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The deletion mutant EGFRvIII significantly contributes to stress resistance typical for the tumour microenvironment

Jan Theys [a,*], Barry Jutten [a], Ludwig Dubois [a], Kasper M.A. Rouschop [a], Roland K. Chiu [b], Younan Li [a], Kim Paesmans [a], Philippe Lambin [a], Guido Lammering [a,1], Bradly G. Wouters [a,c,d,1]

[a] Maastricht Radiation Oncology (MaastRo) Lab, Grow-School for Oncology and Developmental Biology, University of Maastricht, The Netherlands
[b] Department of Health Risk Analysis and Toxicology, Maastricht University, The Netherlands
[c] Ontario Cancer Institute, Princess Margret Hospital, University Health Network, Departments of Radiation Oncology and Medical Biophysics, University of Toronto, Canada
[d] Selective Therapeutics Program, Ontario Institute for Cancer Research, Toronto, Canada

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A B S T R A C T

Background and purpose: The epidermal growth factor receptor (EGFR) is overexpressed or mutated in many tumour types. The truncated, constitutively active EGFRvIII variant has not been detected in normal tissues but is found in many malignancies. In the current study, we have investigated the hypothesis that EGFRvIII contributes to a growth and survival advantage under tumour microenvironment-related stress conditions.

Materials and methods: U373MG doxycycline-regulated isogenic cells expressing EGFRwt or EGFRvIII were created and validated using Western blot, FACS and qRT-PCR. *In vitro* proliferation was evaluated with standard growth assays. Cell survival was assayed using clonogenic survival. Animal experiments were performed using NMRI-*nu*-xenografted mice.

Results: Inducible isogenic cell lines were created and showed high induction of EGFRwt and EGFRvIII upon doxycycline addition. Overexpression of EGFRvIII but not of EGFRwt in this model resulted in a growth and survival advantage upon different tumour microenvironment-related stress conditions *in vitro*. Induction of EGFRvIII increased tumour growth *in vivo*, which was reversible upon loss of expression.

Conclusions: Under conditions where nutrients are limited and stress is apparent, as in the tumour microenvironment, expression of EGFRvIII leads to a growth and survival advantage. These data indicate a potential selection of EGFRvIII-expressing tumour cells under such stress conditions.
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The epidermal growth factor receptor (EGFR) is overexpressed, amplified or altered in various human epithelial tumours [1]. Upon ligand binding and receptor activation, tyrosine residues in their cytoplasmatic domain are phosphorylated, resulting in the activation of downstream substrates that control cell proliferation, differentiation and survival. It is therefore not surprising that overexpression of EGFR has been shown to be associated with tumour aggressiveness and treatment resistance [2,3] and that targeting of EGFR is a potentially promising treatment strategy [4–8].

Several reports have documented rearrangements within the EGFR gene, especially in primary glioblastomas [9]. The most common variant is EGFRvIII, which harbours an in-frame deletion resulting in a truncated receptor that lacks 267 amino acids in the extracellular binding domain. This truncation leads to important functional changes: the receptor cannot bind ligand, but is constitutively active, resulting in uncontrolled pro-oncogenic effects, thereby promoting neoplastic transformation and tumourigenicity. The EGFRvIII variant is found in many malignancies, including breast, head and neck, prostate, lung and brain tumours. In patients with glioblastoma, the EGFRvIII mutation has an overall prevalence of 50–60% in patients whose tumours show amplification of EGFR [10]. Strikingly, EGFRvIII has not been detected in normal tissues. Moreover, expression in tumour tissues *in vivo* is rapidly lost when cultured *in vitro* [11]. This observation suggests that the tumour microenvironment, which is characterized by heterogeneities in nutrient supply and oxygenation, may provide suitable conditions for growth and survival of EGFRvIII-expressing tumour cells. The establishment of such a microenvironment, unlike that of any normal tissue, is a consequence of alterations in the metabolic and proliferative status of tumour cells together with a highly irregular vascular supply [12]. This process is believed to occur early in the development of a tumour, and has been implicated in promoting metastasis, angiogenesis and increased malignancy. Cellular adaptive responses to this environment are

* Corresponding author. Address: Maastricht Radiation Oncology (MAASTRO), GROW – School for Oncology and Developmental Biology, Uns 50/23, P.O. Box 616, Maastricht University, 6200 MD Maastricht, The Netherlands.
E-mail address: jan.theys@maastro.unimaas.nl (J. Theys).
[1] These authors contributed equally to this work.

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critical for the continued growth of tumours. The role of EGFRvIII expression in the context of the tumour microenvironment has only been minimally defined. The present study was performed to investigate the contribution of EGFRvIII expression on tumour growth, as well as its importance in determining the response to microenvironmental stress.

Materials and methods

Plasmids and generation of cell lines

The glioblastoma cell line U373MG (ATCC, Manassas, VA, USA) was cultured in MEMα medium, supplemented with 10% FBS. Isogenic cell lines that can be induced to express either EGFR wild-type (wt) or EGFRvIII were generated using the Flp-In T-Rex Core Kit from Invitrogen (Breda, NL), according to the manufacturer's recommendations. These cell lines were designated U373FlpIn/EGFRvIII and U373FlpIn/EGFRwt, respectively. To induce gene expression, doxycycline (1 μg/ml) was added to the medium.

Antibodies and Western blotting

Western blotting was done according to standard protocols as described [13]. Antibodies used were Sc-03 (Santa Cruz Biotechnology, Huissen, NL) for EGFRwt, L8A4 (a gift from D. Bigner, Duke University, Durham, NC, USA) for EGFRvIII and HRP-linked anti-rabbit (EGFRwt) or anti-mouse (EGFRvIII) secondary antibodies. Immobilized proteins were detected using ECL-plus reagent (Kirkegaard & Perry Laboratories, NL) and by exposing blots to X-ray film.

Quantitative real-time PCR

RNA extraction was performed using the SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA). cDNA was prepared using the iScript cDNA Synthesis kit (BioRad Laboratories, CA, USA). Reactions were carried out in a 25 μl volume using SYBR Green Master Mix (Applied Biosystems, CA) with the ABI Prism 7700 Sequence Detection System. Values for each gene were normalized to expression levels of 18S RNA. The primer sequences used were:

EGFRvIII forward: 5′AAGAAAGGTAATTATGTGGTGACA3′;
EGFRvIII reverse: 5′CCGTCTTCCTCCATCTCATAGC3′;
EGFRwt forward: 5′ACCTGCGTGAAGAAGTGTCC3′; and
EGFRwt reverse: 5′CCGTCTTCCTCCATCTCATAGC3′.

Flow cytometric analysis

FACS analysis with Oregon Green-labeled cetuximab was performed as described [14].

In vitro growth and survival assays

For growth under control aerobic conditions, 2.5×10^5 cells were seeded and incubated in 5% CO_2 for 1–7 days. To monitor growth under stress conditions, cells were seeded and allowed to attach during overnight incubation in 5% CO_2. The following day (day 0), dishes were placed in the hypoxic chamber or medium was replaced according to the appropriate conditions. Total cell numbers were counted after trypsinization using a Coulter Z series particle counter (Beckman, Mijdrecht, NL) and cell numbers normalized to the amount of cells present at day 0. Clonogenic assays were performed as previously described [13].

Tumour xenograft model

In vivo experiments were performed using adult NMRI-*nu* (nu/nu) female mice. Animal facilities and experiments were in accordance with local institutional guidelines. U373 FlpIn/EGFRvIII cells were mixed with matrigel and 3×10^6 cells were subcutaneously injected in the lateral flank. Induction of gene expression was done by giving animals doxycycline (2 g/l) in their drinking water. Tumours were measured with calipers in 3 orthogonal diameters and volumes calculated with the formula $A \times B \times C \times \pi/6$. The tumour doubling time (T_d) was calculated using the equation $T_d = (T - T_0) \times \log 2/[\log(V) - \log(V_0)]$ where $T - T_0$ indicates the length of time between two measurements and V_0 and V denote the tumour volume at two points of measurement.

Statistics

Statistical analysis was carried out using the program GraphPad Prism version 5.01 for Windows (GraphPad Software, 2007, CA, USA). A Mann–Whitney *U* test was used to assess differences in tumour doubling times. Non-linear regression analysis was performed to compare differences between *in vivo* growth curves. Student's *t*-test was applied to determine differences in growth and survival assays.

Results

To investigate the potential influence of EGFRvIII on tumour phenotype, and to compare the effect of EGFRvIII overexpression with that of EGFRwt, we created doxycycline-inducible U373MG glioma isogenic cell lines expressing EGFRvIII or EGFRwt. The unmodified parental U373MG cells express moderate levels of endogenous EGFRwt and have been widely recognized as a valuable *in vitro* and xenograft model. Previously, we constructed a Flp-In T-Rex host acceptor cell line, designated U373FlpIn. Following transfection and selection, we identified clones for both EGFRvIII and EGFRwt. Quantitative PCR revealed induction of mRNA levels of 7.15 ± 0.6 for EGFRvIII and 7.55 ± 0.6 for the EGFRwt in response to doxycycline (Fig. 1A). As shown in Fig. 1B, protein levels of EGFRvIII were strongly induced after exposure to doxycycline, with virtually undetectable background levels. EGFRwt is also induced in the U373FlpIn/EGFRwt cells, but to a lesser extent than EGFRvIII in the U373FlpIn/EGFRvIII cells, presumably due to the already expressed endogenous EGFRwt levels in U373FlpIn. To show that the vIII mutant and the wt receptor were properly processed and located at the cell membrane after doxycycline administration, we performed a flow cytometry experiment using Oregon Green-labeled cetuximab (C225-OG) (Fig. 1C). Without doxycycline induction, both cell lines showed similar levels of C225-OG binding, indicating binding of C225-OG to endogenous EGFRwt receptor. Increased C225-OG binding was observed when doxycycline was added, reaching a 5- to 6-fold increase at 72 h. Taken together, these data demonstrate that the use of this inducible system is feasible and that it results in an increase of correctly processed receptors at the membrane of induced U373FlpIn cells.

To assess the biological relevance of EGFRvIII expression in relation to stress typically found in the tumour microenvironment, we evaluated the growth of U373FlpIn/EGFRvIII and U373FlpIn/EGFRwt inducible isogenic cell lines under conditions of serum starvation, low pH, glucose deprivation, hypoxia (0.2%) and anoxia. As shown in Fig. 2, no effect on growth after induction of EGFRvIII or EGFRwt was observed in medium containing 10% FBS. However, in conditions of serum starvation, addition of doxycycline to overexpress EGFRvIII resulted in a consistent growth advantage over non-induced cells (Fig. 2A). In contrast, no proliferation effect

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