was reported previously (5).

Mice were immunized with NY-ESO-1 fusion protein, and E978 (IgG1) was generated with bacterially synthesized full-length NY-ESO-1 protein of 180 amino acids and mAb ES121 (IgG1) with a truncated protein, which lacked the first 10 amino acids. ES121 cotypes with another NY-ESO-1–specific mAb B9.8 (23). Fig. 1 shows sequences of NY-ESO-1 and LAGE-1, including the protein translation and reported binding sites of the two antibodies (E. Stockert, LICR, New York Branch).

**Immunohistochemistry.** Paraffin-embedded sections were stained with E978 at a concentration of 3 μg/mL, and binding was detected with either a peroxidase-labeled streptavidin biotin-based assay (LSAB2 kit from Dako Corp., Carpinetta, CA) or a Vectastain Elite universal ABC kit (Vector Laboratories, Burlingame, CA), and 3-amino-9-ethyl-carbazole (Sigma-Aldrich, St. Louis, MO) was used as the chromogen. ES121 mAb was used at 9.4 μg/mL and as described previously (5). No antibodies for LAGE-1 were available. For each antibody one section was studied per tumor. Negative and positive controls were always included. These were as follows: omission of the primary antibody and inclusion of a known NY-ESO-1–positive tumor, respectively. Slides were scored by eye as a percentage of tumor cells staining for NY-ESO-1 and assigned to one of four groups: A (>50% cells staining); B (11 to 50% of cells staining); C <11% of cells staining; and D (no staining).

**Peptide ELISA.** Peptides (Abspep Pty. Ltd., Parkville, Australia) NY-ESO-1: AA 73–90; NGCCRCGARGPESRLLEF

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5 Unpublished data.
and AA 91–108: YLAMPFATMEAELARRS; LAGE-1: AA 73–90: DGRCPCGARRPDSRLLQL and AA 91–108: HIT-

Roger Murphy, LICR, Melbourne, Australia) were dissolved at MPFSSPMEAELVRRI, or NY-ESO-1 whole protein (Dr.

73–90: DGRCPCGARRPDSRLLQL and AA 91–108: HIT-

and AA 91–108: YLAMPFATMEAELARRS; LAGE-1: AA

used in each 25-

Sigma-Genosys (Castle Hill, New South Wales, Australia.)

rose gels and the DNA purified with Qiagen gel extraction kits

in a 20-

number in melanoma tissue.

in the qRT-PCR assays for the quantitation of mRNA copy

DNA sequence of both inserts was confirmed by DNA sequence

g). Plasmid preparations were made with Qiagen kits, and the

cloned into pBluescript-KS with a TOPO A cloning kit (Invitro-

amplified from the melanoma cell line SK-MEL-37, with the

Branch). For the LAGE-1 plasmid, a RT-PCR product was

was used to determine the tissue distribution of NY-ESO-1 and

was used to synthesize cDNA in a 20-μL reaction, with 1 μg of random hexamers (Promega, Madison, WI), 1 mmol/L deoxynucleoside triphosphates (Ap-

plied Biosystems, Foster City, CA), 40 units of RNase inhibitor

Madison, WI), 1 mmol/L deoxynucleoside triphosphates (Ap-

plied Biosystems; ref. 25). cDNA was syn-

thesized as above. One μL of cDNA (equivalent to 100 ng of total RNA) served as a template, and the PCR was set up

to distinguish amplified genomic DNA. Primer and probe se-

quences are as follows: NY-ESO-1 primer 1 (forward), 5’-tgcttggatctctagtgc-3’; NY-ESO-1 primer 2 (reverse), 5’-tactggtcggacagcttgca-3’; NY-ESO-1 probe, FAM-aggattgcececatgcc-

ctcctc-TAMRA; LAGE-1 primer 1 (forward), 5’-ggcttgtcgtgc-

cate-3’; LAGE-1 primer 2 (reverse), 5’-ggctgtcgtgcactc-

3’; and LAGE-1 probe, FAM-cgtgcctgctcttgct-TAMRA. The binding sites of these are shown in Fig. 1. A multiplex PCR was set up with the housekeeping gene β-actin to normalize the cDNA samples. It was first determined that the expression of β-actin was consistent in samples of measured amounts of cDNA amplified from 10 different melanoma tissues. Copy numbers of NY-ESO-1 and LAGE-1 were calculated from standard curves of the relevant plasmid and expressed per 10^5 copies of the housekeeping gene β-actin in 100 ng of total RNA. The cycle threshold (Ct) value is defined as the PCR cycle at which a product begins to be amplified, thus the lower the Ct value, the higher the initial copy number.

RESULTS

Comparison of Monoclonal Antibodies ES121 and E978 in Immunohistochemical Examination of Malignant Melanoma

The anti–NY-ESO-1 mAb ES121 has been described previously (5). To validate results obtained with mAb E978, the two were compared for the detection of NY-ESO-1 in paraffin-embedded tissue (Table 1). Forty melanomas were compared with both mAbs, and staining was categorized as described in Materials and Methods. For ES121, five were in group A (>50% cells staining), seven were in group B (11 to 50% cells staining), five were in group C (<11% cells staining), and twenty-three were in group D (no staining) compared with seven, nine, five, and nineteen, respectively, for E978. In four of the tumors that were positive for E978 but did not bind E121, staining was weak, and the proportion of positive cells was small. In one sample that showed no staining with the ES121, staining with E978 was intermediate. For all of the remaining cases, staining patterns were similar. In some, when adjacent sections were compared with both antibodies, the same regions stained; however, staining with ES121 was less intense and tended to stain fewer cells. In general, the mAbs showed similar staining patterns in tumors that had either high levels (Fig. 2, A and B) or heterogeneous expression (Fig. 2, C and D) of NY-ESO-1, and this was independent of magnification (Fig. 2,
On the basis of these results, E978 was used for all additional evaluations described here.

Reactivity of Antibody E978 on NY-ESO-1 and LAGE-1 Peptides by ELISA

The NY-ESO-1 epitopes recognized by mAbs ES121 and E978 have previously been mapped to amino acids 73–90 and 91–108, respectively (E. Stockert, LICR, New York Branch; Fig. 1).\(^6\) Both of these regions have 62% amino acid identity with the LAGE-1 antigen, which differs by seven amino acids in each of these 18 amino acid peptides. Consequently, it was uncertain whether either mAb was capable of binding LAGE-1. To evaluate this, synthetic peptides for the NY-ESO-1 and LAGE-1 sequences were used in ELISA assays. In both cases, mAb bound well to NY-ESO-1 protein and the respective peptides E978: NY-ESO-1\(^{73-90}\) and ES121: NY-ESO-1\(^{91-108}\) (data not shown). In contrast, neither bound to the LAGE-1 peptides at concentrations of ≤1 µg/mL. Although LAGE-1 protein was not available for specific testing, on the basis of these studies, neither antibody appears to be capable of binding to these epitopes when presented as peptides. The isotype control mAb to CD3 did not bind to any of the antigens tested (data not shown).

Validation of NY-ESO-1 and LAGE-1 RT-PCR Products

RT-PCR. The specificity of each set of oligonucleotide primers was evaluated by conventional RT-PCR and qRT-PCR with two plasmids containing either NY-ESO-1 (Fig. 3A) or LAGE-1 (Fig. 3B) cDNA as templates. The NY-ESO-1 primers amplified a single product from the NY-ESO-1 plasmid, but no product was obtained from the LAGE-1 plasmid (Fig. 3A). These products were of the expected size (330 bp; Figs. 1 and 3A). The LAGE-1 primer set amplified a single product from the LAGE-1 plasmid of the correct size (358 bp). However, at a high copy number of NY-ESO-1 plasmid (10\(^{8}\) copies, but not ≤10\(^{7}\)), the LAGE-1 primers did amplify weakly a slightly higher product (~400 bp; Fig. 3B). This high copy number exceeds that seen in any of the clinical samples and is therefore unlikely to confound antigen analysis. In contrast, RT-PCR for NY-ESO-1 was highly specific. These primer sets were then used to amplify cDNA derived from the melanoma cell line SK-MEL-37, which expresses both NY-ESO-1 and LAGE-1.

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* Weak staining.

Validation of NY-ESO-1 and LAGE-1 RT-PCR Products

RT-PCR. The specificity of each set of oligonucleotide primers was evaluated by conventional RT-PCR and qRT-PCR with two plasmids containing either NY-ESO-1 (Fig. 3A) or LAGE-1 (Fig. 3B) cDNA as templates. The NY-ESO-1 primers amplified a single product from the NY-ESO-1 plasmid, but no product was obtained from the LAGE-1 plasmid (Fig. 3A). These products were of the expected size (330 bp; Figs. 1 and 3A). The LAGE-1 primer set amplified a single product from the LAGE-1 plasmid of the correct size (358 bp). However, at a high copy number of NY-ESO-1 plasmid (10\(^{8}\) copies, but not ≤10\(^{7}\)), the LAGE-1 primers did amplify weakly a slightly higher product (~400 bp; Fig. 3B). This high copy number exceeds that seen in any of the clinical samples and is therefore unlikely to confound antigen analysis. In contrast, RT-PCR for NY-ESO-1 was highly specific. These primer sets were then used to amplify cDNA derived from the melanoma cell line SK-MEL-37, which expresses both NY-ESO-1 and LAGE-1.

\(^6\) Unpublished data.

Fig. 2 Comparison of reactivity of two anti–NY-ESO-1 mAbs; ES978 (A, C, and E) and ES121 (B, D, and F) on melanoma tissue tested by immunohistochemistry.
NY-ESO-1 and LAGE-1 Expression in Melanoma

Both primer sets amplified products of the expected size (Fig. 3, A and B) with an additional higher band (~550 bp) amplified with the LAGE primers. The DNA sequences of each product were confirmed by sequence analysis and found to be identical to the published sequences of NY-ESO-1 and LAGE-1, respectively. The higher band from SK-MEL 37 was confirmed to be the LAGE long variant.

**qRT-PCR.** Unique primers and probes designed for qRT-PCR of NY-ESO-1 and LAGE-1 were tested on the NY-ESO-1 and LAGE-1 plasmids and SK-MEL 37 melanoma cell line with NY-ESO-1 primers (A) and LAGE-1 primers (B). Ct values obtained by qRT-PCR on NY-ESO-1and LAGE-1 plasmids with NY-ESO-1 primers and probe (C) and LAGE-1 primers and probe (D).

![Plasmid Copy Number NY-ESO-1 LAGE-1](image)

![NY-ESO-1 LAGE-1](image)

![NY-ESO-1 Plasmid Copy Number CT Value](image)

![LAGE-1 Plasmid Copy Number CT Value](image)

Both primer sets amplified products of the expected size (Fig. 3, A and B) with an additional higher band (~550 bp) amplified with the LAGE primers. The DNA sequences of each product were confirmed by sequence analysis and found to be identical to the published sequences of NY-ESO-1 and LAGE-1, respectively. The higher band from SK-MEL 37 was confirmed to be the LAGE long variant.

**qRT-PCR.** Unique primers and probes designed for qRT-PCR of NY-ESO-1 and LAGE-1 were tested on the NY-ESO-1 and LAGE-1 plasmids. Fig. 3, C and D, shows the cycle threshold (Ct) values obtained on known copy numbers of the plasmids. In contrast to the primers used for conventional RT-PCR, the qRT-PCR reagents were found to be absolutely specific for their respective cDNA (Fig. 3, C and D).

**Analysis of Clinical Malignant Melanoma Samples.** A total of 120 samples was tested by RT-PCR. One was a primary tumor, the remainder were metastases that had been resected from lymph node (n = 48), skin/subcutaneous sites (n = 46), lung (n = 6), brain (n = 2), liver (n = 3), bowel (n = 5), and other sites (n = 9). Of these tumors, 11 were solely positive for NY-ESO-1 by RT-PCR, 15 were solely positive for LAGE-1, and 37 were positive for both (Table 2A). In addition, a low abundance cDNA band was detected in some patient samples (~550 bp). Sequence analysis confirmed that this was a previously described splice variant LAGE-1a (26).

**Reactivity of Antibody E978 on NY-ESO-1 and LAGE-1 Proteins by Immunohistochemistry.** To assess the specificity of the mAb E978 for NY-ESO-1 and LAGE-1, 120 melanomas were immunostained with E978 (Table 2A). Tumors were characterized by the two RT-PCR methods and were characterized as being positive or negative for NY-ESO-1 and LAGE-1. Antibody staining was then evaluated for each of the four combinations: NY-ESO-1+/LAGE-1−, NY-ESO-1+/LAGE-1−, NY-ESO-1−/LAGE-1+, and NY-ESO-1−/LAGE-1−. Of the 120 tumors which were PCR +ve for NY-ESO-1, 48 stained with E978. Fifty one were positive by qRT-PCR and of these 43 stained with E978, so there was good correlation between both of these molecular methods and antibody staining (Table 2A). Thirty-seven samples expressed both antigens by RT-PCR, and of these, 33 (89%) bound E978. Interestingly, of the 57 tumors that were negative for both NY-ESO-1 and LAGE-1, 9 (16%) nonetheless bound E978. Of these, six contained only a very low percentage of cells that were E978 positive (group C, <1% cells staining), and the remaining three were in group B (11 to 50% cells staining). To assess the possibility that E978 might bind to LAGE-1, staining was examined in the 15 tumors that typed negative for NY-ESO-1 and positive for LAGE-1. Of these, two (13%) showed immunostaining, suggesting possible cross-reactivity or alternatively binding to some as yet undefined antigen. Table 2A also shows a parallel analysis done with qRT-PCR to assess antigen expression. All tumors containing >25 copies mRNA were deemed to be positive for antigen expression because above this copy number assays were reproducible. For reasons discussed below, qRT-PCR analysis resulted in fewer LAGE+ tumors, so the numbers in each of the four categories differed. Nonetheless, the trends were the same. None of the 10 tumors that were positive for LAGE but negative for NY-ESO-1 by qRT-PCR stained with E978. Nonetheless, 11 (18%) of tumors that were negative for both antigens bound the antibody.

**Comparison of Each Method for Quantitation of NY-ESO-1 Expression in Tumor Tissues: Immunohistochemistry, RT-PCR, and qRT-PCR.** Melanoma tissue sections that bound the antibody E978 were arbitrarily categorized depending on the extent of staining: (a) group A, tumor contained >50% melanoma cells staining positive for NY-ESO-1; (b) group B, 11 to 50% positive cells; and (c) group C, 1 to 10% positive cells. To evaluate the correlation between immunohistochemistry and the two PCR-based methods, tumors categorized on this basis were assessed by both methods. Table 2 shows the relationship between RT-PCR and immunohistochemistry. The amount of the amplified product was categorized according to the intensity of the band seen on ethidium bromide-stained gels. Of the 120 tumors, 54 stained with E978. Among the 66 that were negative by immunohistochemistry, 5 were positive for NY-ESO-1 gene expression by conventional RT-PCR. Four of these were also positive by qRT-PCR, but copy numbers were low (ranging between 32 and 94 copies). Among the NY-ESO-1 group A tumors (n = 23), there were no major discrepancies between
immunohistochemistry and qRT-PCR, although the range of copy numbers was large (137 to 17,717). The largest number of discrepancies was found in tissues that were classified as having 11–50% positive staining cells or distribution of the target antigen(s) may have important impact on the success of a vaccine, so detailed knowledge about expression of these molecules may be critical. The purpose of this study was to evaluate immunohistochemistry and molecular methods for typing the homologous cancer-testis antigens NY-ESO-1 and LAGE-1 with two different mAbs and two RT-PCR primer sets specific for each antigen. We have detailed a new mAb against NY-ESO-1, E978, and have characterized the advantages of this antibody. The other base substitution detected is within exon 2 where a thymine (present in the LAGE-1 sequence) becomes a guanine (the base at this location in NY-ESO-1). This is a silent substitution and does not result in any amino acid change and does not involve the binding sites of either the LAGE-1 primers or probes (Fig. 1). A third reported substitution (6), which leads to an amino acid change at residue 6, does not occur within the DNA region amplified by any of the RT-PCR primers and so was not detected in this study.

Quantitation of mRNA for NY-ESO-1 and LAGE-1: Relationship between Copy Number of Each Antigen. Because both antigens were commonly co-expressed, we investigated whether there was a relationship between mRNA copy number, which might suggest that a common mechanism underlay expression of each. Fig. 4B shows the correlation based on copy number ($r = 0.0063$). There was no correlation, indicating that any regulatory mechanisms for expression of these antigens are independent of each other.

**DISCUSSION**

For cancer clinical trials that target defined antigens, typing criteria are required for patient selection. The presence, absence or distribution of the target antigen(s) may have important impact on the success of a vaccine, so detailed knowledge about expression of these molecules may be critical. The purpose of this study was to evaluate immunohistochemistry and molecular methods for typing the homologous cancer-testis antigens NY-ESO-1 and LAGE-1 with two different mAbs and two RT-PCR–based methods. This study is the first comprehensive comparison of methods used for typing tumors for these commonly expressed cancer antigens. We have detailed a new mAb against NY-ESO-1, E978, and have characterized the advantages of this antibody.
NY-ESO-1 and LAGE-1 Expression in Melanoma

Furthermore, peptide binding studies (data not shown) with ELISA showed that both antibodies were specific for NY-ESO-1 peptides and were incapable of binding to the corresponding LAGE-1 peptides, indicating that neither was suitable for detection of LAGE-1. Thus, until LAGE-1 specific antibodies become available, RT-PCR–based methods are required for the detection of LAGE-1.

These molecular methods proved to be reliable and specific with several caveats: (a) when NY-ESO-1 plasmid DNA was present at high copy number (≥10^6 copies/10^6 copies of β-actin in 100 ng of RNA), some nonspecific amplification by the LAGE-1 primers occurred (Fig. 3). Because none of the tumors studied here had >10^6 copies of NY-ESO-1 mRNA, this is unlikely to be a practical problem. (b) The qRT-PCR assay detected LAGE-1 with greater stringency; however, polymorphisms in the LAGE-1 gene resulted in some false negative results and confounded this analysis in some patients.

Having characterized E978, it was then important to compare this antibody with the PCR-based methods. As shown in Table 2B, the concordance between the results obtained with the immunohistochemistry and RT-PCR assays for NY-ESO-1 was high, particularly in the tumor group that had high levels of NY-ESO-1 expression. Of the 48 samples that were positive by RT-PCR, most were also positive by immunohistochemistry; however, 5 (9%) were negative. These all had low copy numbers (<94) of NY-ESO-1, and mRNA positivity probably reflects the greater sensitivity, which is conferred by the amplification of PCR. Discrepancies in the results may also reflect sampling methods. Table 2A shows that 11 tumors that stained positive for NY-ESO-1 by immunohistochemistry were negative by conventional RT-PCR or qRT-PCR, respectively. As seen in Fig. 2, antigen expression can be focal, and it was not always feasible to extract RNA and prepare tissue sections from identical parts of the tumor sample. This may also account for the mismatch between results by each method.

For the purpose of quantitation, the qRT-PCR assay provided helpful information about mRNA copy number, and these correlated well with four arbitrary categories of NY-ESO-1 expression as defined by immunohistochemistry. Clearly the RT-PCR–based methods cannot provide information about the heterogeneity of antigen distribution in these tissues or reflect the expression level of tumor antigens in individual cells. It may therefore be desirable to use both immunohistochemistry and qRT-PCR to obtain information about both expression level and antigen distribution.

On a tumor-by-tumor basis, it was clear that NY-ESO-1 and LAGE-1 were not consistently co-expressed. Although they were both present in 31% (37 of 120) of samples as shown by RT-PCR or 30 as shown by qRT-PCR (Table 2A, Fig. 4B), many tumors expressed one or other but not both. Because these antigens can share epitopes, the question arises whether it is really necessary to type for both antigens. This is likely to depend on whether the immunodominant epitopes lie within shared or dissimilar regions of the proteins. Presently, the mapping of NY-ESO-1 and LAGE-1 epitopes is not complete, and very little is known about immunodominance patterns among these epitopes. Nonetheless, for some stretches of the NY-ESO-1 molecule (e.g., amino acids 165–177), several epitopes are known to exist and homology with LAGE-1 is high. If these

![Fig. 4 Antigen distribution. A, relationship between copy number by qRT-PCR and extent of expression of NY-ESO-1 defined by immunohistochemistry (IHC). Shown are the range, median, and 75th and 25th percentiles for mRNA copy number of NY-ESO-1 in each group. The dotted line (25 copies) represents the level above which the qRT-PCR assay was deemed positive. B, relationship between NY-ESO-1 and LAGE-1 mRNA copy number. The hatched box contains those samples that were negative (<26 copies/10^6 copies of β-actin/100 ng RNA). R^2 = 0.0063.](image-url)
turn out to be critical, the presence of either antigen could make a tumor susceptible to killing after vaccination with either one of these antigens. If other less homologous parts of the molecule turn out to be important, it may be more relevant to know precisely which antigen is present.

Because neither the PCR primers nor the antibodies that were used to detect NY-ESO-1 were reliable for detection of LAGE-1, it is clear that antigen-specific reagents would then be required for the confident detection of each antigen.

Lastly, the relationship between clinical outcomes after vaccination and the level of antigen expression needs to be considered. Antigen expression level is important because it may correlate with the likelihood of anticancer responses after successful vaccination. At present, little is known about the impact on clinical response rates when considering the level of antigen expression or the distribution of antigen throughout a tumor. This needs to be studied prospectively in clinical trials where antigen expression is well documented and where robust immune responses are induced, thus making it possible to prospectively evaluate any relationship between antigen expression and clinical benefit. Until these studies are done, it is too early to use these methods to include or rule out patients from participation in a clinical trial.

In vitro studies of tumor cell lines show that the level of protein expression in a tumor cell may influence the ability of a CTL to kill its target (27, 28). This has been assessed for MAGE-1 where it was estimated that only cells expressing >10% of MAGE-1 mRNA level of the reference cell line MZ2-MEL.3.0 were susceptible to cell lysis by CTLs. In a more recent study with a more accurate method of quantitation (28), expression levels of several melanoma antigens, including NY-ESO-1, were analyzed by real-time qRT-PCR on 12 melanoma cell lines. The levels of these transcripts were found to be extremely variable among the cell lines tested. A threshold level occurring CD4+ T cell responses against NY-ESO-1 in cancer patients: a promising major histocompatibility complex class II T-helper epitope recognized by Th1- and Th2-type tumor-reactive CD4+ T cells. Cancer Res 2002;62:213–8.


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Immunohistochemical and Molecular Analysis of Human Melanomas for Expression of the Human Cancer-Testis Antigens NY-ESO-1 and LAGE-1

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