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A novel serum protein signature associated with resistance to epidermal growth factor receptor tyrosine kinase inhibitors in head and neck squamous cell carcinoma

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Abstract ***Background:*** Acquired resistance to tyrosine kinase inhibitors (TKIs) is becoming a major challenge in the treatment of many cancers. Epidermal growth factor receptor (EGFR) is overexpressed in squamous carcinomas, notably those of the head and neck (HNSCC), and can be targeted with several TKIs. We aimed to identify soluble proteins suitable for development as markers of EGFR TKI resistance in cancer patients to aid in early and minimally invasive assessment of therapeutic responses.

Methods: Resistant HNSCC cell lines were generated by exposure to an EGFR TKI, gefitinib, *in vitro*. Cell lines were characterised for their biological behaviour *in vitro* (using growth inhibition assays, flow cytometry, western blots, antibody arrays and/or immunoassays) and *in vivo* (using subcutaneous tumour xenografts). Sera from EGFR-treated and -untreated HNSCC patients were analysed by immunoassay.

Results: Two independent sublines of CAL 27 and a PJ34 subline with acquired resistance to EGFR TKIs (gefitinib, erlotinib and afatinib) were developed. Resistant cells grew as highly aggressive xenografts leading to reduced host survival rates compared with EGFR-TKI sensitive cells. This suggested a link between resistance *in vitro* and poor prognosis *in vivo*. A sig-

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nificant upregulation of proteins linked to tumour angiogenesis and invasion was identified in resistant cells. This 'resistance-associated protein signature' (RAPS) was detected in the sera of a small cohort of HNSCC patients and was associated with reduced survival.

Conclusion: We have identified a protein signature associated with EGFR-TKI resistance that may also be linked to poor prognosis and warrants further investigation as a potential clinical biomarker.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) arises in the mucosae of the oral cavity, tongue, oropharynx, larynx, hypopharynx and paranasal sinuses and, despite innovations in surgery and radiotherapy, overall 5-year survival rates remain poor.[1] The epidermal growth factor receptor (EGFR) is overexpressed in most HNSCCs and is associated with poor prognosis,[2] making it a theoretically compelling drug target. The EGFR-targeted monoclonal antibody cetuximab has been approved for the treatment of HNSCC in combination with either radiotherapy or platinum-based drugs.[3] However, clinical trials in HNSCC of the EGFR tyrosine kinase inhibitors (TKIs) gefitinib or erlotinib, as monotherapies, have yielded response rates of only 5–15%.[4] By contrast, in non-small cell lung cancers (NSCLCs), phase III clinical trials of gefitinib recently yielded response rates of 62% or 74% where EGFR was mutated.[5,6] EGFR mutations are rare in HNSCC[7] and an equivalent biomarker of response has yet to be defined.[8] Nevertheless, interest in targeting EGFR remains high with new strategies (inhibitor combinations, irreversible or multi-targeting inhibitors) currently being evaluated in HNSCC[3] including 35 active/recruiting studies listed in the National Institutes of Health (NIH) database (http://www.clinicaltrials.gov; accessed 8/1/2013).

Even in patients who initially respond to EGFR TKIs, acquired resistance generally arises within 6–12 months.[9] In this manuscript, we describe the use of novel HNSCC cell lines with acquired resistance to multiple EGFR TKIs to identify proteins associated both with resistance and with highly aggressive disease. Further, in pilot studies we detected a resistance-associated protein signature (RAPS) in the sera of HNSCC patients treated with gefitinib and found it to be associated with reduced overall and progression-free survival. This suggests that the RAPS has potential for development as an innovative marker of therapy resistance and/or cancer progression in HNSCC.

2. Materials, methods and patients

2.1. Cell culture

CAL 27 sublines were grown in Dulbecco's modified Eagle's medium and PJ34s in Iscove's Modified Dulbecco's medium supplemented with 10% foetal calf serum, at 37 °C in a 5% CO_2 humidified incubator. Both parental cell lines are negative for human papillomavirus (HPV) infection.[10]

2.2. In vitro growth assays

Cells in 96-well plates were fixed with 10% (w/v) trichloroacetic acid then stained with 0.4% (w/v) sulphorhodamine B (SRB) in 1% (v/v) acetic acid. After washing with 1% acetic acid, SRB was eluted with 10 mM Tris, pH 10 and the absorbance read at 570 nm. For GI_{50} determination, cells were incubated with drug/vehicle for 72 h prior to fixation. For growth rate determination, cells were fixed at 24-hourly intervals.

2.3. Xenograft tumours

Female athymic mice, 6–8 weeks old, (CrTac:NCr-Fox1(nu), Charles River, Ramsgate, UK) were injected bilaterally s.c. with 3 million (CAL^{S2}/CAL^{R2}) or 5 million (CAL^{S}/CAL^{R}, PJ^{S}/PJ^{R}) cells/site. Tumour volumes were calculated from measurements of orthogonal diameters. All experiments were performed in accordance with UK Home Office regulations under the Animals (Scientific Procedures) Act 1986 and National Cancer Research Institute (NCRI) guidelines.[11]

2.4. Antibody arrays

Cells were incubated with 0.1% dimethyl sulfoxide (DMSO) (control) or 0.06 μM (GI_{50} CAL^{S}) gefitinib for 72 h then lysed according to Ref. 12. Lysates were incubated with array membranes (ARY007, Proteome-Profiler™, R&D Systems, Abingdon, United Kingdom (UK)). Bound proteins were visualised with a chemiluminescent substrate and exposure to radiographic film. Scanned images were quantified using ImageQuant software (Non-linear Dynamics, Newcastle-upon-Tyne, UK). Details provided in Supplementary text.

2.5. Immunoassays

72 h cell-conditioned medium was centrifuged (10,000*g*, 10 min, 4 °C) and granulocyte-macrophage colony-stimulating factor (GM-CSF)/interleukin-8 (IL-8)/tissue inhibitor of metalloproteinase-1 (TIMP-1)