Clinical Cancer Research

A Threshold of Systemic MAGE-A Gene Expression Predicting Survival in Resected Non–Small Cell Lung Cancer

Ingo Mecklenburg[1], Wulf Sienel[2], Severin Schmid[2], Bernward Passlick[2], and Peter Kufer[3]

Abstract

Purpose: Quantitative measurement of minimal residual disease 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 $\geq$0.2 in at least one sample of bone marrow or blood at tumor surgery had a significantly reduced overall ($P = 0.007$), cancer-free ($P = 0.002$), and distant metastasis–free survival ($P < 0.001$) versus patients below 0.2 in all samples without significant difference in locoregional recurrence–free survival. The corresponding HRs ($\geq$0.2 vs. <0.2) for death, cancer-related death, and development of distant metastasis were 2.56 [95% confidence interval (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 $\geq$ 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. *Clin Cancer Res; 23(5); 1213–9. ©2016 AACR.*

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 postoperative outgrowth of minimal residual disease (MRD) caused by preoperative dissemination of cancer cells, which 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 hematologic remission, who failed to clear MRD from bone marrow as determined by qRT-PCR, develop a hematologic 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 hypomethylation (12). In addition, other chromatin remodeling events like histone acetylation and methylation further modulate MAGE-A expression. Although little is understood of the physiologic 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,

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

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Corresponding Author: Ingo Mecklenburg, Department of Internal Medicine, Klinikum Landsberg, Bgm.-Dr.-Hartmann-Str. 50, 86899 Landsberg am Lech, Germany. Phone: 49-(0)-8191-333-1007; Fax: 49-(0)-8191-333-1009; E-mail: ingo.mecklenburg@klinikum-landsberg.de

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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 broadly as 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.

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 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 [International Union against Cancer (UICC) stage Ia–IIIa] planned to undergo tumor resection by lobectomy or pneumonectomy with systematic mediastinal lymphadenectomy at the University Hospital Freiburg (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 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 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 a 3-month interval and an additional thoracic computed tomography scan, abdominal ultrasound, and bronchoscopy at a 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 Supplementary Data. 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 clinicopathologic variables, the two-tailed Pearson χ^2 test or Fisher 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 hazards 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

A total of 116 patients with suspected lung cancer were enrolled in the study (a patient flow diagram is depicted in Supplementary Fig. S1 in the Supplementary Data). According to postoperative assessment, 94 patients with histopathologically confirmed NSCLC fulfilled the inclusion criteria. Twenty-two patients dropped out because of a benign histology, such as tuberculosis

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

Characteristic	All study patients $n = 94$	Patients with MAGE-A–positive bone marrow or blood at or above LLOQ $n = 41$ (43.6%)	P^a	Patients with MAGE-A–positive bone marrow or blood at expression level ≥0.2 $n = 29$ (30.9%)	P^a
Tumor extension					
pT1–pT2	64 (21 + 43)	29 (47.5%)		20 (31.2%)	
pT3–pT4	30 (18 + 12)	12 (40.0%)	0.63	9 (30.0%)	0.90
Lymph node status					
pN0–1	72 (52 + 20)	34 (47.2%)		23 (31.9%)	
pN2	22	7 (31.8%)	0.20	6 (27.3%)	0.68
Tumor histology					
Adeno	37	19 (51.4%)		14 (37.9%)	
Squamous	42	18 (42.9%)		13 (31.0%)	
Miscellaneous[b]	15	4 (26.7%)	0.26	2 (13.3%)	0.22
Grading					
G1–G2	40 (3 + 37)	18 (45.0%)		14 (35.0%)	
G3–G4	54 (53 + 1)	23 (42.6%)	0.82	15 (27.8%)	0.45
Age					
≤66 years	50	18 (36.0%)		16 (32.0%)	
>66 years	44	23 (52.3%)	0.11	13 (29.5%)	0.90

Abbreviations: Adeno, adenocarcinoma; LLOQ, lower limit of quantification.

[a]Two-sided *P* values determined by Pearson χ^2 test show possible significance of correlation between detection of MAGE-A transcripts and clinicopathologic parameters.

[b]"Miscellaneous" represents 8 adenosquamous carcinomas and 7 large cell carcinomas.

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. Clinicopathologic 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). Forty-nine patients died within the observation period (55.1%). Table 2 shows treatment failures according to the site of recurrence and MAGE-A expression level in bone marrow or blood.

MAGE-A expression in blood and bone marrow

In total, 1,848 expression profiles of seven *MAGE-A* genes in 264 bone marrow and blood samples of 94 patients were created (Supplementary Table S1). Fifteen and 3 samples dropped out due to vial damage or failure of amplification of the housekeeping marker *PBGD*, respectively. Quantifiable MAGE-A expression, that is, 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 subthreshold 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-

Table 2. Treatment failure according to MAGE-A expression level in bone marrow or blood and site of recurrence

Variable	Total cohort $n = 89$	Patients with MAGE-A–positive bone marrow or blood at expression level ≥0.2 $n = 29$ (32.6%)	Patients with MAGE-A–positive bone marrow or blood at expression level <0.2 $n = 60$ (67.4%)
Disease recurrence	40	20	20
Local recurrence[a]	16	6	10
Distant metastasis[b]	30	16	14
Distant metastasis without prior local recurrence	24	14	10
Death	49	22	27
Death of any cause[c]	49	22	27
Cancer-related death	36	18	18
Event-free outcome	40	7	33

[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

MAGE-A expression threshold	P^a
≥0.05 vs. <0.05	0.013
≥0.1 vs. <0.1	0.002
≥0.2 vs. <0.2	<0.001
≥0.3 vs <0.3	0.004
≥0.5 vs. <0.5	0.013

[a]*P* values of univariate analyses were determined by log-rank test.

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 (Fig. 1A). The corresponding 5-year Kaplan–Meier estimates of distant metastasis–free survival were 87% [95% confidence interval (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; Fig. 1B and C). The corresponding 5-year Kaplan–Meier estimates of overall survival were 59% (95% CI

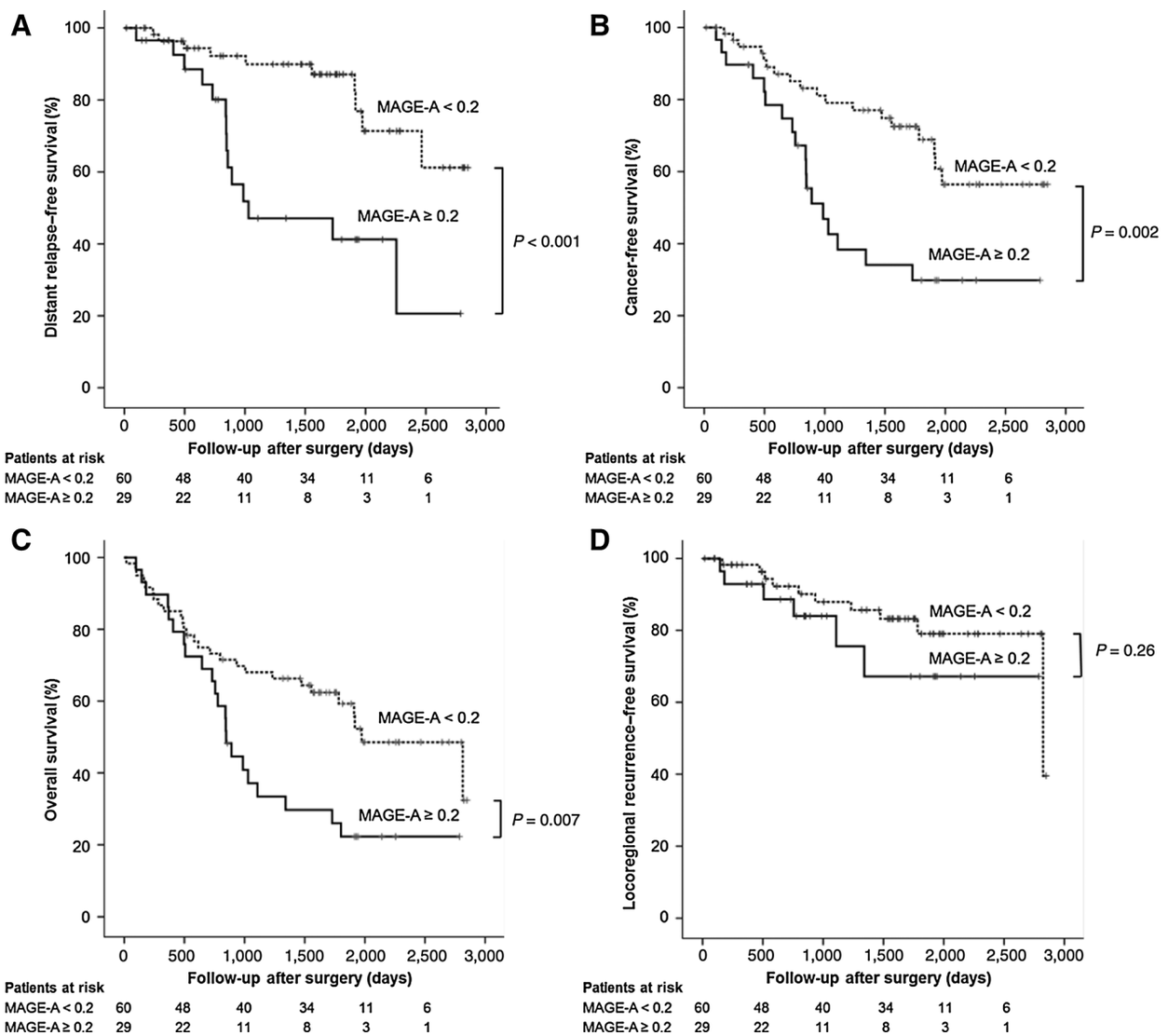

Figure 1.

A–D, Kaplan–Meier estimates of distant relapse–free survival (**A**), cancer-free survival (**B**), overall survival (**C**), and locoregional recurrence–free survival (**D**) 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).

Table 4. Multivariable HRs for overall survival, cancer-free survival, and distant metastasis–free survival

	Overall survival			Cancer-free survival			Distant metastasis–free survival		
Variable	Univariate analysis (*P*)[a]	Multivariate analysis (*P*)[b]	HR (95% CI)	Univariate analysis (*P*)[a]	Multivariate analysis (*P*)[b]	HR (95% CI)	Univariate analysis (*P*)[a]	Multivariate analysis (*P*)[b]	HR (95% CI)
MAGE-A gene expression (≥0.2 vs. <0.2)	0.007	0.002	2.56 (1.42–4.63)	0.002	0.001	3.32 (1.66–6.61)	< 0.001	0.001	4.03 (1.77–9.18)
Tumor size (T3–4 vs. T1–2)	<0.001	0.002	1.71 (1.23–2.38)	0.007	0.016	1.65 (1.11–2.45)	0.51	n.s.	—[c]
Lymph node status (N2 vs. N0–1)	0.005	0.057	1.39 (1.00–1.93)	0.003	0.026	1.56 (1.07–2.29)	0.30	n.s.	—[c]
Grading (G3–4 vs. G1–2)	0.17	n.s.	—[c]	0.30	n.s.	—[c]	0.77	n.s.	—[c]
Patient age (>66 vs. ≤66 years)	0.94	n.s.	—[c]	0.67	n.s.	—[c]	0.84	n.s.	—[c]
Tumor histology (squamous carcinoma vs. adenocarcinoma vs. miscellaneous)	0.60	n.s.	—[c]	0.78	n.s.	—[c]	0.45	n.s.	—[c]

Abbreviation: n.s., not significant.

[a]*P* values of univariate analyses were determined by log-rank test.

[b]Stepwise multivariate analysis was performed using the Cox proportional hazards model.

[c]No estimate of relative risk is given, as the variable was not significant on multivariate analysis.

± 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 (Fig. 1D).

In the subgroup of patients with R1 resection ($n = 7$), 3 patients died due to local relapse. The remaining 4 patients had an uneventful course of disease. Further subgroup analyses on the correlation of MAGE-A expression with survival are found in the Supplementary Data.

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

Multivariate analysis using the Cox proportional hazards 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 an HR (≥0.2 vs. <0.2) of 4.03 (95% CI, 1.77–9.18). HRs for cancer-related death were 3.32 (95% CI, 1.66–6.61) 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%. HRs 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, for example, 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 CT antigens has been evaluated as a 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 current study. Although MAGE-A expression in our study serves only as a marker for the presence of MRD in blood or bone marrow and as a 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 a compartment for the prediction of broadly disseminated disease and systemic tumor load (28, 29). As 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 $\geq$0.2 was associated with the highest increase in relative risk for death of any cause and cancer-related death compared with 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, for example, 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.

Disclosure of Potential Conflicts of interest

P. Kufer is the inventor on a provisional U.S. patent application on a method of treating a patient or exempting a patient from further treatment subsequent to tumor removal. The patent application is fully owned by P. Kufer and is not licensed. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: I. Mecklenburg, B. Passlick, P. Kufer
Development of methodology: I. Mecklenburg, P. Kufer
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I. Mecklenburg, W. Sienel, S. Schmid, B. Passlick
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): I. Mecklenburg, W. Sienel, S. Schmid, P. Kufer
Writing, review, and/or revision of the manuscript: I. Mecklenburg, W. Sienel, B. Passlick, P. Kufer
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): I. Mecklenburg
Study supervision: P. Kufer

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