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Melanoma-associated antigen A11 reduces erlotinib and afatinib efficacy in head and neck cancer

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ABSTRACT

Melanoma-associated antigen A (MAGE-A) proteins are members of the cancer/testis antigens (CTA), and the expression of these proteins is almost exclusively limited to malignant cells, making them an attractive treatment target. MAGE-A expression is correlated with poor overall survival in several cancers, including head and neck squamous cell carcinoma (HNSCC). Among others, MAGE-A11 was found to be associated with resistance to different antineoplastic and targeted compounds, such as epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs). We searched The Cancer Genome Atlas (TCGA) database with a focus on MAGE-A and found that MAGE-A overexpression is a common event in HNSCC (27.5%). Furthermore, MAGE-A overexpression was correlated with significantly reduced overall survival (35.45 months vs. 64.78 months, $P = 0.0173$). In particular, MAGE-A11 overexpression was found in 9% of specimens. We then examined MAGE-A11 expression, the efficacy of EGFR and the EGFR mutational status and the effects of the pan-HER (human EGFR) TKIs erlotinib and afatinib in HNSCC cell lines. Next, we used a model of stable MAGE-A11 overexpression to demonstrate that MAGE-A11 impaired the efficacy of erlotinib and afatinib. In summary, our study provides evidence that MAGE-A11 contributes to erlotinib and afatinib resistance in head and neck cancer cell lines.

1. Introduction

Almost three decades ago, melanoma-associated antigen A (MAGE-A) proteins were discovered and characterized as the first human tumor-associated antigens (van der Bruggen et al., 1991). Together with other members, melanoma-associated antigens belong to the large family of cancer/testis antigens (CTA) (Old and Chen, 1998) . As we and others have reported, MAGE-A proteins can be found in adult male germ cells, fetal keratinocytes, the placenta and a variety of human cancers, including head and neck cancer (Kienstra et al., 2003; Scanlan et al., 2004; Simpson et al., 2005; Muller-Richter et al., 2008). Importantly, recent studies show that MAGE proteins are associated with poor outcomes among patients with different types of solid malignancies (Chen et al., 2017; Hou et al., 2016; Laban et al., 2017; Wu et al., 2017).

In the past, studies from our group documented a correlation between MAGE-A expression and impaired efficacy of several cytotoxic agents, monoclonal antibodies and tyrosine kinase inhibitors (TKIs) (Hartmann et al., 2013, 2014, 2015). Importantly, MAGE-A11 has been associated with the reduced efficacy of different agents. Consistent with this, recent work from our group showed a correlation between MAGE-A11 expression and lymph node metastasis, further underscoring the contribution of MAGE-A11 to a malignant phenotype (Brisam et al., 2016). More recently, a study from our group highlighted a causal role for MAGE-A11 as a contributor to cisplatin resistance in head and neck squamous cell carcinoma (HNSCC) (Hartmann et al., 2017).

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Although the molecular basis for head and neck cancer is complicated, the epidermal growth factor receptor (EGFR) is known to be overexpressed and to act as a driver of carcinogenesis in HNSCC (Grandis and Tweardy, 1993). Based on this information, several clinical trials have shown the temporary success of EGFR-targeting agents, such as cetuximab (Vermorken et al., 2008), erlotinib (Bauman et al., 2017), and afatinib (Machiels et al., 2015) in unselected head and neck cancer patients. However, response rates among these unselected patient cohorts are mixed, and further characterization of patients by predictive markers may improve clinical outcomes. Therefore, our study aimed to investigate the causal role of MAGE-A11 expression in the context of anti-EGFR TKI efficacy in head and neck cancer.

2. Materials and methods

2.1. TCGA data

We reviewed data from The Cancer Genome Atlas (TCGA) for HNSCC using cbioportal.com (Cerami et al., 2012; Gao et al., 2013). The analysis was based on TCGA Head and Neck Squamous Cell Carcinoma Provisional dataset with 530 samples, which is an extended version of the already published database (Cancer Genome Atlas, 2015). We analyzed MAGE-A mRNA levels with a focus on transcripts showing >2-fold upregulation and correlated these findings with the available survival data.

2.2. Cell lines

The human papillomavirus (HPV)-negative HNSCC cell lines FaDu and SCC9 were purchased from the American Tissue Culture Collection (Manassas, VA, USA). As described in former publications, the cells were cultured in a humidified atmosphere of 5% CO_2/95% air at 37 °C and were fed two to three times per week according to the manufacturer's protocol.

2.3. Isolation of RNA, reverse transcription and quantitative real-time polymerase chain reaction (RT-qPCR)

As described elsewhere (Hartmann et al., 2017), the cells were separated from the culture plate, and RNA isolation was achieved using the TRIzol reagent (Ambion, Carlsbad, CA, USA). A Nano Drop 2000 device (ThermoFisher Scientific, Waltham, MA, USA) was used to estimate the RNA concentration. The concentration was determined at a wavelength of 260 nm using fully desalinated water as the blank. All samples were adjusted to a concentration of 0.2 μM of RNA/ml.

The samples were then cleaned of DNA contamination using a gDNA Wipeout Buffer (Qiagen, Venlo, Netherlands) by heating to 42 °C for 2 min and then cooling on ice. Next, reverse transcription was performed using a QuantiTect Reverse Transcription Kit (Qiagen, Venlo, Netherlands). The incubation was performed at 42 °C for 15 min. The following denaturation step was performed at 95 °C for 3 min.

We used a QuantiTect SYBR-Green PCR Kit 200 (Qiagen, Venlo, Netherlands), QuantiTect Primer sets (Table 1) and a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA) for polymerase chain reaction. The quantitative expression levels of the tested genes were calculated as a ratio to the expression level of β-actin.

2.4. MAGE-A11 overexpression model

As recently described by our group (Hartmann et al., 2017), we stably transfected the FaDu cell line with a pcDNA™3.1 (+) Mammalian Expression Vector (ThermoFisher Scientific, Waltham, MA, USA) containing either a synthetic MAGE-A11 sequence or a non-coding control. The cells were selected by Geneticin pressure (G418, ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. The cells were cultured for a period of 3–4 months, and the expression of MAGE-A11 was detected by several RT-qPCR experiments using a sequence-optimized primer (primer for: TTCCAGAGCACCGAGAG; primer rev: CTCTGGGTTCTTCTGGG). MAGE-A11 expression in cultured cells was approximately 48,000 times higher compared to that of the vector control (Hartmann et al., 2017). The primers were purchased from ThermoFisher Scientific (Waltham, MA, USA). The PCR protocol was described in earlier publications from our group.

2.5. Drug treatment and crystal violet assay

A total of 1×10^4 cells from each cell line were seeded per well in a 96-well format and incubated overnight prior to any experiments. Erlotinib and afatinib were purchased from Selleckchem (distributed by Absource Diagnostics GmbH, München, Germany) and stored according to the manufacturer's protocols. The concentrations used in this study (16.66, 5.55, 1.85, 0.61, and 0.21 μM) were derived from a log 3 dilution. Following 24 h of incubation in standard medium, erlotinib or afatinib was added, and the cultures were incubated for another 48 h.

The fraction of surviving cells was determined with a Crystal Violet assay. A 1-g quantity of Crystal Violet was dissolved in 1 L of double-distilled water containing 20% methanol. Then, after removal of the drug-containing medium, 50 μL of Crystal Violet solution was added to the wells. Fifteen minutes later, the 96-well plates were washed with distilled water and dried overnight. The optical density (OD) was measured at a wavelength of 595 nm with a Tecan infinite F50 microplate reader (Tecan Group Ltd., Männedorf, Switzerland). All experiments were performed in triplicate.

2.6. EGFR mutational analysis

To determine the mutation status of EGFR in the cell lines, we performed amplicon sequencing using semiconductor sequencing technology (Ion PGM, ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendations. All coding regions were amplified by multiplex PCR using customized oligos (Ion Ampliseq designer, ThermoFisher Scientific, Waltham, MA, USA) and the Ion AmpliSeq Library Kit 2.0. Subsequently, libraries were templated and enriched with the Ion OneTouch 2 and the Ion OneTouch ES automated systems. Variants were named and analyzed using IonTorrent Somatic VariantCaller version 5.0 and Ion Reporter Software version 5.0 (ThermoFisher Scientific, Waltham, MA, USA).

2.7. Illustrations and statistical analysis

GraphPad Prism 6.04 (La Jolla, CA, USA) was used for the illustration and basic statistical analysis of the *in vitro* data. To test whether MAGE-A11-overexpressing cells showed lower TKI efficacy, we used multiple paired *t*-tests (two-tailed). $P \leq 0.05$ was considered to indicate a statistically significant difference.

Table 1
Primers used in the study.

Gene	Company	Catalog no.
β-Actin	Qiagen	QT01680476
MAGE-A11	Qiagen	QT01004094

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