A threshold of systemic MAGE-A gene expression predicting survival in resected non-small cell lung cancer

Ingo Mecklenburg¹*, Wulf Sienel², Severin Schmid², Bernward Passlick², Peter Kufer³

1 Klinikum Landsberg, Department of Internal Medicine, Landsberg am Lech, Germany
2 University Hospital Freiburg, Department of Thoracic Surgery, Freiburg, Germany
3 Amgen Research Munich, Germany

* Corresponding Author: Dr. Ingo Mecklenburg, Klinikum Landsberg, Department of Internal Medicine, Bgm.-Dr.-Hartmann-Str. 50, 86899 Landsberg am Lech, Germany. Phone: +49-8191-333-1007; FAX: +49-8191-333-1009; Email: ingo.mecklenburg@klinikum-landsberg.de

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Running title: MAGE-A expression predicts survival in NSCLC
**Translational relevance:**

Tools for the reliable quantification of systemic tumor load in individual cancer patients are only available in very few indications such as B-lineage acute lymphoblastic leukemia but are still missing in most solid tumors including resected lung cancer. MAGE-A genes have been described as broadly tumor specific antigens and have been investigated as biomarkers in several *in-vitro* studies. A study was conducted for the evaluation of the clinical relevance of a quantitative multimarker MAGE-A real-time PCR for the quantification of disseminated systemic tumor load in individual patients with resected non-small cell lung cancers. The quantitative measurement of MAGE-A expression in blood and bone marrow is a statistically significant and independent predictor of survival and most strongly correlates with the development of distant metastasis in such patients. Thus, the quantitative measurement of minimal residual disease can help to predict the individual clinical outcome of patients with solid tumors.
ABSTRACT

Purpose: Quantitative measurement of minimal residual disease (MRD) predicting recurrence in individual cancer patients is available only in very few indications such as acute lymphoblastic leukemia but is still missing in most solid tumors including non-small cell lung cancer (NSCLC).

Experimental design: MAGE-A expression levels in blood and bone marrow determined as calibrator normalized relative ratios by quantitative multimarker real-time RT-PCR for transcript amplification of MAGE-A1, -A2, -A3/6, -A4, -A10 and -A12 in 94 patients with completely resected NSCLC were correlated with survival in a clinical study.

Results: Patients with MAGE-A expression levels ≥ 0.2 in at least one sample of bone marrow or blood at tumor surgery had a significantly reduced overall (p = 0.007), cancer-free (p = 0.002), and distant-metastasis-free survival (p < 0.001) versus patients below 0.2 in all samples without significant difference in locoregional-recurrence-free survival. The corresponding hazard ratios (≥ 0.2 vs. < 0.2) for death, cancer-related death and development of distant metastasis were 2.56 (95% CI, 1.42 – 4.63), 3.32 (95% CI, 1.66 - 6.61), and 4.03 (95% CI, 1.77 - 9.18), respectively. Five-year Kaplan-Meier estimates of distant-metastasis-free survival were 43% (MAGE-A ≥ 0.2) versus 87% (MAGE-A < 0.2).

Conclusions: MAGE-A expression in blood or bone marrow at tumor surgery is an independent predictor of survival in resected NSCLC. The reliable prediction of distant metastasis in individual patients with a statistically proven impact on overall survival may help to refine patient selection for adjuvant therapy urgently needed especially in the clinical management of elderly patients.
INTRODUCTION

Lung cancer is the leading cause of cancer-related death in the United States and Europe (1). Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all cases (2). The 5-year survival of patients with resected stage IA NSCLC is only 73% and drops to 24% in resected stage IIIA NSCLC (3). Recurrences in completely resected NSCLC are thought to originate from the post-operative outgrowth of minimal residual disease (MRD) caused by pre-operative dissemination of cancer cells, that remain undetected by conventional staging procedures at the time of surgery. However, quantitative measurement of MRD predicting disease free-survival and/or clinical recurrence in individual patients reliably enough to inform therapeutic decisions has been established only in a few malignancies such as B-lineage acute lymphoblastic leukemia (B-ALL). More than 90% of adult patients with B-ALL in complete hematological remission, who failed to clear MRD from bone marrow as determined by real-time quantitative polymerase chain reaction (PCR) develop a hematological relapse (4). Availability of PCR markers highly specific for the malignant cells such as individual rearrangements of immunoglobulin genes was a key success factor for the advancement of MRD assessment from an exploratory method into an established staging procedure for clinical patient management (5).

For MRD assessment in NSCLC and other solid tumors several members of family A of melanoma-associated antigens (MAGE-A) are available as PCR markers of similarly high tumor specificity (6). The MAGE-A gene family has 15 members located on chromosome Xq28 (7–9). The MAGE-A gene family belongs to the family of Cancer/Testis(CT)-antigens, which are normally restricted in their adult tissue expression to testis and placenta (10) and expressed briefly during early embryonic development (11). In tumor cells, genome-wide epigenetic reprogramming frequently leads to activation of MAGE-A expression through promoter hypo-methylation (12).
addition, other chromatin remodeling events like histone acetylation and methylation further modulate MAGE-A expression. While little is understood of the physiological function of MAGE-A proteins, there is more clarity on their role in promoting malignancy. MAGE-A proteins interfere with two major tumor suppressor mechanisms: By suppressing p53-mediated transcription they inhibit both p53-mediated apoptosis and senescence (13). Moreover, by targeting the p53 pathway, MAGE-A proteins confer resistance to chemotherapeutic drugs that act via p53-mediated apoptosis (14).

Most types of solid tumors including NSCLC frequently express at least one out of several MAGE-A family members (15,16). Therefore we used an established quantitative multimarker real-time reverse transcription (RT)-PCR for transcript amplification of MAGE-A1, -A2, -A3/6, -A4, -A10 and -A12 (17) in a proof-of-concept study designed to investigate whether the MAGE-A expression level in blood or bone marrow at the time of tumor surgery is an independent predictor of survival in patients with resected NSCLC. We tested the hypothesis, that detection of systemic MAGE expression in blood and/or bone marrow aspirates is associated with the formation of distant metastasis and cancer-related death. Furthermore, the protocol was designed to determine a quantitative cut-off value of MAGE expression in patients with disseminated tumor load in NSCLC, as distinct expression levels have not been examined before.
PATIENTS AND METHODS

Trial design

Patients with suspected localized NSCLC (UICC stage Ia-IIla) planned to undergo tumor resection by lobectomy or pneumonectomy with systematic mediastinal lymphadenectomy at the University Hospital Freiburg, Germany, were enrolled consecutively between August 2004 and March 2008. The study protocol was approved by the ethics committee of the University of Freiburg. All patients gave written informed consent. Preoperative staging included computed tomography of the head, chest, and abdomen as well as a bone scintigraphy. Patients with R2-resection, overt distant metastasis, neoadjuvant therapy or a history of further malignant disease were excluded.

For measuring the MAGE-A expression level in blood and bone marrow by quantitative multimarker real-time reverse transcription polymerase chain reaction (RT-PCR), all patients underwent bilateral bone marrow aspiration through an aspiration needle from each anterior iliac crest and donated peripheral blood immediately before thoracotomy. Tumors were classified according to the WHO classification for histologic tumor typing (18). The tumor stage was classified according to the 7th edition of the International Union against Cancer (UICC) tumor-node-metastasis classification (19). Only patients with histologically confirmed NSCLC and complete tumor resection (R0 and R1) were included in the prospective study. Patients with microscopic residual tumor at the bronchial margin (R1 resection) received a recommended adjuvant cisplatin-based chemotherapy according to the IALT-study protocol (20).

Follow-up assessments comprised physical examination, chest X-ray, and blood tests at 3-month interval and an additional thoracic computed tomography scan,
abdominal ultrasound, and bronchoscopy at 6-month interval. In addition, family practitioners were contacted to obtain information about locoregional relapse, distant metastasis, and death. The median observation period was 43 months (range 1 – 95 months).

The primary study endpoint was postoperative distant-metastasis-free survival defined as the postoperative time to distant metastasis without prior locoregional recurrence. Secondary endpoints were locoregional-recurrence-free, cancer-free and overall survival defined as the postoperative time to locoregional recurrence without prior distant metastasis, to any locoregional recurrence or distant metastasis and to death from any cause, respectively.

**Quantitative multimarker MAGE real-time RT-PCR**

The multimarker MAGE real-time RT-PCR was described elsewhere (6,17). The detailed protocol used in this study is found in the supplement. The MAGE-A gene expression level as determined herein is equal to 2.5-times the number of MAGE-A mRNA molecules per PBGD mRNA molecules in the blood or bone marrow sample from a cancer patient relative to/divided through the number of MAGE-A mRNA molecules per PBGD mRNA molecules in the calibrator sample consisting of 2 mL of healthy blood spiked with 10 Mz2-Mel melanoma cells or LB23-SAR sarcoma cells.

**Statistical analysis**

SPSS software (version 21.0 for PC, IBM Inc.) was used for statistical calculations. To analyze a possible association of bone marrow and blood findings with clinicopathological variables, the two-tailed Pearson’s $\chi^2$ test or Fisher’s exact test in frequencies < 5 were used. The threshold for statistical significance was $p < 0.05$. Distant-metastasis-free, locoregional-recurrence-free, cancer-free and overall
survival were characterized using Kaplan-Meier plots and survival distributions were compared by log-rank statistics.

The joint effects of other prognostically relevant variables were further examined using the Cox proportional hazard model. The respective covariables were entered stepwise forward into the model to assess the possible independence of the prognostic value of MAGE-A gene expression. The 0.05 level of significance was used for entering or removing a covariable.
RESULTS

Characteristics of the patients

116 patients with suspected lung cancer were enrolled in the study (a patient flow diagram is depicted in Figure S1 in the supplement). According to postoperative assessment, 94 patients with histopathologically confirmed non-small cell lung cancer (NSCLC) fulfilled the inclusion criteria. 22 patients dropped out because of a benign histology such as tuberculosis or pneumonia (n=5), small-cell lung cancer (n=4) or due to incomplete tumor (R2) resection (n=13). Nine patients were included in whom ipsilateral intrapulmonary secondary lesions were found during tumor surgery, which could be removed in parallel. Clinicopathological characteristics are shown in Table 1. Seven patients had microscopic residual tumor at the bronchial margin (R1 resection). The median age at the time of surgery was 66 years (range, 44 – 82 years). Follow-up information was available for 89 of 94 patients (94.7%). The median observation period was 43 months (range 1 – 95 months). 49 patients died within the observation period (55.1%). Table 2 shows treatment failures according to site of recurrence and MAGE-A expression level in bone marrow or blood.

MAGE-A expression in blood and bone marrow

In total, 1848 expression profiles of seven MAGE-A genes in 264 bone marrow and blood samples of 94 patients were created (Supplementary Table S1). 15 and 3 samples dropped out due to vial damage or failure of amplification of the housekeeping marker PBGD, respectively. Quantifiable MAGE-A expression i.e. expression at or above the lower limit of quantification (LLOQ = 0.01) of at least one MAGE-A gene in at least one sample of bone marrow or blood was detected in 43.6% of patients (n=41). No statistical correlations were found between MAGE-A
expression and tumor extension, grading, histology, lymph node status or age of the patients (Table 1).

**Correlation of MAGE-A expression with survival**

To determine the impact of different MAGE-A expression levels in bone marrow or blood on patients’ clinical outcome according to the primary endpoint, the distant-metastasis-free survival of patients with a MAGE-A expression level at or above a certain threshold value in at least one sample of bone marrow or blood was compared with patients with sub-threshold MAGE-A expression in all samples. Patients with a MAGE-A expression ≥ LLOQ in at least one sample of bone marrow or blood differed only with borderline statistical significance from patients without quantifiable MAGE-A expression in all samples (p = 0.049, log-rank test). However, patients with a MAGE-A expression level ≥ 0.05, 0.1, 0.2, 0.3 and 0.5 in at least one sample of bone marrow or blood showed a statistically significant difference in distant-metastasis-free survival to patients below the respective threshold in all samples with p-values of 0.013, 0.002, <0.001, 0.004 and 0.013, respectively (log-rank test, Table 3). Thus, with a p-value of <0.001 the MAGE-A expression level of ≥ 0.2 in at least one sample of bone marrow or blood versus MAGE-A expression below 0.2 in all samples clearly distinguishes best between patients with a higher risk of developing distant metastasis versus patients with a lower risk (Figure 1, A). The corresponding 5-year Kaplan-Meier estimates of distant-metastasis-free survival were 87% (95%CI ±10%) for patients with MAGE-A expression < 0.2 versus 43% (95%CI ±11%) for ≥ 0.2.

As to the secondary endpoints, log-rank tests revealed a significantly reduced cancer-free survival (p=0.002) and overall survival (p=0.007) between the two patient subgroups (≥ 0.2 vs. < 0.2) (Figure 1, B and C). The corresponding 5-year Kaplan-
Meier estimates of overall survival were 59% (95%CI ±14%) for patients with MAGE-A expression < 0.2 versus 26% (95%CI ±8%) for ≥ 0.2. The corresponding estimates for cancer-free survival were 69% (95%CI ±14%) versus 31% (95%CI ±18%).

In contrast to the strong correlation of MAGE-A ≥ 0.2 in bone marrow or blood with the development of distant metastasis, there was no significant difference in locoregional-recurrence-free survival (p=0.26, log-rank test) between patients with MAGE-A ≥ 0.2 in bone marrow or blood and patients below 0.2 in all samples (Figure 1, D).

In the subgroup of patients with R1 resection (n=7) three patients died due to local relapse. The remaining four patients had an uneventful course of disease. Further subgroup analyses on the correlation of MAGE-A expression with survival are found in the supplement.

**MAGE-A expression in bone marrow or blood is an independent predictor of survival**

Multivariate analysis using the Cox proportional-hazard model revealed that MAGE-A expression in bone marrow or blood at levels ≥ 0.2 is a significant prognostic factor predicting death of any cause (p=0.002), cancer-related death (p=0.001), and development of distant metastasis (p=0.001) independently from standard prognostic factors of survival such as tumor extension, tumor histology, grading, and age of patient at the time of surgery (Table 4). MAGE-A expression ≥ 0.2 in blood or bone marrow was the only significant predictor of distant metastasis with a hazard ratio (≥ 0.2 vs. < 0.2) of 4.03 (95% CI, 1.77 - 9.18). Hazard ratios for cancer-related death were 3.32 (95% CI, 1.66 - 6.61 95%CI) for MAGE-A expression (≥ 0.2 vs. < 0.2), 1.65 (95% CI, 1.11 – 2.45) for tumor size (T3-4 vs. T1-2) and 1.56 (95% CI, 1.07 – 2.29) for lymph node status (N2 vs. N0-1). The false positivity rate of MAGE-A expression ≥ 0.2
for cancer-related death was 18.3%. Hazard ratios for death of any cause were 2.56 (95% CI, 1.42 – 4.63) for MAGE-A expression (≥ 0.2 vs. < 0.2), 1.71 (95% CI, 1.23 – 2.38) for tumor size (T3-4 vs. T1-2) and 1.39 (95% CI, 1.00 – 1.93) for lymph node status (N2 vs. N0-1).
DISCUSSION

This proof-of-concept study demonstrated that MAGE-A expression at levels ≥ 0.2 in blood or bone marrow at the time of tumor surgery is an independent predictor of survival in patients with resected NSCLC. Accordingly, the tested hypothesis, that detection of systemic MAGE expression in blood and/or bone marrow aspirates is associated with the formation of distant metastases and cancer-related death, could be confirmed.

MAGE-A expression in blood or bone marrow was found to have a larger impact on distant metastasis-free survival than on cancer-free and overall survival. This is demonstrated by the lowest p-value in univariate analysis and by the most favorable 5-year Kaplan-Meier estimate e.g. 87% of patients with MAGE-A expression below 0.2 remain free of distant metastasis. This indicates that quantification of MAGE-A expression in bone marrow and blood indeed measures systemic MRD that has the potential to grow out and form distant metastases. This conclusion is supported by the observation that MAGE-A expression in blood or bone marrow does not have an impact on the development of locoregional recurrences.

The expression of MAGE-A and other Cancer/Testis-antigens has been evaluated as biomarker in several studies, however, data on the prognostic relevance is sparse. Although expression of MAGE-A genes in lung cancer tissue has been reported as marker of poor prognosis in adenocarcinoma (21) and squamous tumors (22), no correlations were found between MAGE-A expression in blood or bone marrow and tumor histology in the present study. While MAGE-A expression in our study serves only as a marker for the presence of MRD in blood or bone marrow and as quantitative measure of the systemic tumor load, its expression in the primary tumor may be indicative of a more aggressive quality of the disease as such (23). This may
also be the reason, why patients with expression of MAGE-A and other CT-antigens in the primary tumor tissue benefit from adjuvant chemotherapy (24,25).

Aside from tumor tissue MAGE-A expression has also been detected in regional lymph nodes of patients with lung cancer (26,27). This approach might help in the diagnosis of locally advanced disease, but correlation to the individual prognosis of the patient is missing so far and the association to clinically most important distant metastases is unproven. Therefore, blood and bone marrow have been chosen as compartment for the prediction of broadly disseminated disease and systemic tumor load (28,29). Since distribution of disseminated tumor cells in these systemic compartments may not be homogeneous, usually samples from different sites are taken for MRD assessment. Accordingly, differences in quantitative signals were also found in this study among the three sampled specimens per patient.

By multivariate analysis the expression of MAGE-A in blood or bone marrow was confirmed as highly significant predictor of an unfavorable clinical outcome and the independence of its prognostic value for survival from other prognostic factors was demonstrated. MAGE-A expression ≥ 0.2 was associated with the highest increase in relative risk for death of any cause and cancer-related death compared to the risk factors tumor size (T3-4 vs. T1-2) and lymph node status (N2 vs. N0-1) and the only significant predictor of distant metastasis, for which it is associated with the highest increase in relative risk (4.03-fold) versus cancer-related death (3.32-fold) and death of any cause (2.56-fold).

Thus, MAGE-A based MRD assessment may help to refine patient selection for adjuvant therapy in the future, which is urgently needed especially in the clinical management of elderly patients above 65 years (30).

MAGE-A proteins were originally discovered as target antigens of cytotoxic T cells in malignant melanoma (31) and adjuvant vaccination with a recombinant MAGE-A3
fusion protein in resected MAGE-A3 positive NSCLC is currently in phase III clinical development (32,33). Along this line, measuring MAGE-A expression levels in bone marrow and blood may also serve as a biomarker to monitor reduction or clearance of systemic MRD under adjuvant therapy. This has to be investigated in future studies taking repeated samples at different time points after surgery, thus confirming the results of this proof-of-concept study in a larger patient population and further refining the understanding of the importance of MAGE-A for prognosis in resected NSCLC.

Eventually, frequent expression of MAGE-A genes e.g. in breast, prostate, colorectal, hepatocellular, renal, ovarian and bladder cancer (34), warrants similar studies on the prognostic impact of MAGE-A positive MRD in other tumor types.
FOOTNOTES
We gratefully acknowledge the technical support of Stephanie Petersen, Simone Kiser, Martina Stemmer and Susanne Trimborn. All authors negate any competing financial interests. The study was approved by the ethic committee of the University Freiburg, Germany.

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AUTHORS' CONTRIBUTIONS
I.M. and P.K. conceived the project and designed the experiments. W.S., S.S. and B.P. treated study patients and collected clinical samples and data. I.M. and W.S. analyzed the data. I.M. and P.K. wrote and edited the manuscript.
REFERENCES


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**Table 1:** MAGE-A expression in bone marrow or blood according to clinical and pathological characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All study patients</th>
<th>Patients with MAGE-A positive bone marrow or blood at or above LLOQ</th>
<th>p value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Patients with MAGE-A positive bone marrow or blood at expression level ≥ 0.2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=94</td>
<td>N=41 (43.6%)</td>
<td></td>
<td>N=29 (30.9%)</td>
</tr>
<tr>
<td><strong>Tumor extension</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT1-pT2</td>
<td>64 (21+43)</td>
<td>29 (47.5%)</td>
<td>0.63</td>
<td>20 (31.2%)</td>
</tr>
<tr>
<td>pT3-pT4</td>
<td>30 (18+12)</td>
<td>12 (40.0%)</td>
<td></td>
<td>9 (30.0%)</td>
</tr>
<tr>
<td><strong>Lymph node status</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pN0-1</td>
<td>72 (52+20)</td>
<td>34 (47.2%)</td>
<td>0.20</td>
<td>23 (31.9%)</td>
</tr>
<tr>
<td>pN2</td>
<td>22</td>
<td>7 (31.8%)</td>
<td></td>
<td>6 (27.3%)</td>
</tr>
<tr>
<td><strong>Tumor histology</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adeno</td>
<td>37</td>
<td>19 (51.4%)</td>
<td></td>
<td>14 (37.9%)</td>
</tr>
<tr>
<td>Squamous</td>
<td>42</td>
<td>18 (42.9%)</td>
<td>0.26</td>
<td>13 (31.0%)</td>
</tr>
<tr>
<td>Miscellaneous&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15</td>
<td>4 (26.7%)</td>
<td></td>
<td>2 (13.3%)</td>
</tr>
<tr>
<td><strong>Grading</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1-G2</td>
<td>40 (3+37)</td>
<td>18 (45.0%)</td>
<td></td>
<td>14 (35.0%)</td>
</tr>
<tr>
<td>G3-G4</td>
<td>54 (53+1)</td>
<td>23 (42.6%)</td>
<td>0.82</td>
<td>15 (27.8%)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 66 years</td>
<td>50</td>
<td>18 (36.0%)</td>
<td>0.11</td>
<td>16 (32.0%)</td>
</tr>
<tr>
<td>&gt; 66 years</td>
<td>44</td>
<td>23 (52.3%)</td>
<td></td>
<td>13 (29.5%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Two-sided p values determined by Pearson’s Chi square test show possible significance of correlation between detection of MAGE-A transcripts and clinicopathological parameters. LLOQ = lower limit of quantification.

<sup>b</sup> “Miscellaneous” represents 8 adenosquamous carcinomas and 7 large cell carcinomas.
Table 2: Treatment failure according to MAGE-A expression level in bone marrow or blood and site of recurrence

<table>
<thead>
<tr>
<th>Variable</th>
<th>Total Cohort</th>
<th>Patients with MAGE-A positive bone marrow or blood at expression level ≥ 0.2</th>
<th>Patients with MAGE-A positive bone marrow or blood at expression level &lt; 0.2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=89</td>
<td>N=29 (32.6%)</td>
<td>N=60 (67.4%)</td>
</tr>
<tr>
<td>Disease recurrence</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Local recurrencea</td>
<td>16</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Distant metastasisb</td>
<td>30</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>Distant metastasis without prior local recurrence</td>
<td>24</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>Death</td>
<td>49</td>
<td>22</td>
<td>27</td>
</tr>
<tr>
<td>Death of any causec</td>
<td>49</td>
<td>22</td>
<td>27</td>
</tr>
<tr>
<td>Cancer-related death</td>
<td>36</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Event-free outcome</td>
<td>40</td>
<td>7</td>
<td>33</td>
</tr>
</tbody>
</table>

a Locoregional tumor recurrence occurred as first relapse event in all cases.

b In 6 cases distant metastasis developed after locoregional relapse.

c In 13 cases death was unrelated to the malignant disease.
Table 3: Univariate analysis of distant metastasis-free survival with different threshold levels for MAGE-A expression in bone marrow or blood

<table>
<thead>
<tr>
<th>MAGE-A expression threshold</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 0.05 vs. &lt; 0.05</td>
<td>p = 0.013</td>
</tr>
<tr>
<td>≥ 0.1 vs. &lt; 0.1</td>
<td>p = 0.002</td>
</tr>
<tr>
<td>≥ 0.2 vs. &lt; 0.2</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>≥ 0.3 vs &lt; 0.3</td>
<td>p = 0.004</td>
</tr>
<tr>
<td>≥ 0.5 vs. &lt; 0.5</td>
<td>p = 0.013</td>
</tr>
</tbody>
</table>

* p-values of univariate analyses were determined by log-rank test.
Table 4: Multivariable hazard ratios for overall survival, cancer-free survival and distant metastasis-free survival

<table>
<thead>
<tr>
<th>Variable</th>
<th>Overall survival</th>
<th>Cancer-free survival</th>
<th>Distant metastasis-free survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>univariate analysis (p Value)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>multivariate analysis (p Value)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Hazard Ratio (95% CI)</td>
</tr>
<tr>
<td>MAGE-A gene expression (≥ 0.2 vs. &lt; 0.2)</td>
<td>0.007</td>
<td>0.002</td>
<td>2.56 (1.42 – 4.63)</td>
</tr>
<tr>
<td>Tumor size (T&lt;sub&gt;3-4&lt;/sub&gt; vs. T&lt;sub&gt;1-2&lt;/sub&gt;)</td>
<td>&lt;0.001</td>
<td>0.002</td>
<td>1.71 (1.23 – 2.38)</td>
</tr>
<tr>
<td>Lymph node status (N&lt;sub&gt;2&lt;/sub&gt; vs. N&lt;sub&gt;0-1&lt;/sub&gt;)</td>
<td>0.005</td>
<td>0.057</td>
<td>1.39 (1.00 – 1.93)</td>
</tr>
<tr>
<td>Grading (G3-4 vs. G1-2)</td>
<td>0.17</td>
<td>n.s.</td>
<td>- c</td>
</tr>
<tr>
<td>patient age (&gt; 66 vs. ≤ 66 years)</td>
<td>0.94</td>
<td>n.s.</td>
<td>- c</td>
</tr>
<tr>
<td>Tumor histology (squamous carcinoma vs. adenocarcinoma vs. Miscellaneous)</td>
<td>0.60</td>
<td>n.s.</td>
<td>- c</td>
</tr>
</tbody>
</table>

<sup>a</sup> P-values of univariate analyses were determined by log-rank test.

<sup>b</sup> Stepwise multivariate analysis was performed using the Cox proportional-hazard model.

<sup>c</sup> No estimate of relative risk is given, since the variable was not significant on multivariate analysis.

n.s. = not significant
Figure Legend

Figure 1: Kaplan-Meier estimates of (A) distant-relapse-free survival, (B) cancer-free survival, (C) overall survival, and (D) locoregional-recurrence-free survival in percentage among patients with a MAGE-A expression level ≥ 0.2 versus < 0.2 in blood and bone marrow samples against the follow-up after surgery (in days)
Figure 1:

A) Distant-recurrence-free survival (%)

B) Cancer-free survival (%)

C) Overall survival (%)

D) Locoregional-recurrence-free survival (%)

Patients at Risk
MAGE-A < 0.2: 60 48 40 34 11 6
MAGE-A ≥ 0.2: 29 22 11 8 3 1

Follow-up after surgery (days)

Follow-up after surgery (days)

Follow-up after surgery (days)

Follow-up after surgery (days)

p < 0.001

p = 0.002

p = 0.26

p = 0.007

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A threshold of systemic MAGE-A gene expression predicting survival in resected non-small cell lung cancer


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