

# Melanoma-associated antigen expression and the efficacy of tyrosine kinase inhibitors in head and neck cancer

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**Abstract.** Melanoma-associated antigen (MAGE) has been identified in a variety of types of cancer. The expression of several MAGE subgroups is correlated with poor prognosis and chemotherapeutic resistance. One target of chemotherapeutic treatment in head and neck cancer is the epidermal growth factor receptor (EGFR). The efficacy of tyrosine kinase inhibitors (TKI) in the context of melanoma-associated antigens is discussed in the present study. Five human squamous cell carcinoma cell lines were treated with the EGFR TKIs, erlotinib and gefitinib. The efficacy of these agents was measured using a crystal violet assay. Furthermore, the expression levels of MAGE-A1, -A5, -A8, -A9, -A11 and -A12 were determined by reverse transcription-quantitative polymerase chain reaction. The association between TKI efficacy and MAGE-A expression was analyzed by linear regression. The cell lines revealed inhomogeneous expression patterns for the MAGE-A subgroups. Four of the five cell lines demonstrated a good response to erlotinib and gefitinib. However, treatment with erlotinib induced better results than those of gefitinib, and revealed a concentration-dependent effect. The expression of MAGE-A5 and -A11 were significantly correlated with lower efficacy of erlotinib and gefitinib. By contrast, MAGE-A12 was associated with a superior response to these two drugs. One cell line, which expressed all investigated MAGE-A subgroups, was entirely resistant to the two TKIs. These results revealed a notable correlation between MAGE-A5 and -A11 and lower efficacy of EGFR TKIs. Pretreatment analysis of MAGE-A status may therefore aid improvement of chemoprevention using erlotinib and gefitinib in head and neck cancer.

## Introduction

Over 20 years ago, the melanoma-associated antigens (MAGEs) were identified by van der Bruggen *et al* (1). MAGEs belong to the group of cancer/testis antigens (CTA), which includes multiple proteins, for example NY-ESO-1, sinovial sarcoma X and G antigen 1 (2). It is known that these proteins are found in adult male germ cells, fetal keratinocytes, the placenta and a variety of human malignancies, including head and neck cancer (3-6). At present, the large group of MAGE tumor antigens consists of ~60 proteins. Their specific expression in the majority of malignancies, coupled with their immunogenicity, makes these proteins promising targets for anticancer therapies (7). However, little is known about the function of MAGE-A tumor antigens. Studies by Doyle *et al* (8) and Yang *et al* (9) demonstrated the negative effect of MAGE expression on p53 levels. Notably, studies by Ries *et al* (10,11) provided clear evidence that MAGE-A expression serves as predictor of malignant transformation in oral leukoplakia. Furthermore, there is evidence that MAGE-A is expressed in dysplastic leukoplakia and carcinoma *in situ*, but not in oral lichen planus, oral ulcers or leukoplakia without dysplasia (12). Another study revealed that MAGE-A tumor antigens are the most frequently expressed CTAs in head and neck cancer (13). Notably, there is no correlation between MAGE-A expression and clinicopathological characteristics in head and neck cancer. Recently, Laban *et al* (14) published data indicating a marked correlation between poor prognosis in a subset of patients with head and neck cancer and the expression of MAGE-A antigens. In addition, a previous study by our group identified a correlation between MAGE-A5 and -A8 expression and poorer responses to anti-epidermal growth factor receptor (EGFR) therapy *in vitro* (15). Of note, MAGE-A11 expression is also correlated with poorer responses to cisplatin, 5-fluorouracil, docetaxel and paclitaxel (16).

In head and neck cancer, targeted therapy has an expanding role. This therapeutic approach is largely based on the EGFR and its associated pathways. Downstream signaling molecules of the EGFR, mediate the invasion, growth, progression and survival of tumor cells (17). The majority of head and neck cancer specimens demonstrate overexpression of the EGFR, and alterations in the copy number of this receptor are associated with poor prognosis (18). In general, EGFR-targeted therapies

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for head and neck cancer are performed with cetuximab, a chimeric antibody directed against the EGFR. Targeting the EGFR by tyrosine kinase inhibitors is another well-studied field of oncology, particularly in the case of EGFR-mutated non-small cell lung cancer (NSCLC), in which erlotinib and gefitinib serve as useful drugs that aid the improvement of progression-free survival and overall survival (19). Unfortunately, in an unselected cohort of head and neck cancer patients, erlotinib failed to improve progression-free survival when used in combination with cisplatin and radiotherapy (20). The role of erlotinib in head and neck cancer may be more efficacious in the field of chemoprevention. Using a combined approach of erlotinib and sulindac, data from Shin *et al* (21) demonstrated effective chemoprevention in preclinical and clinical models. These findings are discussed in a study recently published by Gross *et al* (22).

The present study therefore aimed to investigate whether the expression of MAGE-A tumor antigens was associated with poor efficacy of erlotinib and gefitinib.

## Materials and methods

**Cell lines.** The cell lines used in the present study (Table I) were established at the Cancer Institute of the University of Pittsburgh (Pittsburgh, PA, USA) (23). As described previously (15,24), the cells were cultured in a humidified atmosphere of 5% CO<sub>2</sub>/95% air at 37°C and were fed 2-3 times per week with Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, GmbH, Darmstadt, Germany) with low glucose, 10% fetal calf serum (Life Technologies, GmbH), 1% penicillin/streptomycin (Life Technologies, GmbH) and 1% L-glutamine (Biochrom KG, Berlin, Germany).

**RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of MAGE-A.** The protocol and quantification of MAGE-A expression by RT-qPCR was conducted as previously described by our group (15,16).

RNA isolation was executed using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. Isolated RNA was stored at -80°C prior to reverse transcription. Complementary (c)DNA was synthesized from identical quantities of total RNA (1 µg) with the M-MLV RT RNase H(-) point mutant using the buffer system provided (Promega Corp., Mannheim, Germany), according to the manufacturer's instructions.

MAGE-A expression profiles were quantitatively analyzed by RT-qPCR using the FastStart DNA Master Plus SYBR-Green I (Roche Diagnostics GmbH, Mannheim, Germany). Each reaction mixture (20 µl) was comprised of 0.5 µl cDNA, 1 µl forward primer (20 µM), 1 µl reverse primer (20 µM) (both from TIB MOLBIOL GmbH, Berlin, Germany), 4 µl LightCycler DNA Master SYBR-Green I, 1 µl dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) and 12.5 µl water. The cycling conditions for RT-qPCR in the LightCycler 2.0 (Roche Diagnostics, Mannheim, Germany) were as follows: Initial denaturation at 95°C for 10 min, followed by 45 cycles of amplification with denaturation at 95°C for 10 sec, primer annealing at 62-67°C for 3-4 sec and elongation at 68-72°C for 3-4 sec (specific temperature and

elongation indicated in Table II). Following completion of this protocol, a melting range analysis was conducted. The protocol comprised one cycle at 95°C for 20 sec, followed by one cycle at 60°C for 20 sec with continuously measured fluorescence. The values measured were analyzed by the LightCycler Relative Quantification Software 1.0 (Roche Diagnostics GmbH), which provided efficiency-corrected, calibrator-normalized relative quantification results. The relative concentrations were normalized to β-actin messenger RNA levels.

**Drug treatment and crystal violet assay.** Cells of each cell line were seeded at 10,000 cells/well, respectively. Erlotinib and gefitinib were purchased from Selleckchem (distributed by Absource Diagnostics GmbH, München, Germany) and stored according to the manufacturer's instructions. The concentrations used in the present study (4.94, 1.65, 0.55, 0.18 and 0.06 µM) were derived from a log 3 dilution starting at 400 µM (data not shown). The control cells were cultured in medium as described above (without TKIs). These concentrations were chosen based on the clinically relevant maximum serum concentration of the selected tyrosine kinase inhibitors (TKIs), of ~1 µM (25). Following 24 h of incubation in standard medium, erlotinib or gefitinib was added, and the cultures were incubated for a further 72 h. Crystal violet (1 g; Carl Roth GmbH, Karlsruhe, Germany) was dissolved in 1 l double-distilled water containing 20% methanol. Following the removal of the drug-containing medium, 50 µl crystal violet was added to each well and incubated for 15 min. The 96-well plates were then washed with distilled water and the optical density (OD) was measured at 595 nm using a RainBow microplate reader (Tecan, Maennedorf, Swiss). All experiments were performed in triplicate. The mean was calculated from at least three independent experiments and used in further analyses.

**Statistical analysis.** The association between chemosensitivity and MAGE-A expression status was analyzed using a linear regression model. This model allows evaluation of the correlation between the viable fraction of the cell culture and the concentration of the drug, as well as the expression level of MAGE-A subgroups during drug treatment. However, since the linear regression model based on 6 MAGE-A subgroups is only able to include 4 possible variables, MAGE-A1 and -A9 were excluded by SPSS.  $P \leq 0.05$  was considered to indicate a statistically significant difference. Statistical analysis of the data was performed using Microsoft Excel 2010 (Microsoft, Redmond, WA, USA) and SPSS Statistics 22 (IBM SPSS, Armonk, NY, USA) and was supported by the Department of Statistics, University of Würzburg. GraphPad Prism 6.04 (GraphPad Software Inc., La Jolla, CA, USA) was used to generate graphical illustrations.

## Results

**MAGE-A expression varies between squamous cell carcinoma cell lines.** MAGE-A expression was detected in all five of the cell lines. The minimum number of expressed subgroups was two (in PCI-68). The lowest quantities of MAGE-A tumor antigens were also detected in PCI-68. In contrast to PCI-68, all MAGE-A tumor antigen subgroups examined were expressed by PCI-52.

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

Cell line	Origin	Patient gender	TNM
PCI-1	Laryngeal carcinoma of the glottis	male	pT2N0M0G2
PCI-9	Primary carcinoma at the base of the tongue	male	pT4N3M0G2
PCI-13	Oral squamous cell carcinoma of the retromolar triangle	male	pT4pN1M0G3
PCI-52	Primary carcinoma of the aryepiglottic fold	male	pT2N0M0G2
PCI-68	Primary tongue carcinoma	male	pT4N0M0G1

Table II. Sequences, base pair lengths, annealing temperatures and elongation times of the primers used.

Gene	Sequence, 5'-3'	Base pairs	Annealing, °C/sec	Elongation, °C/sec
β-actin	F: CCAACCGCGAGAAGATGA R: CCAGAGGCGTACAGGGATAG	97	65/3	68/4
MAGE-A1	F: GGCCGAAGGAACCTGACC R: GTCCTCTGGGTTGGCCTGT	69	67/3	72/4
MAGE-A5	F: GCCCTAGAGGAGCACCAAAG R: CGCAACAGGCAGGAGTGT	80	62/4	72/3
MAGE-A8	F: AAAGGTTTCGCAGAGAACAGG R: GTCAGGGCAGCAGGAGAGT	119	65/3	72/3
MAGE-A9	F: GGCCTTGGTCTGAGACAGTG R: GTCCTCCTGGTTAGCCTGT	97	65/3	72/3
MAGE-A11	F: ACAGGAGTCCCAGGAGAACC R: CTGTGGGAAATATCTGGGTGA	81	67/3	72/4
MAGE-A12	F: GTCGGTGGAGGGAAGCAG R: AGGGCAGCAGGTAGGAGTG	104	65/3	72/3

MAGE, melanoma-associated antigen; F, forward; R, reverse.

Furthermore, the highest expression of the subgroups -A1, -A9 and -A11 was also detected in PCI-52. The highest expression of MAGE-A5 was detected in PCI-9, while PCI-13 exhibited the highest levels of MAGE-A8, and the greatest expression of MAGE-A12 was identified in the PCI-1 cell line (Fig. 1).

*Efficacy of erlotinib treatment varies between squamous cell carcinoma cell lines.* Four of the five cell lines (PCI-1, PCI-9, PCI-13 and PCI-68) exhibited a concentration-dependent response to 72 h of erlotinib treatment (Fig. 1). At the minimum concentration of 0.06  $\mu$ M, the fraction of viable cells ranged from 69.51 (PCI-1) to 91.71% (PCI-68), compared with that of the control. The intermediate concentration (0.55  $\mu$ M) of erlotinib resulted in a viable fraction of 54.07% in the PCI-1 cell line, whereas this concentration resulted in a viable fraction of 83.93% in PCI-9 cells. The maximum concentration of erlotinib (4.94  $\mu$ M) resulted in a viable fraction of 42.15% in PCI-1 cells, while in PCI-9 cells, this concentration resulted in a viable fraction of 74.90%. By contrast, the PCI-52 cell line demonstrated no concentration-dependent response to erlotinib. Erlotinib concentrations of 0.06, 0.18, 0.55 and 1.65  $\mu$ M yielded no significant effect on viability (resulting in 102.78, 102.85, 101.14 and 102.60%, respectively). Only the highest concentration of 4.94  $\mu$ M erlotinib resulted in a small decrease in viability compared with that of the control (94.03%).

*Erlotinib treatment efficacy is affected by MAGE-A expression.* A linear regression model was produced using erlotinib concentration and MAGE-A expression levels as independent variables (Table III), with the viable fraction compared with the control as the dependent variable. The model produced an r-value of 0.848, indicating a notable adaptation. MAGE-A1 and -A9 were excluded as independent variables. However, potential effects of MAGE-A1 and -A9 may be determined using a larger regression model with more cell lines. Negative values of the standardized coefficients represent an inhibitory effect on the fraction of viable cells. By contrast, positive values of the standardized coefficients indicate a beneficial effect on the fraction of viable cells. Increasing concentrations of erlotinib significantly decreased the viability of cells ( $P<0.001$ ). MAGE-A12 was also associated with a decrease in the viable fraction during erlotinib treatment ( $P=0.009$ ), while MAGE-A5 ( $P=0.015$ ) and -A11 ( $P<0.001$ ) were correlated with a higher fraction of viable cells following erlotinib treatment.

*Efficacy of gefitinib treatment varies between squamous cell carcinoma cell lines.* Analogously with the results described for erlotinib treatment, four of the five cell lines (PCI-1, PCI-9, PCI-13 and PCI-68) exhibited a concentration-dependent response to 72 h of gefitinib treatment (Fig. 1). The minimum concentration of 0.06  $\mu$ M resulted in cell viabilities of 57.56% in PCI-1,

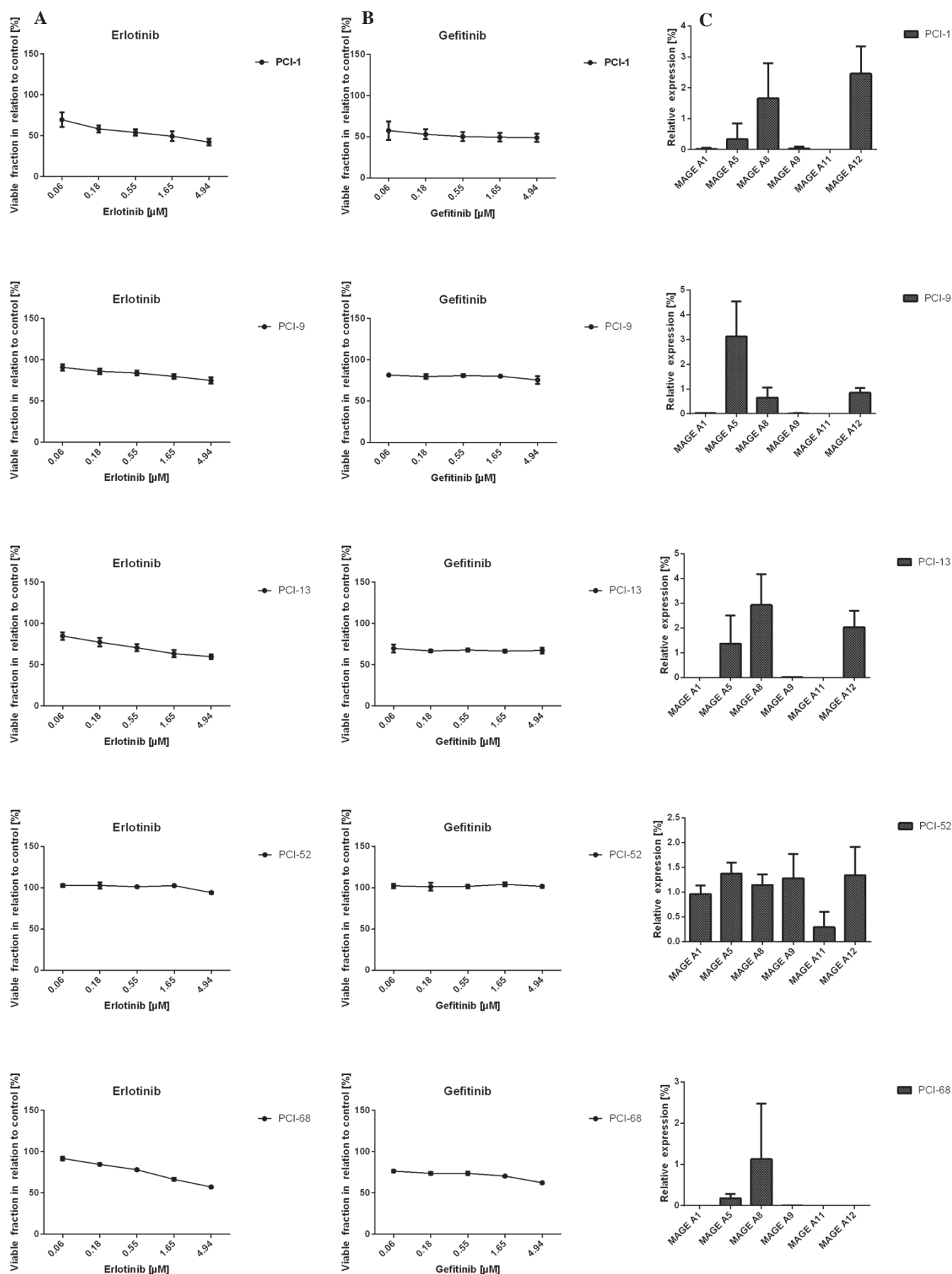


Figure 1. Effects of erlotinib and gefitinib on cell viability and MAGE-A subgroup expression profiles of head and neck squamous cell carcinoma cell lines. Fraction of viable cells compared with that of the control (100%) following 72 h of (A) erlotinib or (B) gefitinib treatment. With the exception of the PCI-52 cell line, all cell lines demonstrated a marked decrease in viable cells following drug treatment. Standard deviation is illustrated by error bars extending above and below the data points. (C) Cell-specific MAGE-A subgroup expression profile of each cell line, relative to  $\beta$ -actin expression. MAGE, melanoma-associated antigen.



Table III. Linear regression of erlotinib/MAGE-A subgroup following 72 h of treatment.

Independent variable	Standardized coefficient	P-value
MAGE-A5	<b>0.299</b>	<b>0.015</b>
MAGE-A8	0.168	0.274
MAGE-A11	<b>0.574</b>	<b>&lt;0.001</b>
MAGE-A12	<b>-0.398</b>	<b>0.009</b>
Erlotinib concentration	<b>-0.482</b>	<b>&lt;0.001</b>

Bold text indicates statistically significant results. MAGE, melanoma-associated antigen.

Table IV. Linear regression of gefitinib/MAGE-A subgroup following 72 h of treatment.

Independent variable	Standardized coefficient	P-value
MAGE-A5	<b>0.300</b>	<b>0.028</b>
MAGE-A8	0.156	0.364
MAGE-A11	<b>0.661</b>	<b>&lt;0.001</b>
MAGE-A12	<b>-0.371</b>	<b>0.027</b>
Gefitinib concentration	<b>-0.255</b>	<b>0.046</b>

Bold text indicates statistically significant results. MAGE, melanoma-associated antigen.

81.57% in PCI-9, 69.77% in PCI-13 and 76.38% in PCI-68 cells, indicating a markedly greater efficacy of gefitinib than that of the lowest concentration of erlotinib. At the intermediate concentration (0.55  $\mu$ M) of gefitinib, the fraction of viable cells ranged from 50.34% in PCI-1 cells to 80.87% in PCI-9 cells. Compared with the control, the maximum concentration of 4.94  $\mu$ M gefitinib resulted in viable fractions of 48.87 and 75.51% in PCI-1 and PCI-9 cells, respectively. Notably, compared with the lowest concentration of erlotinib, gefitinib produced greater responses in the PCI-1, PCI-9, PCI-13 and PCI-68 cell lines. Conversely, erlotinib treatment at the intermediate and highest concentrations yielded better results than those of gefitinib. As previously observed following erlotinib treatment, PCI-52 did not demonstrate a concentration-dependent response to gefitinib treatment. Furthermore, even the highest concentration of gefitinib (4.94  $\mu$ M) failed to reduce cell viability compared with that of the control (101.59%).

*Gefitinib treatment efficacy is affected by MAGE-A expression.* The linear regression model was constructed using the gefitinib concentration and MAGE-A expression levels as independent variables (Table IV), with the viable fraction compared with the control as the dependent variable. The model generated an r-value of 0.805. Analogously to the results obtained in the analysis of erlotinib, negative standardized coefficient values represented an inhibitory effect on the fraction of viable cells, while positive values indicated a beneficial effect on the fraction of viable cells. The increasing concentration of gefitinib significantly reduced the fraction of viable cells ( $P=0.046$ ), demonstrating a concentration-dependent response of the cell lines to gefitinib treatment (Table IV). MAGE-A12 was also associated with a decreased number of viable cells following gefitinib treatment ( $P=0.027$ ). Cell lines expressing MAGE-A11 demonstrated the lowest response to gefitinib treatment, as indicated by the large fraction of viable cells ( $P<0.001$ ). MAGE-A5 expression was also correlated with a significantly poorer efficacy of gefitinib treatment ( $P=0.028$ ), while MAGE-A8 expression did not significantly alter the fraction of viable cells ( $P=0.364$ ). MAGE-A1 and -A9 were again excluded as independent variables.

## Discussion

Due to the high rate of recurrence and a poor overall survival rate of ~50%, the treatment of head and neck squamous

cell carcinoma (HNSCC) remains challenging. Therefore, improvements to patient selection in terms of non-surgical treatment approaches may help to enhance clinical outcomes. One technique for the identification of high-risk patients may be via the evaluation of MAGE-A expression status. There is an increasing understanding that the expression of certain CTAs, including MAGE-A, is associated with markedly poorer survival amongst patients with head and neck cancer (14). The present study revealed inhomogeneous expression of MAGE-A1, -A5, -A8, -A9, -A11 and -A12 in the HNSCC cell lines tested. Furthermore, treatment with erlotinib and gefitinib at clinically relevant concentrations yielded differential response rates in the various HNSCC cell lines. While four of the five cell lines (PCI-1, PCI-9, PCI-13 and PCI-68) exhibited concentration-dependent responses to erlotinib and gefitinib, TKI treatment of PCI-52 cells was completely ineffective. The PCI-1 and PCI-52 cell lines were comparable in terms of their TNM status; however, PCI-52 was the only cell line to express all of the MAGE-A subgroups investigated, while PCI-1 expressed three out of the six subgroups. Notably, the PCI-1 cell line was the most responsive to erlotinib and gefitinib treatment, whereas PCI-52 was entirely resistant to these two agents. These results highlight an urgent need for the identification of additional molecular markers, including MAGE-A tumor antigens.

The linear regression models of erlotinib and gefitinib revealed marked adaptation (r-values, 0.848 and 0.805, respectively) and significant effects of agent concentration on the fraction of viable cells, providing evidence for a useful experimental setup. Correlation analysis of MAGE-A subgroups and erlotinib treatment revealed MAGE-A5 and -A11 as significant negative predictors of treatment success. Of note, MAGE-A11 was previously shown to act as a proto-oncogene in prostate cancer by serving as a transcriptional activator of the androgen receptor (26). By contrast, MAGE-A12 was associated with an improved outcome of erlotinib administration. In a previous study by our group, MAGE-A5 and -A8 were reported as negative predictors of anti-EGFR therapy using panitumumab (15). In agreement with a recent study by our group, MAGE-A12 was correlated with a greater response to panitumumab (15). Mollaoglu *et al* (27) previously described MAGE-A12 as a predictor of improved prognosis in oral squamous cell carcinoma. As described by their group, N0 cervical lymph node

status was found more frequently in MAGE-A12-positive patients than that of the MAGE-negative controls. Furthermore, MAGE-A11 was reported to have a negative impact on treatment with cisplatin, 5-fluorouracil, paclitaxel and docetaxel (16). In the same study, MAGE-A5 was demonstrated to be associated with improved outcomes following paclitaxel therapy. Statistical analysis revealed that treatment with gefitinib or erlotinib were comparable in the context of MAGE-A expression. MAGE-A5 and -A11 were correlated with poorer outcomes of gefitinib therapy. Similarly to the effects observed following erlotinib treatment, improved outcomes of gefitinib treatment were associated with MAGE-A12 expression. A potential reason for the negative influence of MAGE-A11 on EGFR-directed therapies may be due to its association with the expression of other transmembrane receptors. Recently, Hou *et al* (28) demonstrated a significant correlation between MAGE-A9 and -A11 expression and Her2/neu expression in breast cancer. There is a broad consensus that evasion of EGFR-targeted therapy is facilitated by the activation of alternative receptors, including Her2/neu, as well as their downstream signaling pathways (29,30). Even if TKI treatment combined with cisplatin and radiotherapy failed to improve progression-free survival in unselected cohorts, erlotinib and gefitinib may function as chemopreventive drugs (31). One significant consideration in the treatment of patients with head and neck cancer is the phenomenon of field cancerization (32). Therefore, the possibility of reducing the incidence of malignant transformation to dysplastic oral mucosa may be a valuable tool, based on the findings regarding the chemopreventive properties of TKIs (31,32). MAGE-A subgroup analysis may help to identify high-risk patients, thus aiding the development of personalized therapies and follow-up.

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