Detection of MAGE-A Transcripts in Bone Marrow Is an Independent Prognostic Factor in Operable Non–Small-Cell Lung Cancer

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Abstract Purpose: MAGE-A gene expression in humans is mostly restricted to tumor cells, and the role of MAGE-A transcripts and peptides as diagnostic markers and therapeutic targets is currently under investigation. Thus far, the clinical relevance of MAGE-A transcripts as marker for disseminated tumor cells in bone marrow of patients with operable lung cancer without overt metastases is still unclear.

Experimental Design: Preoperative bone marrow aspirates from 50 consecutive patients with operable non–small-cell lung cancer free of distant metastases (i.e., pT 1-4 pN0-2 M0 R0) were admitted to the study. Each bone marrow sample was divided and examined using multimarker MAGE-A reverse transcription-PCR (RT-PCR) and immunocytochemical staining with the antipancytokeratin antibody A45-B/B3. Multimarker MAGE-A RT-PCR consisted of multiple subtype-specific nested RT-PCRs with primers for MAGE-A1, MAGE-A2, MAGE-A3/6, MAGE-A4, and MAGE-A12. The median follow-up duration was 92 months (range, 18-110 months).

Results: Twenty-six (52%) lung cancer patients harbored MAGE-A transcripts in their bone marrow, as opposed to none of the 30 healthy controls tested. In all 7 patients with immunocytochemically positive bone marrow, MAGE-A transcripts were also detected. All different MAGE-A subtypes (MAGE-A1, MAGE-A2, MAGE-A3/6, MAGE-A4, and MAGE-A12) were observed. Sixty-five percent of patients with MAGE-A transcripts in bone marrow exhibited only one subtype. Univariate (P = 0.03, log-rank-test) and multivariate survival analysis showed that MAGE-A transcripts in bone marrow were associated with poor outcome in pN0 patients (P = 0.02; relative risk, 7.6).

Conclusions: Detection of MAGE-A transcripts in bone marrow predicts an unfavorable outcome in patients with early-stage operable lung cancer. This finding indicates that MAGE-A transcripts are clinically relevant markers of micrometastatic spread in lung cancer and supports further investigation of MAGE-A as potential future therapeutic target.

The failure to reduce mortality of operable solid tumors most likely results from early dissemination of cancer cells, which is usually missed by conventional staging procedures at the time of surgery. Because adjuvant therapy aims to eradicate occult disseminated tumor cells (DTC) before metastatic disease becomes clinically evident, the early detection of tumor cell dissemination could identify patients who might benefit from such treatment (1). Over the past 10 years, various assays have been developed to detect micrometastatic spread at the single cell level in patients with solid tumors including non–small-cell lung cancer (NSCLC; refs. 2–6). However, the specificity and clinical significance of these assays are still under investigation. Moreover, markers that also represent potential therapeutic targets might be of broader clinical interest to facilitate not only the detection of micrometastatic spread but also its eradication to prevent metastatic relapse.

Since the identification of the first MAGE gene in 1991 (7), the MAGE family has expanded to more than 25 genes that have been identified in humans (8). The functional role of most family members remains uncharacterized although they may play a role in embryonal development and tumor transformation or in certain aspects of tumor progression (9–14). Despite their normally restricted expression, many of the MAGE genes are expressed in a wide variety of histologically distinct tumors, likely because of transformation-dependent loss of the DNA methylation that normally silences these genes (15, 16). Thus, MAGE transcripts seem to be ideal markers for occult tumor cells that could be detected by a reverse transcription-PCR (RT-PCR) assay.
Among the MAGE families, the cluster MAGE-A, which has 12 subtypes (MAGE-A1-A12), has been observed most frequently in malignant tumors and has been characterized as a possible marker of tumor cells (6); however, most carcinomas express at least one MAGE-A subtype (5, 6). Therefore, recent studies for the detection of occult tumor cells used either PCR primers that can simultaneously detect multiple MAGE-A subtypes in one reaction (5, 17) or multimarker RT-PCR settings with different MAGE-A primer pairs (6, 18). Using these MAGE-A RT-PCR assays, DTCs in bone marrow have been detected in 11 of 33 (33%) examined lung cancer patients without overt metastases (cM0; ref. 6), in 22 of 136 (16%) examined breast cancer patients without overt metastases (cM0; ref. 5), and in 18 of 30 (60%) of prostate cancer patients without distant metastases (cM0; ref. 6). However, the key question on whether DTCs detected by MAGE-A RT-PCR are associated with a poor clinical outcome has not been investigated thus far. Therefore, we analyzed the survival in operable NSCLC, which is the most aggressive entity of the cancers mentioned above, and compared the detection of MAGE-A transcripts in bone marrow to the observed cancer-related survival. Bone marrow is known to be a common homing organ of disseminated epithelial tumor cells (1) and a frequent site of metastasis in lung cancer. We focused on operable patients treated by complete resection without neoadjuvant therapy to minimize treatment bias on survival. Because of the severe prognostic effect of manifest distant or lymph node metastases in lung cancer (19), we focused on operable pN0 cM0 lung cancer patients. Here, we report for the first time that the detection of MAGE-A transcripts in bone marrow is an independent prognostic factor in early-stage operable NSCLC.

Patients and Methods

Patients and follow-up. The study was activated in January 1996 after approval by the ethical committee of the University of Munich. Patients who gave written informed consent were enrolled consecutively until March 1997. During this time, bone marrow aspirates from 50 patients with operable NSCLC were collected at the time of surgery. All operations were done in the Department of Thoracic Surgery, Asklepios Fachkliniken München-Gauting. The latest follow-up was updated in October 2005. Basic data of 32 patients of the present study cohort have been published in a work describing the method used in the present study, but the prognostic effect of MAGE-A transcripts in bone marrow of those patients has not been analyzed thus far.

In general, a lobectomy or pneumonectomy with systematic mediastinal lymphadenectomy was done. The resected tumors were classified according to the current International Union Against Cancer (UICC) tumor-node-metastasis (TNM) classification (19). To prevent interobserver bias, grading of all tumors was assessed by one and the same pathologist. Because grading was initially assessed according to the UICC classification of 1986 (20), all tumors were re-reviewed by the same pathologist to update grading to the current UICC classification. The WHO classification was applied for histologic tumor typing (21). Only patients with completely resected primary NSCLC as assessed by histopathologic examination and without overt distant metastases (pT1-2 pN0-1 cM0 R0) were admitted to the study. Preoperative staging included computed tomography of the head, chest, liver, and adrenal glands, as well as a bone scintigraphy, and did not show any suspicion of metastases. The final study population consisted of 10 female and 40 male patients eligible for MAGE-A RT-PCR. The median age at the time of surgery was 68 years (range, 44-88 years). Patients whose primary tumors were classified as pT1 or pT2 received adjuvant percutaneous radiotherapy of the tumor bed, and patients with mediastinal lymph node involvement (pN+) received percutaneous radiotherapy of the entire mediastinum. Systemic neoadjuvant or adjuvant therapy was not done. Follow-up studies included 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 local relapse, distant metastasis, and death. Information on cancer-related death was obtained from 45 of the 50 NSCLC patients. The remaining 5 patients had to be excluded from survival analyses because of perioperative death (n = 3) or an unclear status of relapse and survival (n = 2). The median observation period was 92 months (range, 18-110 months). In addition, 30 healthy allogeneic bone marrow donors (age, 18-49 years; 21 male, 9 female) were included in the study to analyze their bone marrows as negative controls.

Bone marrow preparation. At the time of primary operation, 5-ml bone marrow was taken through an aspiration needle from each anterior iliac crest in general anesthesia before thoracotomy. The obtained bone marrow, yielding between 2 \times 10^7 and 3 \times 10^7 (mean, 2.2 \times 10^7) mononuclear cells, was divided and 2 ml of each native bone marrow aspirate were immediately mixed with 5 ml of nucleic acid extraction buffer [4 mol/L guanidine isothiocyanate, 0.5% sarcosyl (N-laurylsarcosine sodium salt), 25 mmol/L sodium citrate (pH 7.0), 0.7% 2-mercaptoethanol] and stored at -20°C until needed for MAGE-A RT-PCR. Another 2 ml of the remaining bone marrow of each aspirate were centrifuged through 10-ml Ficoll-Hypaque for 30 min at 1,200 \times g. The interface layer, which contains mononuclear cells, was collected and brought to a final concentration of 10^7 cells/ml. These interphase cells were cytocentrifuged (Hettich) on six glass slides at 200 \times g for 3 min and dried overnight for immediate immunocytochemical staining or storage at -80°C. Coagulation of bone marrow resulted in exclusion of 5 patients from immunocytochemical staining, leaving 45 patients eligible for immunocytochemistry and 50 patients for MAGE-A RT-PCR.

Immunocytochemistry. DTCs in bone marrow cytopsins were detected by immunocytochemical staining with the monoclonal antibody A45-B/B3 (Micromet) that binds to an antigen on cytokeratins 8, 18, and 19 (22). High sensitivity and specificity of the immunocytochemical detection of DTCs using the antibody A45-B/B3 has previously been shown (23). For visualization of antibody binding, the alkaline phosphatase anti–alkaline phosphatase technique combined with the Neufuchsins method (24) was used as previously reported (23). In all experiments, an isotype-matched, irrelevant murine monoclonal antibody (MOPC 21, immunoglobulin G1; Sigma) served as negative control on one glass slide per patient. Occurrence of disseminated cancer cells in bone marrow was assessed by examination of five glass slides per patient, yielding \(-5 \times 10^7\) mononuclear cells per slide. All cytopsins were examined independently by two observers who were unaware of the clinical data. Slides with discrepant evaluations were reevaluated and a consensus was reached (n = 3). Cytomorphologic features like clearly enlarged nuclear size, strong or irregular cytoplasmic immunostaining, partially or total covering of nucleus, and high nuclear-to-cytoplasmic ratio were used to identify DTC among cytokeratin-positive cells as previously suggested (25). All immunocytochemically positive cells met all of these criteria and were considered as DTC. Samples with at least one DTC were considered positive for DTCs.

Nested multimarker MAGE-A RT-PCR. RNA was isolated from the bone marrow mixed with nucleic acid extraction buffer according to the method of Chomczynski and Sacchi (26). RNA resulting from this protocol was reverse transcribed to cDNA by use of an equimolar mixture of outer antisense primers of MAGE-A1, MAGE-A2, MAGE-A3/6, MAGE-A4 and MAGE-A12 and the histone, \(\beta_2\)-microglobulin, or \(\alpha_5\) antisense primers, as previously described (6). After reverse transcription, nested multimarker MAGE-A PCR was done as previously
described (6). The presence of cDNA was monitored by a control PCR amplifying histone, β2-microglobulin, or p53. Adequate precautions to prevent cross-contamination and negative control reactions that included mock preparations were routinely done. PCR products were separated on a 1.8% agarose gel and stained with ethidium bromide (Fig. 1). Analysis of ethidium bromide gels was done in a double-blinded fashion without knowledge about immunocytochemical or clinical data. Negative as well as positive findings were confirmed by repeating the assay with a second aliquot of each original total RNA sample. Bone marrow aspirates from 30 healthy allogeneic bone marrow donors served as negative controls.

**Statistical analysis.** A presudy sample size estimation showed that a sample size of 49 patients will be sufficient to detect a survival difference at a two-sided 0.05 significance level, if the hazard ratio of DTC in bone marrow of patients with resected pN0 NSCLC is 2.5 as shown before (2) and the median survival in pN0 NSCLC is 22 months as previously shown (27). This estimation was based on the assumption that the accrual period will be 12 months and the follow-up period will be at least 60 months.

All statistical analyses were done using SPSS software version 13.0 for PC (SPSS, Inc.). To analyze a possible association of bone marrow findings with clinicopathologic variables, the two-tailed Pearson’s χ² test or Fisher’s exact test in frequencies <5 was used. The threshold for statistical significance was P < 0.05. For analysis of follow-up data, life table curves were calculated using the Kaplan-Meier method and survival distributions were compared by log-rank statistics. The primary end point was cancer-related death, as measured from the date of surgery to death. Data of patients who were still alive and without evidence of disease at the end of the study (n = 14) or who died of causes not related to cancer (n = 3) were censored. 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 possible independence of the prognostic value of MAGE-A transcripts. The 0.05 level of significance was used for entering or removing a covariable from this model.

**Results**

**Detection of MAGE-A transcripts, sensitivity, and specificity.** Bone marrow aspirates of 50 consecutive patients with newly diagnosed and completely resected (R0) primary NSCLC who showed no clinical signs of overt distant metastases (pT1-4 pN0-2 M0) were examined according to the presence of MAGE-A transcripts. At the time of initial tumor resection, 26 (52%) patients harbored MAGE-A transcripts in the bone marrow. Of the 45 patients eligible for immunocytochemical bone marrow examination, 7 (16%) patients had cytokeratin-positive cells in the bone marrow. The median frequency of those occult cancer cells per bone marrow sample was 1 cell per 2.5 × 10⁶ mononuclear cells analyzed (range, 1-4). Sensitivity of MAGE-A RT-PCR to detect DTC was calculated in comparison with anti-cytokeratin immunocytochemistry of the same bone marrow samples serving as a kind of reference standard for the detection of DTC (1). In all 7 patients with immuno-cytocytochemically positive bone marrow, MAGE-A transcripts were also detected. Thus, sensitivity of MAGE-A RT-PCR according to the detection of DTC was 100%. All bone marrow aspirates from 30 healthy allogeneic bone marrow donors serving as negative controls showed no MAGE-A transcripts. Thus, no false-positive results were observed, showing a 100% specificity of MAGE-A RT-PCR according to the detection of DTC.

**MAGE-A profile in bone marrow.** Among the 26 patients with MAGE-A transcripts in the bone marrow, the MAGE-A1...
subtypewasobservedinbonemarrowof10(38%)patientsand
correspondedmostfrequentlytopositiveMAGE-Aamplifications.
ThesecondmostfrequentMAGE-Asubtypesinthebonemarrow
wereMAGE-A2(n=8;31%),MAGE-A3/6(n=8;31%),and
MAGE-A12(n=8;31%).MAGE-A4wasobservedin6(23%)
patients.Bonemarrowsamplesof17(65%)patientsexhibited
onlyoneMAGE-Asubtype.Oneadditionalpatientshowedall
fivemAGE-Asubtypesinhisbonemarrow;anotherpatient,four
MAGE-A subtypes (MAGE-A1, MAGE-A3/6, MAGE-A4, and
MAGE-A12); and seven additional patients, two MAGE-A
subtypes (MAGE-A3/6 and MAGE-A4, n=2; MAGE-A1 and
MAGE-A3/6, n=2; MAGE-A1 and MAGE-A12, n=3).

Tumor characteristics and clinicopathologic variables. Primary
wereclassifiedaspt1in3(6%)patients, pt2in32
(64%) patients, pt3 in 3 (6%) patients, and pt4 in 12 (24%)
patients. Although tumor extension is a standard risk factor used
for prognostic evaluation in NSCLC, we found that the incidence
of MAGE-A transcripts and immunocytochemically cytokeratin-
positive cells in the bone marrow was similar in patients with
smaller (pt1-pt2) and larger (pt3-pt4) tumor extensions (P = 0.46
and P = 0.42, respectively, χ2 test; Table 1). Routine histopath-
ologic lymph node examination exhibited pN0 status in 17 (34%)
patients, pN1 status in 17 (34%) patients, and pN2 lymph node
involvement in 16 (32%) patients. Ten (59%) of the pN0 patients,
7 (41%) of the pN1 patients, and 9 (56%) of the pN2 patients
harbored MAGE-A transcripts in their bone marrow. Immunocy-
tochemically cytokeratin-positive cells were detected in bone
marrow of 4 of 15 (27%) examined pN0 patients, 2 of 16 (12%)
examined pN1 patients, and 1 of 14 (7%) examined pN2 patients.
The lymph node status was associated neither with MAGE-A
transcripts (P = 0.49, χ2 test; Table 1) nor with immunocyto-
chemically positive cells (P = 0.19, Fisher's exact test) in bone
marrow aspires. Further characteristics like tumor stage, tumor
histology, age of patient, and sex did not correlate with detection
of MAGE-A transcripts (Table 1) or immunocytochemically
positive cells (data not shown). Interestingly, MAGE-A transcripts
in bone marrow samples occurred significantly more frequently
(87%) in better differentiated tumors (G1-G2) than in poorer
differentiated ones (22%; G3-G4; P = 0.001, Fisher's exact test;

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. patients</th>
<th>No. patients with MAGE-A transcripts in the bone marrow, n (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>50</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Tumor extension</td>
<td></td>
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<td>0.46</td>
</tr>
<tr>
<td>pT1-pT2</td>
<td>35</td>
<td>17 (65)</td>
<td></td>
</tr>
<tr>
<td>pT3-pT4</td>
<td>15</td>
<td>9 (35)</td>
<td></td>
</tr>
<tr>
<td>Lymph node involvement</td>
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<td>0.49</td>
</tr>
<tr>
<td>pN0</td>
<td>17</td>
<td>10 (38)</td>
<td></td>
</tr>
<tr>
<td>pN1-pN2</td>
<td>33</td>
<td>16 (62)</td>
<td></td>
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<tr>
<td>Tumor stage</td>
<td></td>
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<tr>
<td>Stages Ia-IIb</td>
<td>29</td>
<td>14 (54)</td>
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<tr>
<td>Stages IIIa-IIIb</td>
<td>21</td>
<td>12 (46)</td>
<td></td>
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<tr>
<td>Tumor histology</td>
<td></td>
<td></td>
<td>0.14</td>
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<tr>
<td>Adenocarcinoma</td>
<td>15</td>
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<tr>
<td>Squamous cell carcinoma</td>
<td>30</td>
<td>17 (65)</td>
<td></td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>5</td>
<td>4 (15)</td>
<td></td>
</tr>
<tr>
<td>Grading</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>G1-G2</td>
<td>23</td>
<td>20 (77)</td>
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<tr>
<td>G3-G4</td>
<td>27</td>
<td>6 (23)</td>
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<td>Age, y</td>
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</tr>
<tr>
<td>&lt;68</td>
<td>26</td>
<td>14 (54)</td>
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</tr>
<tr>
<td>&gt;68</td>
<td>24</td>
<td>12 (46)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
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<tr>
<td>Female</td>
<td>10</td>
<td>4 (15)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>40</td>
<td>22 (85)</td>
<td></td>
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</table>

*Amplification of at least one MAGE-A subgroup was considered
positive for multimarker MAGE-A RT-PCR.

Two-sided P values determined by Pearson's χ2 test or by Fisher’s
exact test in frequencies <5 show possible significance of
correlation between detection of MAGE-A transcripts and clinicopatologic variables.

Pathologic stages were classified according to the UICC TNM
classification (19).

“Miscellaneous” represents three mixed histologies and two
large-cell carcinomas.

subtype was observed in bone marrow of 10 (38%) patients and
contributed most frequently to positive MAGE-A amplifications.
The second most frequent MAGE-A subtypes in the bone marrow
were MAGE-A2 (n = 8; 31%), MAGE-A3/6 (n = 8; 31%), and
MAGE-A12 (n = 8; 31%). MAGE-A4 was observed in 6 (23%)
patients. Bone marrow samples of 17 (65%) patients exhibited
only one MAGE-A subtype. One additional patient showed all
five MAGE-A subtypes in his bone marrow; another patient, four
MAGE-A subtypes (MAGE-A1, MAGE-A3/6, MAGE-A4, and
MAGE-A12); and seven additional patients, two MAGE-A
subtypes (MAGE-A3/6 and MAGE-A4, n = 2; MAGE-A1 and
MAGE-A3/6, n = 2; MAGE-A1 and MAGE-A12, n = 3).

Tumor characteristics and clinicopathologic variables. Primary
tumors were classified as pt1 in 3 (6%) patients, pt2 in 32
(64%) patients, pt3 in 3 (6%) patients, and pt4 in 12 (24%)
patients. Although tumor extension is a standard risk factor used
for prognostic evaluation in NSCLC, we found that the incidence
of MAGE-A transcripts and immunocytochemically cytokeratin-
positive cells in the bone marrow was similar in patients with
smaller (pt1-pt2) and larger (pt3-pt4) tumor extensions (P = 0.46

Fig. 2. Cumulative cancer-related survival of operable NSCLC patients with
MAGE-A transcripts in bone marrow (BM) compared with patients without
MAGE-A transcripts in bone marrow samples. Five patients were excluded due to
perioperative death (n = 3) or an unclear status of relapse and survival (n = 2).
A, analysis of all eligible patients (n = 45). Patients with MAGE-A transcripts in bone
marrow aspires showed a tendency toward unfavorable outcome (P = 0.14,
log-rank test). B, survival of pN0 patients (n = 16). MAGE-A transcripts in bone
marrow were associated with a significant unfavorable outcome (P = 0.03,
log-rank test).
In bone marrow of the 26 examined patients with poorer differentiated tumors (4%; G3-G4; \( P = 0.03 \), Fisher’s exact test). Among the 45 patients eligible for follow-up analysis, 58\% (26/45) with relapse died of cancer-related causes. The median follow-up duration was 92 months (range, 18-110 months). Within the observation period, a total of 30 (67\%) patients developed a relapse. Local recurrence occurred in 8 patients, distant metastasis in 4 patients, and both local recurrence and distant metastasis in 18 patients. Local recurrence was observed in 3 of 21 (14\%) patients with MAGE-A–negative bone marrow samples and in 5 of 24 (21\%) patients with MAGE-A transcripts in the bone marrow (\( P = 0.48, \) log-rank test). The occurrence of distant metastasis was slightly but not significantly increased in patients with MAGE-A transcripts in the bone marrow (\( n = 3; 12\% \)) compared with patients with MAGE-A–negative bone marrow aspirates (\( n = 1 \) (5\%); \( P = 0.39, \) log-rank test). Presence of MAGE-A transcripts in the bone marrow was also associated with a tendency toward relapse in the 45 eligible patients. Relapse occurred in 11 (52\%) patients with MAGE-A–negative bone marrow and in 19 (79\%) patients with MAGE-A transcripts in the bone marrow (\( P = 0.15, \) log-rank test). All but 2 patients (\( n = 28; 93\% \)) with relapse died of cancer-related causes. Among the patients with early lymph node status (pN0), the prognostic effect of MAGE-A transcripts in the bone marrow was statistically significant (\( P = 0.03, \) log-rank test; \( n = 16 \) eligible patients; Fig. 2B). Coagulation of bone marrow samples resulted in exclusion of additional 4 patients from survival analysis, leaving 41 patients eligible for a prognostic assessment of the immunocytochemical bone marrow status. There was no correlation between immunocytochemically detected cytokeratin-positive cells in the bone marrow and cancer-related survival in the total population (\( P = 0.55, \) log-rank test), but among patients with pN0 lymph node status, there was a tendency toward decreased cancer-related survival in case of cytokeratin-positive cells in the bone marrow. Cancer-related survival was 25\% in pN0 patients with immunocytochemically positive bone marrow and 43\% in pN0 patients with immunocytochemically negative bone marrow (\( P = 0.67, \) log-rank test; \( n = 14 \) eligible patients; Fig. 3).

In the group of pN0 patients, a multivariate analysis was conducted to evaluate whether the correlation of MAGE-A transcripts in bone marrow with shortened cancer-related survival resulted from an association of MAGE-A transcripts with other prognostic factors or whether MAGE-A detection could maintain its own prognostic value (Table 2). Detection of MAGE-A transcripts in bone marrow and standard prognostic variables such as tumor stage, tumor extension, tumor histology, and tumor differentiation were included in a multivariate analysis with a proportional hazards model (Table 2).

### Table 2. Univariate and multivariate analyses of cancer-related survival in pN0 patients

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection of MAGE-A transcripts ( ^{\dagger} )</td>
<td>0.027 ( \dagger )</td>
<td>7.6 (1.0-61.8) 0.017</td>
</tr>
<tr>
<td>Bone marrow negative vs positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor stage</td>
<td>0.043</td>
<td>-</td>
</tr>
<tr>
<td>Ia-Iib vs IIIa-IIIib</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor extension</td>
<td>0.035</td>
<td>-</td>
</tr>
<tr>
<td>pT1 vs pT2 vs pT3-pT4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor histology</td>
<td>0.322</td>
<td>-</td>
</tr>
<tr>
<td>Adenocarcinoma vs squamous cell carcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vs miscellaneous</td>
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<td></td>
</tr>
<tr>
<td>Grading</td>
<td>0.547</td>
<td>-</td>
</tr>
<tr>
<td>G1-G2 vs G3-G4 ( ^{\dagger} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>0.652</td>
<td>-</td>
</tr>
<tr>
<td>Female vs male ( ^{\dagger} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.765</td>
<td>-</td>
</tr>
<tr>
<td>68 vs &gt;68 y</td>
<td></td>
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</tr>
</tbody>
</table>

Abbreviation: 95\% CI, 95\% confidence interval.

\( ^{\dagger} \) Unclear status of relapse and survival resulted in exclusion of one patient, leaving 16 pN0 patients available for the analysis of possible joint effects of prognostic variables.

\( P \) values of univariate analyses were determined by log-rank test.

Stepwise multivariate analysis was done using the Cox proportional hazard model.

\( ^{\dagger} \) Amplification of at least one MAGE-A subgroup was considered positive for multimarker MAGE-A RT-PCR.

No estimate of relative risk is given because the variable was not significant on multivariate analysis.

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**Fig. 3.** Cumulative cancer-related survival of patients with completely resected pN0 NSCLC and immunocytochemical examination of bone marrow. DTCs in bone marrow were detected immunocytochemically with the anti-cytokeratin antibody A45-B/B3 (Micomet). Coagulation of bone marrow resulted in exclusion of 2 patients in the pN0 group, leaving 14 patients eligible for survival analyses. Patients with cytokeratin-positive cells in bone marrow aspirates showed a tendency toward unfavorable outcome that was not statistically significant (\( P = 0.67, \) log-rank test).
histology, grading, and age of patient at the time of operation were tested for joint effects of a possible prognostic value in pN0 patients. It was not feasible to consider therapy as an extra variable in the multivariable analysis because adjuvant systemic therapy was not given and radiotherapy was done in all patients with pT3 and pT4 tumors, resulting in equal values for therapy and pT status. A preceding univariate analysis revealed that besides detection of MAGE-A transcripts in bone marrow, also tumor stages and pT status were significant prognostic variables (P < 0.05; Table 2). The multivariate regression analysis showed that MAGE-A transcripts in the bone marrow were a significantly (P = 0.02) independent prognostic predictor for shortened cancer-related survival. Because detection of MAGE-A transcripts in bone marrow was associated with grading (Table 1), the prognostic effect of grading itself was analyzed to evaluate a possible prognostic joint effect of both variables. Univariate analysis showed that the grading did not influence cancer-related survival (P = 0.55, log-rank test), and multivariate analysis showed that it did not have a prognostic joint effect to MAGE-A transcripts in bone marrow (P = 0.14). The relative risk for cancer-related death was 7.6-fold increased in case of MAGE-A transcripts in the bone marrow.

Discussion

Here we show for the first time that detection of MAGE-A transcripts in the bone marrow is an independent prognostic factor in NSCLC patients without metastases (pN0 cM0). This result suggests that the measured MAGE-A transcripts could be derived from viable occult DTCs with the potential to initiate a later metastatic relapse. RT-PCR of MAGE-A transcripts has previously been used for detection of DTCs in bone marrow of lung cancer, breast cancer, and prostate cancer patients (5, 6), but the prognostic effect of positive MAGE-A RT-PCR findings has not been investigated thus far. Kufer et al. (6) showed a correlation of MAGE-A transcripts in bone marrow of prostate cancer patients with established risk factors of developing systemic disease such as a tumor size ≥pT3, an initial serum PSA ≥20 ng/mL, and a Gleason score of 8 to 10 (P = 0.02), but they did not publish any follow-up data. Thus, this is the first study showing survival data of patients with MAGE-A transcripts in bone marrow.

The multimarker MAGE-A RT-PCR detected occult bone marrow involvement in 26 (52%) and conventional anticytokeratin immunocytochemistry in 7 (16%) patients in this study, suggesting a superior sensitivity of the multimarker MAGE-A RT-PCR compared with conventional immunocytochemistry. This discrepancy between RT-PCR and immunocytochemistry has also been described in previous works (6, 28) and might result from methodologic differences during sample processing such as the risk of tumor cell loss or cell damage during sample collection and shipping or Ficoll-Hypaque isolation for immunocytochemistry. For example, immunocytochemical examination may be limited by coagulation of bone marrow during collection and shipping as it was the case in five patients in this study. This limitation is excluded in RT-PCR because, here, bone marrow samples are immediately mixed with nucleic acid extraction buffer, which confers cellular lysis and stabilization of RNA for shipping and storage.

Immunocytochemical analysis of bone marrow cytospins requires screening of a large number of slides (23) and thus is laborious, time-consuming, and observer dependent. In contrast, RT-PCR provides the possibility to analyze the same amount of bone marrow in one reaction. In addition, RT-PCR is an automated, rapid, and versatile method that is observer independent (29). Detection of vital cancer cells in bone marrow was approached by the amplification of MAGE-A transcripts, which are selectively expressed in cancer cells but not in normal bone marrow. Immunocytochemical DTC assays have the advantage that the tumor cell can be envisaged and selected for further molecular characterization (30, 31). However, the currently available antibodies against MAGE-A antigens are not specific for individual MAGE-A proteins. The possibility that the MAGE-A transcripts in bone marrow may result from hematogenously circulating transcripts derived from the primary tumor or testicular germ cells (11) is unlikely because free RNA is usually rapidly degraded in blood (32).

Because of the sample loss by coagulation of bone marrow, leaving 45 patients eligible for immunocytochemistry and 50 patients for MAGE-A RT-PCR, the prognostic values of both methods cannot be compared in this study. Among patients with pN0 lymph node status, cancer-related survival was 25% in patients with immunocytochemically positive and 43% in patients with immunocytochemically negative bone marrow, but this tendency was not statistically significant. However, immunocytochemistry was only intended to serve as a kind of reference standard for the detection of DTC and not to verify a prognostic significance of immunocytochemically positive bone marrow samples, which has already been shown in larger studies (2, 3, 33, 34). There are also studies showing no prognostic significance of immunocytochemically positive bone marrow samples (35, 36), but those studies had limitations by short follow-up durations and availability of only overall survival data instead of cancer-related or disease-free survival data. One possible reason for the prognostic insignificance of cytokeratin-positive cells in this study could be the exclusion of five patients from immunocytochemical staining due to coagulated bone marrow. Another possible explanation could be that a larger patient number is needed for a prognostic significance of immunocytochemical examination but not of MAGE-A RT-PCR.

Previous studies observed MAGE-A transcripts in blood of 22 of 136 (16%) breast cancer patients (5), in bone marrow of 18 of 30 (60%) prostate cancer patients (6), in sputum of 5 of 15 (33%; ref. 18) and 26 of 55 (47%; ref. 17) lung cancer patients, and in bone marrow of 11 of 33 (33%; ref. 6) lung cancer patients. These studies showed a significant correlation to pT and pN status in breast cancer (5) or cM status in lung cancer (6), but have never investigated the biological or prognostic effect of occult DTCs detected by MAGE-A RT-PCR. The present study shows that MAGE-A transcripts in bone marrow correlate with survival (Fig. 2) and opposes any association of DTCs detected by MAGE-A RT-PCR. The present study shows that MAGE-A transcripts in bone marrow correlate with survival (Fig. 2) and opposes any association of DTCs detected by MAGE-A RT-PCR or immunocytochemistry with pN status in cM0-NSCLC. The latter fact is not surprising because hematogenous bone marrow dissemination and lymphatic dissemination are different anatomic routes. Furthermore, it has been shown that there is no relationship between immunocytochemical detection of DTCs in bone marrow or lymph nodes (36, 37). The observation that DTCs detected by immunocytochemistry or MAGE-A RT-PCR did not correlate with the pT status in this study is explainable in the light of a recently suggested hypothesis that tumor cell dissemination
might be an early event in cancer evolution that may occur early during the growth of the primary tumor (1, 38–40).

The independence of the prognostic effect of MAGE-A transcripts from the known prognostic influence of primary tumor characteristics such as tumor stage, tumor extension, histology, or grading (Table 2) is comparable to previous works about DTC in bone marrow showing a similar prognostic independence (2, 41). These consistent findings indicate that the detection of DTC is feasible for additional risk stratification besides TNM staging, and that it might enable a more personalized lung cancer treatment in the future. One explanation for the prognostic independence of DTCs could be the recently postulated early time point of dissemination during cancer development and subsequent parallel evolutions of primary tumor and DTCs (38, 40).

If dissemination is an early event (38), occult tumor cells in bone marrow more likely represent the expression profile at time of dissemination than the locally growing primary tumor. Bone marrow examination with multimarker MAGE-A RT-PCR consisting of multiple subtype-specific RT-PCRs enabled us to analyze the MAGE-A expression profile of occult tumor cells in bone marrow. Because all different MAGE-A subtypes were observed and 65% of patients with MAGE-A transcripts in bone marrow exhibited only one subtype, the use of a multimarker assay covering all examined MAGE-A subtypes is important to avoid false-negative findings.

Concerning treatment of occult tumor cells, the MAGE-A gene family is a promising target for a specific immunotherapy because MAGE-A is expressed in cancer cells but not in normal tissue, except male germ line cells, which are devoid of MHC molecules and therefore do not present MAGE-A antigens (42, 43). Thus far, anti–MAGE-A3 immunization trials in metastatic malignant melanoma showed limited success (44, 45). However, immunotherapy should be more effective in patients with low tumor burden (refs. 46, 47; e.g., after primary tumor resection in patients with occult tumor cells in bone marrow). Because DTC may remain in a nonproliferative state (48) and thus might be resistant to conventional antiproliferative chemotherapy-based adjuvant therapy in operable NSCLC, immunotherapy could be an approach to target particularly remaining occult DTCs after resection of NSCLC. Adjuvant anti–MAGE-A immunotherapy has recently been investigated in a double-blind, randomized phase II study using recombinant MAGE-A3 protein and an immunologic adjuvant in 182 patients with completely resected MAGE-A3–positive stages IB/II NSCLC. The vaccination was well tolerated and, after a median follow-up duration of 21 months, the relapse rate tended to be improved in the immunotherapy group (30%) compared with the placebo group (41%; P = 0.14; ref. 49). Because 65% of patients with MAGE-A transcripts in bone marrow exhibited single different MAGE-A subtypes, the present study might suggest that anti–MAGE-A immunotherapy of occult DTCs in NSCLC could be improved by targeting multiple MAGE-A subtypes.

In summary, the present study reveals that multiple different MAGE-A subtypes are detectable in bone marrow and correlate with an unfavorable outcome in pN0 NSCLC patients. Although the observed prognostic effect must be confirmed in a larger patient cohort, this study may provide a basis for the preselection of patients and for MAGE-A antigens to be included in future adjuvant immunotherapy trials after surgical resection of NSCLC.

References


Detection of MAGE-A Transcripts in Bone Marrow Is an Independent Prognostic Factor in Operable Non–Small-Cell Lung Cancer

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