MAGE-A expression clusters and antineoplastic treatment in head and neck cancer

STEFAN HARTMANN, TILL J. MEYER, ROMAN C. BRANDS, IMME R. HAUBITZ, CHRISTIAN LINZ, AXEL SEHER, ALEXANDER C. KÜBLER and URS D.A. MÜLLER-RICHTER

Department of Oral and Maxillofacial Plastic Surgery, University Hospital of Würzburg, D-97070 Würzburg, Germany

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Abstract. The nonsurgical treatment of head and neck squamous cell carcinoma (HNSCC) usually consists of radiation and chemotherapy. In general, the treatment efficacy of chemotherapy in head and neck cancer is limited. Apart from the placenta, testis and fetal keratinocytes, melanoma-associated antigens-A (MAGE-A) are only found in malignancies. Even though their molecular role remains unclear, several subgroups have been found to contribute to resistance to different chemotherapeutic agents. In the present study, established human squamous cell carcinoma cell lines were incubated with various concentrations of cisplatin, 5-fluorouracil, paclitaxel, docetaxel, cetuximab and panitumumab for 5, 10, 20 and 40 h. The treatment efficacy was measured dynamically by real-time cell analysis (RTCA). In addition, we determined the expression of all known MAGE-A subgroups (MAGE-A1 to MAGE-A12, excluding pseudogene MAGE-A7) by reverse transcription quantitative polymerase chain reaction. Of note, one cell line showed only a marginal expression of MAGE-A antigens, whereas another cell line showed a distinct expression of almost all the MAGE-A subgroups. The expression pattern varied in the other cell lines. MAGE-A4 was the most highly expressed of all the subgroups, and MAGE-A8 could not be detected. With the exception of MAGE-A6, -A8, -A9 and -A10, the expression levels differed significantly between the cell lines. Factor analysis suggested simplifying the MAGE-A expression level into two groups. Spearman's rank correlation revealed a significant association between MAGE-A expression and treatment efficacy for 20.8% (25/120) of the experiments. In 100% of these cases (25/25), Spearman's Rho revealed a positive correlation between clustered MAGE-A expression and poor treatment efficacy. Our data highlight the fact that higher a MAGE-A expression correlates with a poorer outcome of antineoplastic treatment. Clustered MAGE-A expression analysis may help to identify patients who are at a higher risk of antineoplastic treatment failure.

Correspondence to: Dr Stefan Hartmann, Department of Oral and Maxillofacial Plastic Surgery, University Hospital of Würzburg, Pleicherwall 2, D-97070 Würzburg, Germany
E-mail: hartmann_s2@ukw.de

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Introduction

The treatment of head and neck cancer remains challenging. Due to the high rate of locally advanced and metastatic tumors, intensified adjuvant treatment regimes, including radiotherapy and chemotherapy, are performed. However, local recurrence is a common occurrence, and the cumulative 5-year overall survival is approximately 50% (1). In addition to TNM staging and histological grading, molecular markers may aid in the identification of high-risk patients. Melanoma-associated antigens-A (MAGE-A) proteins belong to the very large family of cancer/testis antigens (CTA) (2). With the exception of the placenta, testis and fetal keratinocytes, MAGE-A expression is restricted to malignant tissues. In general, MAGE-A expression has been reported in malignant melanoma (3), breast cancer (4), lung cancer (5), urothelial carcinoma (6), colorectal carcinoma (7), hepatocellular carcinoma (8) and head and neck cancer (9). Typically, the different subgroups are co-expressed, but there is no unique expression pattern for specific tumor entities (10). Remarkably, MAGE-A expression has been found in oral carcinoma *in situ* and leukoplakia with dysplasia, but has not been detected in oral ulcers, oral lichen planus and leukoplakia without dysplasia (11). Recently, Han *et al* reported that a high MAGE-A9 expression is a poor prognostic marker in laryngeal squamous cell carcinoma (12). Of note, in their study, MAGE-A9 expression did not correlate with the TNM status, but a highly significant correlation was observed with grading and overall survival, indicating that the MAGE-A9 expression status may thus be another way of identifying high-risk patients, apart from TNM. Our group previously demonstrated a correlation between the treatment efficacy of several drugs and the expression of certain MAGE-A subgroups (13,14). This correlation may be mediated, at least in part, by MAGE-induced p53-inhibition (15) and/or MAGE-associated expression of taxol resistance-associated gene-3 (TRAG-3) (16). Additionally, MAGE-A3 expression has been shown to contribute to an increased tumor size and the size of metastatic foci in an animal xenograft model of thyroid cancer (17). As antigens, MAGE proteins can be recognized

by T-cells and thus lead to immune activation. This property makes them interesting players in terms of tumor vaccination. For example, in non-small cell lung cancer (NSCLC) patients, MAGE-A3 vaccine has been shown to contribute to the reduction of recurrence rates compared with the control group (18).

This study was designed to evaluate the prognostic value of all known MAGE-A antigens in terms of response to chemotherapy. As the evaluation of the distinct functional role of all MAGE-A antigens is almost unmanageable, the total and clustered MAGE-A expression in the context of chemoresistance was examined.

Materials and methods

Cell lines. We used cell lines that were originally established (Table I) at the Cancer Institute of the University of Pittsburgh (19) and used in our previous studies (13,14,20). As previously described, the cells were cultured in a humidified atmosphere of 5% CO_2/95% air at 37˚C and fed 2 to 3 times/week (14,20).

Drugs. For the antineoplastic treatment of the cell lines, we selected agents that are widely used in different protocols for induction or for neoadjuvant or adjuvant chemotherapy in head and neck cancer. Cisplatin was purchased from Teva (Radebeul, Germany) and 5-fluorouracil was purchased from Medac (Hamburg, Germany). Paclitaxel, docetaxel, cetuximab and panitumumab were provided by the pharmacy of the University Hospital of Würzburg, Germany.

Measurement of cytotoxicity by real-time cell analysis (RTCA). For the determination of cytotoxicity, we used an impedance-based, non-colorimetric and interference-free RTCA system (xCELLigence RTCA SP System; Roche, Mannheim, Germany). Measurement intervals were 30 min. This method is established and has been previously described for cytotoxicity measurement in head and neck cancer cell lines (21). We seeded 10,000 cells/well of each cell line. Following overnight incubation, cisplatin (25-400 μM), 5-fluorouracil (0.75-12 mM), docetaxel (1.56-25 nM), paclitaxel (1.56-25 nM), cetuximab (0.01-100 μg/ml) and panitumumab (0.01-100 μg/ml), all dissolved in 20 μl Dulbecco's modified Eagle's medium (Life Technologies GmbH/ThermoFisher Scientific, Darmstadt, Germany), were added to the cultures, which were incubated for an additional 40 h. All of the experiments were performed in triplicate, and further evaluations used mean values.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). For RNA isolation, the cells were detached from the culture plate and dissolved in TRIzol reagent (Life Technologies GmbH/ThermoFisher Scientific). After repeated centrifugations (at 12,000 x g), the RNA pellet was resolved in 20 μl of fully desalinated water. Subsequently, the RNA concentration was determined using a NanoDrop 2000 system (Thermo Fisher Scientific, Waltham, MA, USA). The absorption was measured at a wavelength of 260 nm and was normalized to the absorption of the fully desalinated water control at the same wavelength. All specimens were diluted to a concentration of 0.2 μg RNA/ml.

In order to remove the DNA contaminants, the RNA was heated up to 42˚C for 2 min and then chilled on ice. This step was performed in the presence of a gDNA Wipeout Buffer (Qiagen, Venlo, The Netherlands) (Table II). Reverse transcription was carried out by incubation at 42˚C for 15 min using a Reverse Transcription kit from QuantiTect (Qiagen). The reverse transcriptase was denatured by heating the sample to 95˚C for an additional 3 min (Table III). Polymerase chain reaction was conducted using a QuantiTect SYBR-Green PCR kit 200 (Qiagen) and a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The protocol and the primers (Qiagen) differ from previous publications by our group and are described in Tables IV-VI. Quantification of expression was calculated relative to β-actin.

Statistical analysis. For statistical analysis, we used MEDAS software (version 2014; Grund, Margetshöchheim, Germany). Global treatment efficacy is represented as the mean number of viable cells at different drug concentrations and incubation times for all cell lines used. We analyzed the influence of the concentration of each drug at a fixed incubation time with the cell lines as repeats with Friedman 2-way ANOVA by ranks. To evaluate significant differences among MAGE-A subgroup expression, we also used the Friedman test. To simplify expression analysis, factor analysis was performed. Afterwards, the factors were correlated with treatment efficacy by Spearman's rank correlation. Spearman's Rho is the correlation coefficient and expresses the relationship between the two investigated parameters. A Rho value of 1 represents a perfect correlation between both investigated parameters, whereas a Rho value of -1 means a perfect inverse correlation of both parameters. Rho $\geq$0.7 is considered a high (positive) correlation, whereas Rho values $\leq$-0.7 are considered a high inverse correlation. In our analysis, we correlated the total and the clustered MAGE-A expression with the results of the RTCA experiments. A high number of viable cells is represented by a high RTCA cell index and indicates a low efficacy of the drug used in the experiment. The significance level was set at $p \leq 0.05$.

Results

Treatment efficacy. A brief overview on the efficacy of cisplatin incubation is provided in Fig. 1A. Of note, the viable fraction after 5 h of incubation is higher than the control (25/50/100/200 μM cisplatin). The same effect can be observed after 10 and 20 h of incubation for concentrations of 25/50/100 μM and 25/50 μM, respectively. After 40 h of incubation, the viable fraction of all concentrations was <100%. The effect of cisplatin on the number of viable cells was significantly associated with the incubation time. Based on all incubation intervals, the concentration of cisplatin was not significantly associated with the viable fraction (Table VII).

Fig. 1B provides an overview of the effects of 5-fluorouracil treatment on the cell lines. After an initial increase in the number of viable cells, the viable fraction at all concentrations after 40 h of incubation is clearly below that of the controls. The effect of 5-fluorouracil on the viable fraction is significantly associated with the incubation time. Like cisplatin, the concentration of 5-fluorouracil is not significantly associated with the viable fraction (Table VII).

Table I. Name, origin and TNM status of the 5 cell lines used in this study.

Name	Origin	TNM classification
PCI-1	Laryngeal carcinoma of the glottis of a male patient	pT2N0M0G2
PCI-9	Primary carcinoma at the base of the tongue of a male patient	pT4N3M0G2
PCI-13	Male patient diagnosed with oral squamous cell carcinoma of the retromolar triangle	pT4pN1M0G3
PCI-52	Primary carcinoma of the aryepiglottic fold of a male patient	pT2N0M0G2
PCI-68	Primary tongue carcinoma of a male patient	pT4N0M0G1

Table II. Composition of the gDNA Wipeout Buffer with RNA.

gDNA Wipeout Buffer, 7X	2 μl
Template RNA	5 μl
RNase-free water	7 μl

Table III. Composition of the reverse transcription reaction with purified RNA.

Quantiscript reverse transcriptase	1 μl
Quantiscript RT buffer, 5X	4 μl
RT Primer mix	1 μl
Purified RNA	14 μl

Table IV. Polymerase chain reaction protocol.

1x	Polymerase activation	15 min	95˚C
40x	Denaturation	15 sec	94˚C
	Annealing	30 sec	50-60˚C
	Elongation	30 sec	72˚C

Table V. Composition of the polymerase chain reaction mixture.

SYBR Green Master Mix	12.5 μl
QuantiTect Primer assay	2.75 μl
cDNA (concentration of 10 ng/μl)	2.5 μl
Water	7.25 μl

Table VI. MAGE-A primers.

Hs_ACTB_2_SG	#QT01680476
Hs_MAGEA1_2_SG	#QT01669430
Hs_MAGEA2_2_SG	#QT01668688
Hs_MAGEA2B_1_SG	#QT01033529
Hs_MAGEA3_1_SG	#QT00064799
Hs_MAGEA4_1_SG	#QT00008862
Hs_MAGEA5_3_SG	#QT01849750
Hs_MAGEA6_1_SG	#QT00059129
Hs_MAGEA8_1_SG	#QT00094668
Hs_MAGEA9_1_SG	#QT00230874
Hs_MAGEA10_1_SG	#QT00005376
Hs_MAGEA11_1_SG	#QT01004094
Hs_MAGEA12_1_SG	#QT00033873
Hs_SPP1_1_SG	#QT01008798

All MAGE-A primers used in this study were purchased from QuantiTect. The name and the order number of all primers is shown. MAGE-A, melanoma-associated antigens-A.

The characteristics of paclitaxel treatment are shown in Fig. 1C. Cells incubated with 1.56 and 3.12 nM of paclitaxel grew faster than the controls after 5, 10, 20 and 40 h. Higher concentrations of paclitaxel (6.25, 12.5 and 25 nM) lead to a smaller viable fraction than the control cells after 10, 20 and 40 h. The effect of paclitaxel on the viable fraction is significantly associated with the incubation time. The concentration of paclitaxel is not significantly associated with the viable fraction (Table VII).

The effects of docetaxel treatment are comparable to those of paclitaxel treatment and are shown in Fig. 1D. With the exception of 1.56 nM of docetaxel after 5 and 10 h, all other concentrations and time intervals showed a smaller viable fraction than the controls. The effect of docetaxel on the viable fraction is significantly associated with the incubation time. The concentration of docetaxel is not significantly associated with the viable fraction (Table VII).

The effects of cetuximab treatment are shown in Fig. 1E. Cetuximab treatment at all concentrations and time intervals led to a higher fraction of viable cells compared with the controls. This effect is time-dependent but not concentration-dependent (Table VII).

An overview on panitumumab treatment efficacy is provided in Fig. 1F. Panitumumab has no relevant cytostatic effect. However, as with the other drugs, there was a significant association between treatment efficacy and time but not concentration (Table VII).

MAGE-A expression. MAGE-A expression analysis showed distinct differences among the cell lines (Fig. 2). Of note, one cell line (PCI-68) showed only a marginal expression of MAGE-A antigens, whereas another cell line (PCI-52) showed a distinct expression of almost all MAGE-A subgroups (10/12). The expression pattern of the other cell lines ranged between that of these cell lines. MAGE-A4 showed the highest expression level of all subgroups, and MAGE-A8 could not be detected. PCI-52 had the highest levels of MAGE-A1 (0.206),

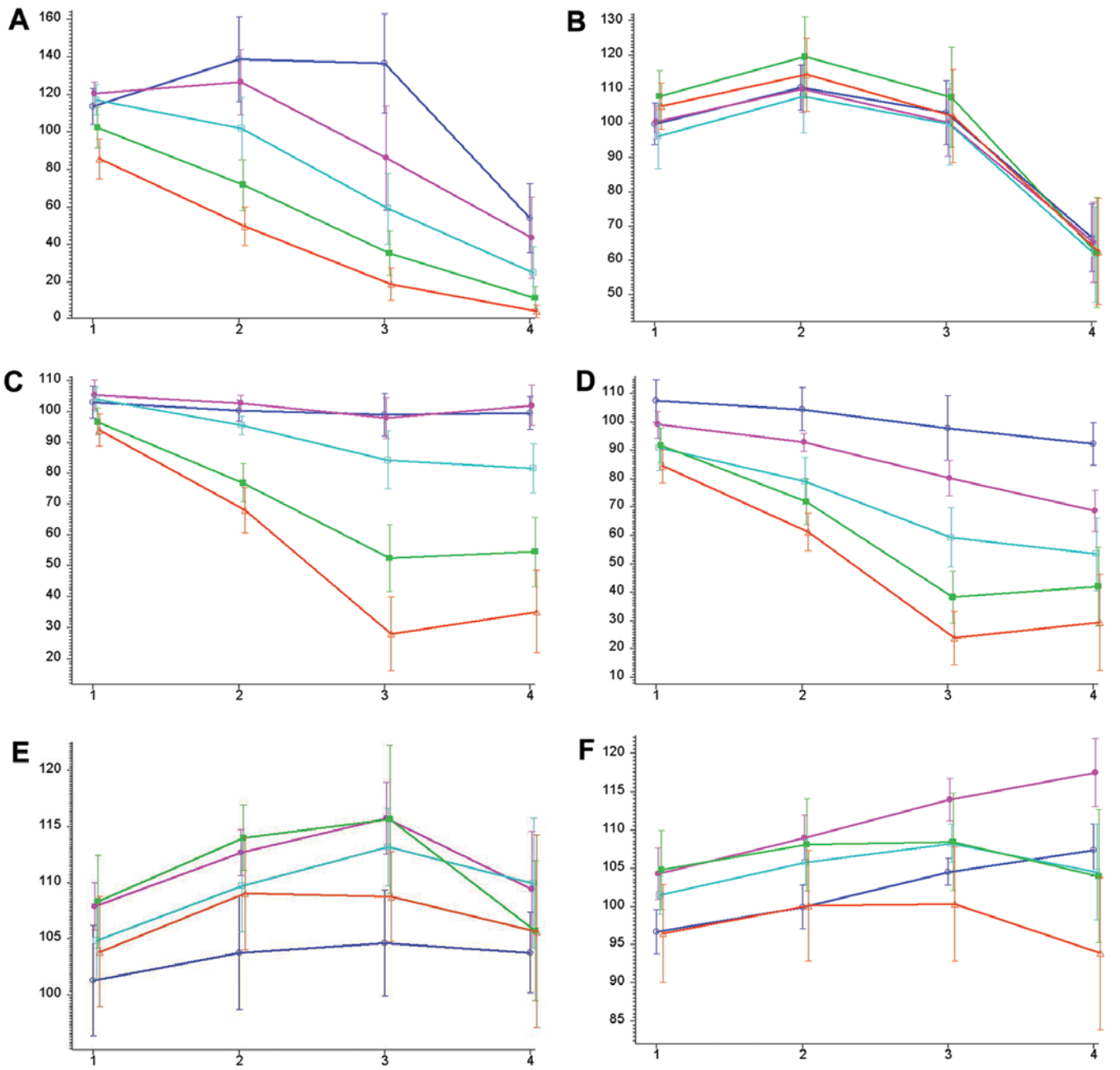

Figure 1. The y-axis represents the viable fraction compared with the control (100). The x-axis shows different time intervals (1, 5 h; 2, 10 h; 3, 20 h; 4, 40 h). The bars indicate the standard deviation of the mean. (A) Cumulative treatment efficacy of cisplatin. The concentrations are as follows: blue, 25 μM; pink, 50 μM; turquoise, 100 μM; green, 200 μM; red, 400 μM. (B) Cumulative treatment efficacy of 5-fluorouracil. The concentrations are as follows: blue, 750 μM; pink, 1.5 mM; turquoise, 3 mM; green, 6 mM; red, 12 mM. (C) Cumulative treatment efficacy of paclitaxel. The concentrations are as follows: blue, 1.56 nM; pink, 3.12 nM; turquoise, 6.25 nM; green, 12.5 nM; red, 25 nM. (D) Cumulative treatment efficacy of docetaxel. The concentrations are as follows: blue, 1.56 nM; pink, 3.12 nM; turquoise, 6.25 nM; green, 12.5 nM; red, 25 nM. (E) Cumulative treatment efficacy of cetuximab. The concentrations are as follows: blue, 0.01 μg/ml; pink, 0.1 μg/ml, turquoise, 1 μg/ml; green, 10 μg/ml; red, 100 μg/ml. (F) Cumulative treatment efficacy of panitumumab. The concentrations are as follows: blue, 0.01 μg/ml; pink, 0.1 μg/ml; turquoise, 1 μg/ml; green, 10 μg/ml; red, 100 μg/ml.

-A4 (1.062), -A5 (0.271), -A9 (0.108) and -A11 (0.079). PCI-13 showed the strongest expression of MAGE-A2 (0.242), -A3 (0.643), -A6 (0.641) and A12 (0.969). PCI-1 showed the highest levels of MAGE-A2b (0.822), and PCI-9 had the strongest expression of MAGE-A10 (0.149). With the exception of MAGE-A6, -A8, -A9 and -A10, the expression levels differed significantly among the cell lines (Table VIII).

Factor analysis. The factor analysis suggests the clustering of MAGE-A expression into 4 groups (Table IX). Cluster 1 consists of MAGE-A1, -A4, -A5, -A9 and -A11. Cluster 2 consists of MAGE-A2, -A2b, -A3, -A6 and -A12. MAGE-A8 represents cluster 3, and MAGE-A10 represents cluster 4. Due to the low expression level of the MAGE-A subgroups -A8 and -A10, clusters 3 and 4 were excluded from further analyses.

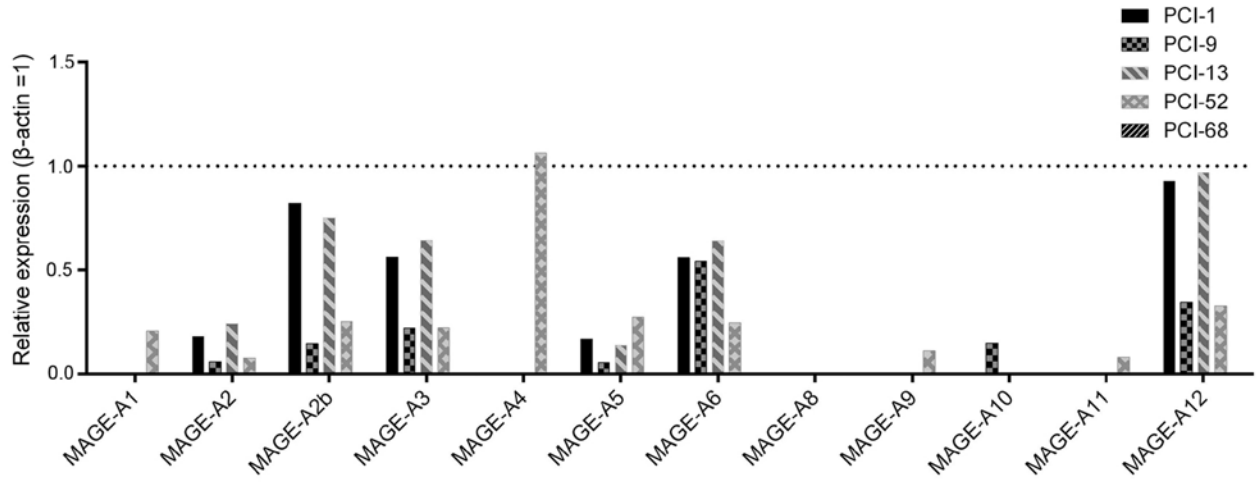

Figure 2. Melanoma-associated antigens-A (MAGE-A) expression profile in the analyzed cell lines. The expression is shown as a relative fraction compared with β-actin (=1).

Table VII. Association between incubation time or concentration and the treatment efficacy for all drugs tested.

Drug	p-value (incubation time)	p-value (concentration)
Cisplatin	<0.000005[a]	0.19
5-Fluorouracil	0.00000[a]	0.92
Paclitaxel	<0.000005[a]	0.11
Docetaxel	<0.000005[a]	0.098
Cetuximab	0.00005[a]	0.72
Panitumumab	0.00005[a]	0.51

Tested drugs were cisplatin, 5-fluorouracil, paclitaxel, docetaxel, cetuximab and panitumumab. The results of Friedman test are shown. [a]p<0.001, indicates extremely significant results.

Table VIII. Differences in MAGE-A subgroup expression among the cell lines analyzed by the Friedman test.

Subgroup	p-value
MAGE-A1	0.017[a]
MAGE-A2	0.024[a]
MAGE-A2b	0.012[a]
MAGE-A3	0.032[a]
MAGE-A4	0.016[a]
MAGE-A5	0.019[a]
MAGE-A6	0.088
MAGE-A8	0.22
MAGE-A9	0.068
MAGE-A10	0.080
MAGE-A11	0.025[a]
MAGE-A12	0.036[a]

MAGE-A, melanoma-associated antigens-A. [a]p<0.05 to 0.01, indicates significant results.

Due to the suggested clustering, 96.61% of the variance was represented, and an excellent fitting was achieved.

Correlation of MAGE-A expression and treatment efficacy. In total, 120 experiments (6 drugs, 5 concentrations, 4 time intervals) represent the treatment efficacy of the drugs in each cell line.

First, we analyzed the correlation of total MAGE-A expression level and efficacy of all tested drugs at all concentrations after all time periods. Of note, there was no significant correlation between total MAGE-A expression and treatment efficacy of all tested drugs.

In the next step, we correlated the clustered MAGE-A expression (cluster 1 or 2) with the treatment efficacy of all drugs. High correlations (Rho ≥0.7) between MAGE-A expression (group 1 or 2) and lower treatment efficacy were observed in 38.3% (46/120) of the experiments. The analysis revealed a significant correlation between cluster 1 (-A1, -A4, -A5, -A9 and -A11) or cluster 2 (-A2, -A2b, -A3, -A6 and -A12) expression and several concentrations of cisplatin, 5-fluorouracil, docetaxel, paclitaxel, cetuximab and panitumumab (Table X). Overall, a significant correlation between clustered MAGE-A expression and treatment efficacy was observed in 20.8% (25/120) of the experiments.

In all 25 cases, a significant correlation Rho was between 0.9 and 1.0, indicating a positive correlation between MAGE-A expression and the RTCA cell index. The RTCA cell index represents the number of viable cells. A higher cell index indicates a minor effective chemotherapeutic treatment. In summary, a higher cluster 1 or 2 MAGE-A expression correlated with a minor efficacy of the tested drugs.

By contrast, a high inverse correlation (Rho ≤-0.7) between MAGE-A expression and treatment efficacy was not observed (data not shown). An inverse correlation (Rho ≤0) was found in 19.2% of the cases (23/120). However, none of these correlations were significant (data not shown).

Table IX. Results of factor analysis.

MAGE	Factor 1	Factor 2	Factor 3	Factor 4	Commonality
A1	-0.108	0.990[a]	-0.055	-0.041	0.997
A2	0.967[a]	-0.048	-0.029	-0.071	0.943
A2b	0.885[a]	-0.133	-0.265	-0.081	0.877
A3	0.986[a]	-0.047	-0.026	0.002	0.975
A4	-0.098	0.989[a]	-0.056	-0.043	0.992
A5	0.548	0.791[a]	-0.095	-0.033	0.936
A6	0.776[a]	-0.077	0.570	-0.162	0.960
A8	-0.120	-0.093	0.081	0.984[a]	0.998
A9	-0.126	0.970[a]	-0.074	-0.056	0.965
A10	-0.143	-0.173	0.955[a]	0.121	0.977
A11	-0.117	0.989[a]	-0.025	-0.039	0.995
A12	0.987[a]	-0.068	-0.011	-0.042	0.980
Variance	4.65	4.57	1.34	1.03	11.593
Variance (%)	38.76	38.12	11.13	8.60	96.61
Factor loading	5	5	1	1	12

The first factor consists of MAGE-A2, -A2b, -A3, -A6 and A12. The second factor consists of MAGE-A1, -A4, -A5, -A9 and -A11. Factors 3 and 4 consist of MAGE-A10 and -A8, respectively. The 4 factor model represents 96.61% of the variance. MAGE-A, melanoma-associated antigens-A. 'a' highlights the MAGE-A subgroups being included in the different factors of factor analysis.

Table X. Significant correlations between group 1 or 2 MAGE-A expression and treatment efficacy.

Drug	Concentration	Time	Rho	p-value
Cisplatin	100 μM	5 h	1.0000	0.00000[b]
5-Fluorouracil	750 μM	5 h	0.9000	0.037[a]
5-Fluorouracil	3 mM	5 h	0.9000	0.037[a]
5-Fluorouracil	3 mM	20 h	1.0000	0.00000[b]
5-Fluorouracil	12 mM	20 h	1.0000	0.00000[b]
5-Fluorouracil	12 mM	40 h	0.9000	0.037[a]
Paclitaxel	6.25 nM	20 h	0.9000	0.037[a]
Paclitaxel	12.5 nM	5 h	0.9000	0.037[a]
Paclitaxel	25 nM	20 h	1.0000	0.00000[b]
Docetaxel	1.56 nM	5 h	0.9000	0.037[a]
Docetaxel	3.12 nM	20 h	1.0000	0.00000[b]
Docetaxel	3.12 nM	40 h	0.9000	0.037[a]
Docetaxel	6.25 nM	5 h	0.9000	0.037[a]
Docetaxel	12.5 nM	10 h	1.0000	0.00000[b]
Cetuximab	0.01 μg/ml	5 h	0.9000	0.037[a]
Cetuximab	0.01 μg/ml	20 h	0.9000	0.037[a]
Cetuximab	1 μg/ml	5 h	1.0000	0.00000[b]
Cetuximab	10 μg/ml	40 h	1.0000	0.00000[b]
Cetuximab	100 μg/ml	5 h	0.9000	0.037[a]
Cetuximab	100 μg/ml	20 h	0.9000	0.037[a]
Cetuximab	100 μg/ml	40 h	0.9000	0.037[a]
Panitumumab	0.01 μg/ml	40 h	0.9000	0.037[a]
Panitumumab	0.1 μg/ml	5 h	1.0000	0.00000[b]
Panitumumab	1 μg/ml	5 h	0.9000	0.037[a]
Panitumumab	1 μg/ml	10 h	0.9000	0.037[a]

Analyzed by the Spearman's rank correlation. MAGE-A, melanoma-associated antigens-A. [a]$p<0.05$ to 0.01, indicates significant results; [b]$p<0.001$, indicates extremely signficant results.

Discussion

There is a general agreement that MAGE genes and proteins are often overexpressed in cancer tissues and contribute to the progression of malignancies (2,17,22). In addition, there is growing evidence that MAGE expression is related to a lower efficacy of systemic antitumor treatment and a poorer prognosis of cancer patients. In particular, MAGE-A proteins appear to play a role as regulator of transcription factors, such as p53 (15,23,24). Our group previously described several correlations between MAGE-A expression patterns and treatment efficacy in head and neck cancer (13,14,20). In terms of head and neck cancer, simultaneous nuclear and cytoplasmic expression of MAGE-A proteins is described as an independent marker for poor survival (9). Based on these findings and due to the complexity of different roles of all MAGE-A subgroups, the aim of this study was to investigate the role of total and clustered MAGE-A expression in the context of chemotherapeutic treatment. To the best of our knowledge, this study is the first to examine the correlation of all known MAGE-A subgroups with the impact of antineoplastic treatment.

As expected, the treatment efficacy showed a wide range in the different cell lines. For all drugs, we noticed a significant time-dependent effect. However, treatment with cetuximab and panitumumab did not lead to relevant cytostatic effects. This was previously described by our group (14).

Regarding the MAGE-A levels, our analysis yielded an inhomogenous expression pattern in the different cell lines. One cell line (PCI-52) expressed 10 out of 12 of the investigated MAGE-A subgroups. By contrast, PCI-68 showed only a marginal expression of the MAGE-A subgroups. In our previous studies, we detected MAGE-A8 expression in this cell line panel (14). However, in this study, we did not observe relevant MAGE-A8 expression. This may be explained by the

fact that another primer set was used in our previous study. The MAGE-A2 gene is found on two different loci on the X chromosome (MAGE-A2 and MAGE-A2b). Of note, in all cell lines expressing MAGE-A2, we observed higher levels of MAGE-A2b than MAGE-A2. This indicates that PCR-based quantitative MAGE-A expression analysis should always include MAGE-A2 and MAGE-A2b. MAGE-A4 showed the highest levels of all investigated subgroups. With the exception of PCI-68, all of the cell lines expressed multiple MAGE-A subgroups. This phenomenon has been reported in the literature and was described in detail in the study by Ries *et al* in 2008 (25). Further statistical analysis revealed significantly different expression levels for A1, A2, A2b A3, A4, A5, A11 and A12, indicating that a diversified panel of cell lines was used.

Since the MAGE-A proteins are considered to be contributors to malignancy, the total amount of protein expression may correlate with a poorer outcome of antineoplastic treatment. To our surprise, total MAGE-A expression was not significantly associated with any of the antineoplastic treatments used in our experiments. This may be explained by complex interactions between pro- and anti-apoptotic functions of different MAGE-A subgroups. Remarkably, the cell line (PCI-52) that was least vulnerable to all agents had the highest cumulative MAGE-A expression. Nevertheless, this cell line was the only one expressing MAGE-A4. Of note, Bandić *et al* described MAGE-A4 as a positive predictor of survival in women with invasive ductal breast cancer (26). Furthermore, Sakurai *et al* showed that MAGE-A4 interacts with Myc-interacting zinc finger protein 1 (Miz1), thus leading to apoptosis in a panel of human cancer cell lines (27). On the other hand, PCI-52 is the only cell line expressing relevant amounts of MAGE-A9 and -A11. A negative influence of MAGE-A9 on the prognosis of breast cancer (28), hepatocellular carcinoma (8), renal cell carcinoma (29) and head and neck cancer (12) has been extensively described. MAGE-A11 expression also seems to contribute to malignancy. Xia *et al* demonstrated that MAGE-A11 distinctly enhances the proliferation of breast cancer cells (30). A study by Lian *et al* showed that MAGE-A11 expression is a poor prognostic factor for overall survival in breast cancer (31). Taken together, these findings may explain how different MAGE-A subgroups antagonize each other, thus making it impossible to perform a cumulative analysis of all subgroups.

The finding that total MAGE-A expression showed no significant correlation with treatment efficacy led us to perform a cluster analysis of the MAGE-A subgroups. Factor analysis suggested clustering MAGE-A expression into two clusters. The first cluster consists of A1, A4, A5, A9 and A11, whereas A2, A2b, A3, A6, A12 represent the second cluster. MAGE-A8 and -A10 were excluded due to low expression levels. Of note, a high correlation was observed between clustered expression and minor treatment efficacy in 38.3% of the experiments (46/120). Remarkably, this finding was significant in 54.3% (25/46) of the cases. This result is quite unexpected as MAGE-A4, -A9 and -A11 were clustered in one group but may have opposite functional roles in terms of apoptosis regulation. Factor analysis revealed a contrast between PCI-13 and PCI-52. These two cell lines showed the highest expression levels of specific MAGE-A subgroups and also had the lowest response to the antineoplastic drugs. Hence, these findings indicate that MAGE-A expression, in general, is related to a poorer efficacy of antineoplastic treatment. Additional analysis is required to clarify why particular MAGE-A subgroups are clustered together and others are not.

Even if the majority (7/25) of significant correlations between high MAGE-A expression and a lower impact of antineoplastic treatment was found in patients treated with cetuximab, we were unable to draw any specific conclusions for clinical use. This notion is supported by the fact that cetuximab and panitumumab treatment, in general, showed no relevant effect, and our statistical findings regarding the EGFR antibodies should be considered an artifact. In clear contrast, high correlations (Rho ≤-0.7) between strong MAGE-A cluster expression and a better treatment efficacy were not observed in any of the cases (0/120). Taken together, these results provide further evidence that the co-expression of MAGE-A subgroups contributes to the minor efficacy of antineoplastic treatment.

Since this was a pilot study, there are no direct clinical implications for antineoplastic treatment at this time. Further analysis using tissue samples and an *in vitro* investigation of the possible interactions among several subgroups are warranted to clarify the role of the MAGE-A expression landscape as a predictor for prognosis in different types of cancer. However, cluster analysis rather than individual subgroup analysis may provide more rapid results and may be less expensive in the future.

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