Molecular Immunology 99 (2018) 124–133

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Targeted delivery of CD44s-siRNA by ScFv overcomes *de novo* resistance to cetuximab in triple negative breast cancer

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ARTICLE INFO

Keywords:
De novo resistance
EGFR
TNBC
RNAi

ABSTRACT

The overexpression of EGFR often occurs in TNBC, and the anti-EGFR receptor antibody cetuximab is used widely to treat metastatic cancer in the clinic. However, EGFR-targeted therapies have been developed for TNBC without clinical success. In this study, we show that impaired EGFR degradation is crucial for resistance to cetuximab, which depends on the cell surface molecule CD44. To further investigate the role of CD44 in EGFR signaling and its treatment potential, we developed a targeting fusion protein composed of an anti-EGFR scFv generated from cetuximab and truncated protamine, called Ce-tP. CD44 siRNA can be specifically delivered into EGFR-positive TNBC cells by Ce-tP. Efficient knockdown of CD44 and suppression of both EGFR and downstream signaling by the Ce-tP/siRNA complex were observed in EGFR-positive TNBC cells. More importantly, our results also showed that targeted delivery of siRNA specific for CD44 can efficiently overcome resistance to EGFR targeting in TNBC cells both in vitro and *in vivo*. Overall, our results establish a new principle to achieve EGFR inhibition in TNBC and limit drug resistance.

1. Introduction

The HER receptor tyrosine kinase family comprises four members: epidermal growth factor receptor (EGFR, ErbB1/HER1), HER2/neu (ErbB2), HER3 (ErbB3) and HER4 (ErbB4) (Arteaga, 2001; Hu et al., 2015a). EGFR is a 170 kDa transmembrane protein that comprises an extracellular domain, which is also used for ligand binding; a transmembrane region; and an intracellular kinase domain. To date, two ligands that bind and activate EGFR have been identified: epidermal growth factor (EGF) and transforming growth factor-alpha (TGF-α). Ligands bind to the extracellular domain of EGFR, resulting in homo- or heterodimerization of the receptor. This heterodimerization can happen between EGFR and one of the HER family of receptor tyrosine kinases. Dimerization then induces autophosphorylation of the tyrosine kinase domain (McCune and Earp, 1989), which serves as a binding site for the recruitment of signal transducers and activators of intracellular substrates. The major signaling routes for EGFR are the Ras-Raf mitogen-activated protein kinase pathway and the phosphatidyl inositol 3′ kinase and Akt pathway, which control important biologic processes such as cellular proliferation, angiogenesis and inhibition of apoptosis (de Bono and Rowinsky, 2002). Cumulative evidence has shown that the dysregulation of EGFR as well as the HER receptor family plays a significant role in tumorigenesis and the progression of cancer (De Pourcq et al., 2012; Hu et al., 2016). Basic and translational studies of the HER receptor family have focused on the approach for converting therapeutic biomolecules from bench to bedside, including the anti-EGFR antibodies panitumumab and cetuximab, the anti-HER2 antibodies trastuzumab and pertuzumab (Baselga et al., 2012; Sliwkowski and Mellman, 2013) and the EGFR tyrosine kinase inhibitors erlotinib and gefitinib.

Breast cancer is a common cancer in women, and its incidence is increasing worldwide (Kamangar et al., 2006). However, the mortality rate in developed countries has declined during the past three decades owing to the implementation of screening, improvements in the local management of early breast cancer, and, most importantly, the introduction of adjuvant systemic treatments (Andergassen et al., 2017). Moreover, in some subgroups of breast cancer, such as HER+ breast cancer, further improvements will be made in the near future as targeted treatments gradually emerge. Triple-negative breast cancers (TNBCs) belong to a subgroup of breast cancers that do not express ER,

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https://doi.org/10.1016/j.molimm.2018.05.010
Received 20 February 2018; Received in revised form 28 April 2018; Accepted 10 May 2018

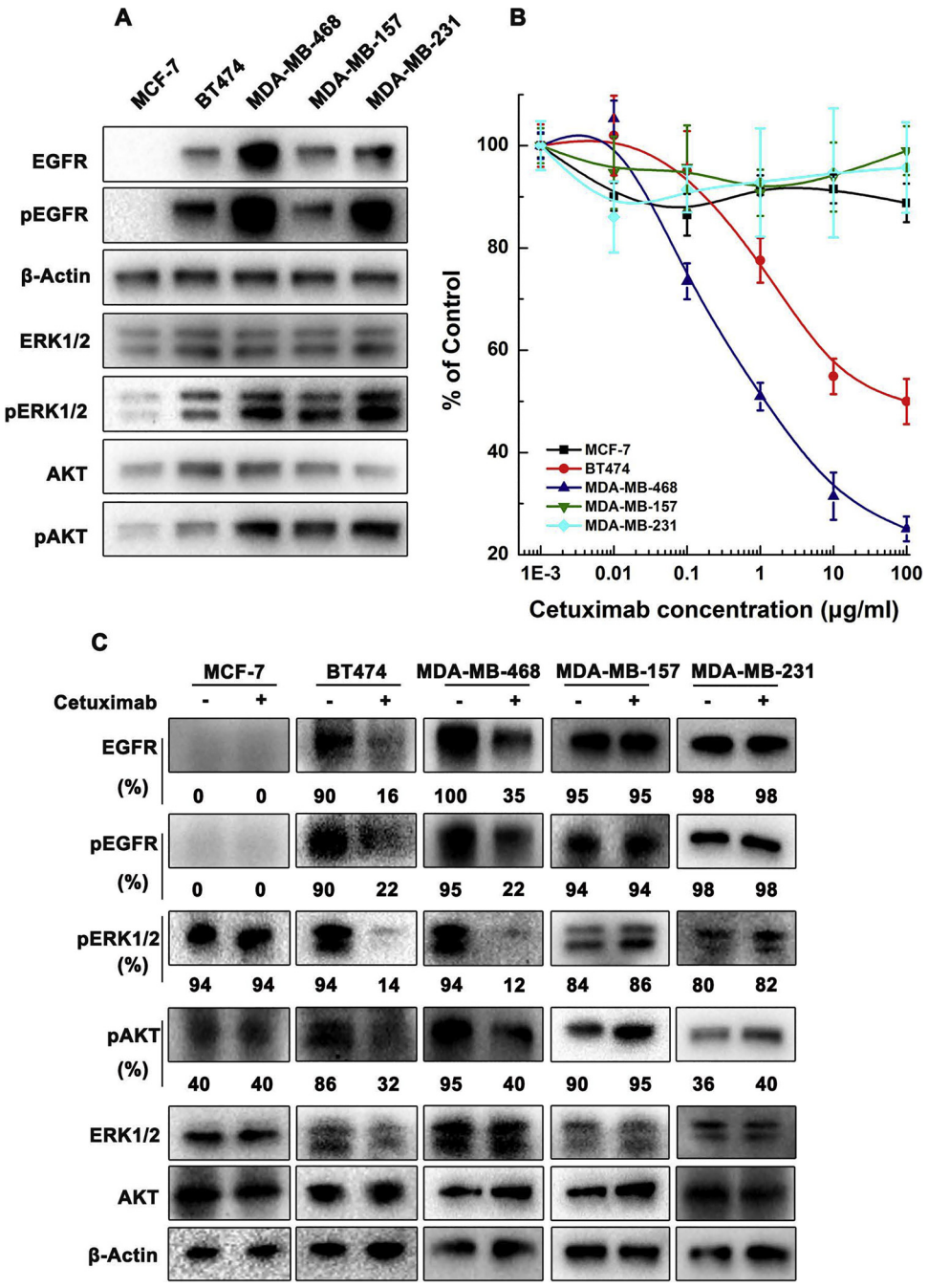

Fig. 1. Impaired EGFR degradation is crucial for the resistance to cetuximab. (A) Immunoblots comparing EGFR expression and downstream signaling between the indicated cancer cell lines. (B) Different cancer cells were treated with the indicated concentrations of cetuximab in the presence of 1% serum-containing growth medium. Cell proliferation was measured after 4 days using AlamarBlue staining. Data are shown as the mean ± SEM. (C) Immunoblots evaluating the effects of 10 μg/ml of the cetuximab pretreatment on EGFR expression and downstream ERK and AKT activation in the indicated breast cancer cell lines. The quantification value of EGFR, pEGFR, pERK1/2 and p-AKT was normalized to the loading control Actin, respectively, and shown below the panel.

PR, or HER2. TNBC accounts for 10–20% of all breast cancers, totaling as many as 40,000 new cases per year in the United States alone (Andergassen et al., 2017). Compared to HER+ or hormone receptor-positive breast tumors, TNBC has a poorer prognosis and shorter progression-free (PFS) and overall survivals (OS) and are at higher risk of relapse even with optimal treatment (Mustacchi and De Laurentiis, 2015).

Among patients diagnosed with resectable (stage I-III) TNBC completing tri-modality therapy (neoadjuvant chemotherapy or surgery ± radiotherapy + adjuvant), as many as 50% of patients undergo disease recurrence, and as many as 37% die in the first 5 years following surgical treatment (Liedtke et al., 2008). Moreover, for patients presenting with metastatic TNBC, cytotoxic chemotherapy such as ixabepilone, capecitabine, anthracyclines and taxanes is the standard-of-care treatment (NCCN, 2016). A retrospective analysis of 111 cases treated with monotherapy or combinations (Kassam et al., 2009) showed that the mean duration of the response was 12 weeks after first-line treatment, 9 weeks after second-line treatment, and 4 weeks after third-line treatment, demonstrating that the duration of response to chemotherapy of TNBC is usually short. So far, TNBC is also the only major type of breast cancer for which no specific FDA-approved targeted therapy is available to improve patient outcomes; it is resistant to targeted therapies such as hormonal therapy and HER2-targeting therapy. The overexpression of EGFR often occurs in TNBC. Although EGFR-targeted therapies show efficacy in patients with solid tumors (e.g., colorectal, head and neck, and non-small cell lung cancer), EGFR-targeted therapies have been developed for TNBC without success despite a preclinical rationale suggesting their efficacy (Bonner et al., 2010; Heinemann et al., 2014; Costa et al., 2017).

In the current report, we show that impaired EGFR degradation is crucial for the resistance to cetuximab in breast cancer cell lines, which is associated with the cell surface molecule CD44. CD44 was recently