Assessment of MAGE-A Expression in Resected Non–Small Cell Lung Cancer in Relation to Clinicopathologic Features and Mutational Status of EGFR and KRAS

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Abstract

Non–small cell lung cancer (NSCLC) is a major public health problem, accounting for more cancer-related deaths than any other cancer. Both immunotherapy, based on the expression of tumor-specific antigens, and targeted therapy, based on the presence of oncogenic mutations, are under development for NSCLC. In this study, we analyzed the expression of MAGE-A, a cancer–testis antigen, in tumors from a cohort of patients with resected NSCLC with respect to their clinicopathologic characteristics and their mutational status for the EGFR and KRAS genes. We found MAGE-A expression by IHC in 43% of the tumors. MAGE-A expression was significantly more frequent in squamous tumors than in adenocarcinomas, did not correlate with disease stage, but was correlated significantly with high tumor grade and worse survival. EGFR and KRAS mutations were present in adenocarcinomas, but not in squamous tumors. Whereas the presence of EGFR mutations did not seem to affect survival, the presence of KRAS mutations was associated with early-stage disease and better survival. MAGE-A expression was absent from adenocarcinomas with KRAS mutations, but not significantly different in tumors with or without EGFR mutations. Together, the reported results provide guidance for the design of combination therapies in patients with NSCLC. Cancer Immunol Res 2(10): 943–8. ©2014 AACR.

Introduction

Despite recent advances in treatment, non–small cell lung cancer (NSCLC) remains a major public health problem, accounting for more cancer-related deaths than any other cancer, in both male and female patients (1). Because symptoms usually appear late, the disease is frequently diagnosed at the locally advanced and metastatic stages, when it is difficult to treat. To complement the main treatment modalities (surgery, radiotherapy, and chemotherapy), targeted therapies are being developed that may be particularly effective in subgroups of patients with appropriate characteristics. For instance, tyrosine kinase inhibitors (TKI) have proved effective in patients with metastatic tumors that harbor the mutated EGFR gene (2, 3), whereas the presence of mutations in the KRAS gene has been proposed to correlate with resistance to treatment with TKI (4, 5). However, the number of patients for whom targeted therapy is available is small, and it is becoming clear that effects of these therapies may not always be long lasting due to the development of resistance (6, 7). Therefore, additional therapeutic approaches are urgently needed both for treating larger numbers of patients, including those with early-stage disease, and for inducing stable clinical responses and extended survival.

On the basis of the knowledge that NSCLC expresses tumor-specific antigens and is immunogenic, one emerging treatment approach is immunotherapy (8). The MAGE-A family of tumor-specific cancer–testis antigens includes 15 genes clustered on Xq28 (9). MAGE-A-encoded antigens are expressed in various cancers, including NSCLC (10–12). The frequency of MAGE-A expression, however, is variable among tumor types and subtypes (13); whereas expression of MAGE-A is known to be regulated through epigenetic mechanisms (14), the molecular factors that account for this variability remain undefined. The physiologic function of MAGE-A proteins and their role in cancer have not been elucidated, although there is increasing evidence that some MAGE-A proteins may correlate with poor clinical outcome (11, 15). MAGE-A antigens, nonetheless, are immunogenic and are important targets of emerging immunotherapy approaches in NSCLC (16). New therapies using potent immunomodulatory antibodies are being assessed in NSCLC, and have yielded the first evidence of clinical efficacy (17, 18). The next few years will witness increasing application

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of immunotherapies and combination therapies in NSCLC (19). However, our knowledge of the expression of tumor-specific antigens in this heterogeneous patient population, and of the potential relationship between tumor-antigen expression, clinical outcome, and mutational status of the tumors, is limited. To address these questions and to help improve the design of future clinical studies, we used IHC to assess the expression of MAGE-A antigens in tumors from a large cohort of patients with resected NSCLC, and correlated the results with the patients' clinicopathologic characteristics and their tumors’ KRAS and EGFR mutational status.

Materials and Methods

Patients and tumors

Archival surgical tumor specimens were obtained from the Pathology Department of the Hospital General Universitario Gregorio Marañon (Madrid, Spain). The samples correspond to surgically resected stage I–IIIA NSCLC from patients who have undergone surgery between 1993 and 2008, and for whom an adequate amount of archived tumor was available for analysis. Clinicopathologic and clinical follow-up data were retrieved from patients’ clinical records. Samples were obtained with written informed consent from patients and approval of the hospital’s Ethical Committee for Clinical Research. Samples were fixed in buffered neutral formalin for 24 hours and automatically embedded in paraffin.

MAGE-A expression analysis

Sections measuring 5 μm were cut using a rotary microtome and mounted on xylanized slides. Sections were deparaffinized and rehydrated in graded alcohols. Deparaffinized samples were stained using MAGE-A–specific mAb (6C1; 0.3 μg/mL; Abcam). Counterstaining was performed with a hematoxylin solution. The detection of nuclear and/or of cytoplasmic staining in any percentage of tumor cells was considered positive. MAGE-A+ samples were further scored based on the percentage of MAGE-A+ tumor cells (1, <10%; 2, 10%–25%; 3, 25%–50%; and 4, >50%). Testis tissue was used as positive control.

EGFR and KRAS mutation analyses

Genomic DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissue samples using the QIAamp DNA FFPE Tissue Kit (Qiagen) according to the manufacturer’s recommendations, and EGFR (exons 18–21) and KRAS (exon 2) genes were amplified by PCR. EGFR gene mutations were determined by qRT-PCR using the Therascreen EGFR Mutation Test Kit (Qiagen) designed to detect the most commonly reported EGFR mutations [19 deletions in exon 19, 3 insertions in exon 20, and point mutations G719X (exon 18), S768I and T790M (exon 20), and L858R and L861Q (exon 21)]. Data were analyzed using the Rotor-Gene Q series software version 2.0.2. Mutational analysis of KRAS exon 2 was carried out by direct sequencing of PCR products. Briefly, PCR products were purified using Exol/SAP (37°C for 15 minutes, then 85°C for 15 minutes), and sequenced directly on both strands using the BigDyeH Terminator v1.1 cycle sequencing Kit (Applied Biosystems) according to the manufacturer’s protocol, and the ABI 3500 Genetic Analyzer (Applied Biosystem).

Statistical analysis

Statistical significance of the correlation between MAGE-A expression, EGFR mutations or KRAS mutations in tumors, and

Figure 1. Assessment of MAGE-A antigen expression in NSCLC. MAGE-A expression was assessed by IHC staining of FFPE NSCLC surgical tumor specimens using the specific mAb 6C1. Examples of MAGE-A− (A and B) and MAGE-A+ (C–F) squamous tumors (A and C), adenocarcinomas (B and D), adenosquamous (E), and large-cell tumors (F) are shown (magnification, ×20).
clinicopathologic features was determined using the \( \chi^2 \) or Fisher exact test. Survival analyses were performed using Kaplan–Meier curves taking into account death due to lung cancer (disease-specific survival), and statistical significance was assessed with the log-rank test (Prism; GraphPad Software Inc.).

**Results and Discussion**

We assessed tumor samples from a cohort of 216 patients with resected NSCLC (stage I–IIIA). Surgical procedures included lobectomy \((n = 153)\), bilobectomy \((n = 7)\), pneumonectomy \((n = 34)\), and other procedures \((n = 22)\). MAGE-A expression was assessed by IHC using the 6C1 mAb that recognizes MAGE-A1, -2, -3, -4, -6, -10, and -12 (20). Examples of staining results are shown in Fig. 1. Overall, we found expression of MAGE-A in 43% of the tumors (Table 1). It is noteworthy that although MAGE-A expression was heterogeneous, the majority of positive samples had a high expression score, with >50% of the tumor cells in the sample expressing MAGE-A (Supplementary Table S1). MAGE-A expression was significantly more frequent in patients aged younger than 60 years and in tumors from men versus those from women. Expression did not seem to be significantly related to the smoking history of the patients (Table 1).

The frequency of expression was variable among histologic subtypes and was about 4 folds more frequent in squamous than in adenocarcinomas (Table 1). We also found frequent expression in other histologic subtypes, including adenosquamous and large-cell tumors. The significant difference in MAGE-A expression between histologic subtypes is important

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<th>Table 1. MAGE-A expression and KRAS and EGFR mutations in NSCLC and correlation with clinicopathologic features</th>
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Abbreviation: ns, not statistically significant.

aMAGE-A expression was assessed by IHC as shown in Fig. 1.

bStatistical significance of the correlation between MAGE-A expression and KRAS and EGFR mutations in tumors and clinicopathologic features was determined using the \( \chi^2 \) or Fisher exact test.

cTumors’ KRAS and EGFR mutational status was assessed in PCR fragments amplified from genomic DNA extracted from FFPE tissue.

dNumbers between parentheses indicate the percentage of MAGE-A+ tumors within the subgroups defined according to the indicated clinicopathologic features.

eAge at diagnosis.

fStatistical analyses were performed taking into account the squamous cell carcinoma and adenocarcinoma tumor groups only.

gTumors of unknown grade were not included in statistical analyses.

hAmerican Joint Committee on Cancer (AJCC) 2007 stage.
for the design and evaluation of MAGE-A–based immunotherapy of NSCLC. The molecular mechanisms underlying the difference, however, remain unknown and deserve further investigation.

Expression was correlated significantly with high tumor grade (Table 1), consistent with the concept that the presence of MAGE-A identifies a subgroup of aggressive tumors. However, the frequency of expression was similar in tumors at early or at more advanced stages, a result that supports the view that MAGE-A–based immunotherapy can be applied to patients at early stages of the disease.

To evaluate the relationship between MAGE-A expression and clinical outcome, we performed a Kaplan–Meier analysis of disease-specific survival. When analyzing the entire cohort, we found no significant difference in patients' survival based on differences in histologic subtype, disease stage, or tumor grade (Supplementary Fig. S1). It is noteworthy that the lack of correlation between disease stage and patients' survival could be attributable to the high proportion of patients with squamous tumors in the cohort, whose survival was independent of disease stage (stage I vs. stages II and III; \( P = 0.261 \)), in agreement with previous reports (21). In contrast, survival of patients with adenocarcinomas significantly correlated with disease stage, being better for patients with early-stage disease (stage I vs. stages II and III; \( P < 0.001 \)). Interestingly, we found that patients with MAGE-A–expressing tumors exhibited worse survival than those with MAGE-A–negative tumors (Fig. 2A).

To address whether MAGE-A expression differentially affected the survival of patients with tumors of different histologic subtype, stage, or grade, we analyzed patient survival with respect to MAGE-A expression and to each of these variables. For patients with squamous tumors, we found no significant difference in survival between patients with MAGE-A–expressing or MAGE-A–negative tumors (Fig. 2A). In contrast, for patients with adenocarcinomas or tumors of other histologic subtypes, survival was significantly worse for patients with MAGE-A–expressing tumors. There was no

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**Figure 2.** Correlation between MAGE-A expression and EGFR and KRAS mutations in NSCLC tumors and patient survival. Analysis of disease-specific survival of patients was assessed using Kaplan–Meier curves, and statistical significance of the difference between groups was determined using the log-rank test. The median duration of follow-up of patients in the cohort was 57 weeks (range, 1–169 wk). A–C, patient survival in relation to MAGE-A expression in tumors was analyzed in the total cohort (A) or in subgroups according to tumor histologic type (A), tumor grade (B), and disease stage (C). D and E, survival was analyzed for patients with adenocarcinomas, in relation to EGFR and KRAS mutational status of the tumors (D) and to disease stage and tumor grade (E).
significant association between MAGE-A expression and survival with respect to histologic grade (Fig. 2B). However, we found that expression of MAGE-A was significantly associated with worse survival in patients with early-stage disease, but not in those with more advanced stages of disease (Fig. 2C). Thus, whereas MAGE-A expression is not an independent prognostic factor in NSCLC, it could be a valuable prognostic factor within certain tumor subtypes and disease stages. This conclusion, however, should be corroborated by further analyses of larger cohorts.

Next, we investigated the association between expression of MAGE-A and the presence of EGFR and KRAS mutations. To this end, we initially assessed the EGFR and KRAS mutational status in all tumors from the cohort (Table 1 and Supplementary Table S2). We did not detect EGFR or KRAS mutations in squamous tumors. In contrast, we found that about 21% of the adenocarcinomas harbored EGFR mutations and about 27% of them harbored KRAS mutations. Adenosquamous tumors exhibited distinct features, as frequently they harbored EGFR mutations (50%), but not KRAS mutations and, vice versa, large-cell tumors frequently harbored KRAS mutations (31%), but not EGFR mutations. EGFR and KRAS mutations were mutually exclusive (Fig. 3).

The presence of EGFR mutations was significantly associated with female gender, whereas that of KRAS mutations was significantly associated with nonsmoker status (Table 1). We also found a significant association between the presence of KRAS mutations and low tumor histologic grade as well as early-stage disease. Consistent with previous data in resected NSCLC (22), the presence of EGFR mutations was not associated with significant differences in patient survival (Fig. 2D). In contrast, we found that patients with tumors harboring KRAS mutations had significantly prolonged survival (Fig. 2D). This result was in line with the frequent presence of patients with early-stage disease among the ones bearing KRAS mutated tumors, a feature that, within the adenocarcinoma group, was significantly associated with better survival (Fig. 2E).

The impact of KRAS mutations on the clinical outcome of NSCLC has thus far remained unclear. Some studies have identified KRAS mutations as a poor prognostic factor, but others have not confirmed these results (23, 24). The discrepant results obtained in this study with respect to some previous studies are likely due to the fact that most of the latter included patients with advanced lung cancers, in which KRAS mutations generally coexist with other alterations. Our data, however, are in line with the recently proposed concept that KRAS mutations occur at very early stages of NSCLC carcinogenesis, and, in the absence of additional alterations, rarely lead to tumor progression (25).

Finally, we aimed at assessing the relationship between MAGE-A expression and the mutational status of the tumors (Fig. 3). In adenocarcinomas, we found MAGE-A expression in a similar proportion of EGFR wild-type (WT) or mutated tumors (13.6% vs. 25%, not statistically significant). In contrast, we found that MAGE-A expression was negatively associated with the presence of KRAS mutations (0% vs. 21.8%, P = 0.02). In contrast, in NSCLC tumors of other histologic subtypes, we found no significant association between MAGE-A expression and the presence of EGFR (50% vs. 20%, not statistically significant) or KRAS (46.7% vs. 44.4%, not statistically significant) mutations.

Together, these results indicate that MAGE-A expression in resectable NSCLC identifies a heterogeneous subgroup of patients with rather aggressive tumors that can harbor or not harbor EGFR or KRAS mutations and are associated with poor survival. Several conclusions can be drawn from these results with regard to the development of MAGE-A–based immunotherapy in NSCLC, alone or in combination with targeted therapies. First, the frequent expression of MAGE-A in early stages of the disease encourages the development of MAGE-A–based immunotherapy for this group of patients, who can most benefit from it. Second, the frequent expression of MAGE-A in squamous tumors, for which only conventional therapy is presently available, encourages the selection of these patients for assessing new approaches combining conventional therapy and immunotherapy. In adenocarcinomas, expression of MAGE-A is found in tumors with both WT and mutated KRAS and EGFR mutations.
Expression of the MAGE-A family of genes in cancer has been extensively studied in recent years. MAGE-A proteins are expressed in a variety of cancer types, including melanoma, lung cancer, and breast cancer. The expression of these genes is often associated with poor prognosis, and their targeting has been explored as a potential therapeutic strategy.

EGFR, which could also be selected for immunotherapy, in the latter case in combination with TKI. Finally, the correlation between MAGE-A expression and poor survival in patients with NSCLC tumors of histologic subtypes other than squamous cell carcinoma and adenocarcinoma warrants further investigation, both with respect to its possible use for stratification of this heterogeneous subgroup and the inclusion of these patients in immunotherapy protocols.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Writing, review, and/or revision of the manuscript: M. Ayyoub, E. Álvarez-Fernández, D. Valmori
Study supervision: M. Ayyoub, D. Valmori

References

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Analysis and interpretation of data (e.g., statistical analysis, bio-statistics, computational analysis): M. Ayyoub, L. Memeo, E. Álvarez-Fernández, R. Costanzo, E. Aiello, D. Martinetti, D. Valmori
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