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Original Articles

Elevated RET expression enhances EGFR activation and mediates EGFR inhibitor resistance in head and neck squamous cell carcinoma

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ABSTRACT

Background and aim: Co-activation of EGFR by alternative receptor tyrosine kinases (RTKs) might mediate resistance to EGFR inhibition in head and neck squamous cell carcinoma (HNSCC). Here we found a novel mechanism to improve the efficacy of EGFR inhibitor erlotinib on HNSCC.
Method: Immunohistochemistry, western blot, cell migration and invasion assays, cell proliferation, cell cycle analysis and *in vivo* serial transplantation assays were used to evaluate the role of RET on HNSCC cells.
Results: The elevated levels of a rearranged during transfection (RET) are observed in HNSCC and that high levels of RET correlate with increased tumor size, advanced tumor stage and decreased overall survival rate. The HNSCC cell proliferation and invasion were inhibited by RET knockdown *in vitro* and *in vivo*. The inhibition of RET expression markedly reduced EGFR phosphorylation and downstream EGFR signaling. The inhibition of RET signaling significantly increased the sensitivity of HNSCC cells to the EGFR inhibitor erlotinib in both *in vitro* and *in vivo* models.
Conclusion: Our results offer a preclinical proof-of-concept supporting a role for RET signaling inhibition in a targeted therapeutic approach to improve the efficacy of EGFR inhibition in HNSCC.

Introduction

Rearranged during transfection (RET) is a transmembrane receptor tyrosine kinase (RTK) activated by the glial-derived neurotrophic factor (GDNF) family of ligands [1]. GDNF ligands form binary complexes with GDNF family receptor-α, which then bind to RET, recruit it to cholesterol-rich membrane subdomains and activate RET signaling [2,3]. In addition, crosstalk between RET and other RTKs, including MET and TrkB, enhances RET activity and RET-associated downstream signaling [4,5]. The activation of the RET signaling pathway is essential to the maintenance and self-renewal of sperm atogonial stem cells [6] and to the development and maturation of neurons in the peripheral and central nervous systems [7,8].

RET signaling contributes not only to the biological processes of cell survival, proliferation and migration but also to the development of multiple types of cancer. RET is an oncogene that drives the pathogenesis of various thyroid tumors, and different RET mutations lead to specific tumor types [9]. Recently, the expression and activation of wild-type RET was observed in several tumor types, including pancreatic carcinoma [10–12] and breast cancer [13–15]. RET activity increases regional tumor invasion and perineural spread in pancreatic carcinoma. The RET ligands GDNF and ARTN, which are secreted by intra- and extra-pancreatic nerves, phosphorylate components of the RET–Ras–mitogen activated protein kinase (MAPK) pathway, thereby inducing the migration of RET-expressing tumor cells to the perineural space [11,12]. RET activity also promotes breast cancer cell proliferation, migration and the development of resistance to endocrine therapies [13,14]. Given that the RET ligand GDNF and Persephin are over-expressed in head and neck squamous cell carcinoma (HNSCC) tissues, GDNF family ligands might stimulate the invasion and migration of HNSCC cells by phosphorylating RET [16,17]. Information regarding RET expression and activity, and its potential role in the progression of HNSCC remains largely unknown.

The epidermal growth factor receptor (EGFR) is an RTK expressed in up to 90% of HNSCC cases, and increased EGFR expression

Abbreviations: RET, rearranged during transfection; EGFR, epidermal growth factor receptor; RTK, receptor tyrosine kinase; TKI, tyrosine kinase inhibitors; GDNF, glial-derived neurotrophic factor; MAPK, mitogen activated protein kinase; HNSCC, head and neck squamous cell carcinoma.

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is significantly associated with advanced tumor stage and poor clinical outcomes [18,19]. The role of EGFR in activating key signaling pathways associated with proliferation, invasion and angiogenesis makes it an attractive therapeutic target [20]. EGFR small molecule tyrosine kinase inhibitors (TKIs), including erlotinib and gefitinib, selectively inhibit the kinase domain of the EGFR tyrosine kinase domain by binding to the EGFR ATP-binding region. Despite the success of TKIs in various preclinical HNSCC models, EGFR-targeted chemotherapy has limited efficacy in HNSCC patients [21]. Low response rates (4–15%) have been observed in clinical trials with HNSCC patients treated with EGFR TKIs such as erlotinib [22]. Multiple mechanisms associated with poor response rates and resistance to EGFR inhibitors have been proposed. In the context of HNSCC, the co-activation of other RTKs and the activation of alternative signal pathways have been implicated in the limited efficacy of TKIs [23–25]. However, the precise mechanisms mediating EGFR-TKI sensitivity in HNSCC have not been fully characterized.

Here, we demonstrate that elevated RET protein levels are observed in HNSCC and that increased RET levels correlate with poor clinical outcomes. Moreover, inhibition of RET expression markedly reduced EGFR phosphorylation and downstream signaling. RET inhibition significantly sensitizes HNSCC cells to the EGFR inhibitor erlotinib in both *in vitro* and *in vivo* models. Our results suggest that RET plays a significant role in tumor growth, and its potential role as a novel adjuvant therapeutic target in combination with EGFR-targeted therapies merits further investigation.

Materials and methods

HNSCC patients and tissue samples

A total of 145 HNSCC paraffin-embedded tissue samples were randomly selected from the Department of Oral and Maxillofacial Surgery-Head and Neck Oncology, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine between September 2007 and June 2009. The pathological tumor stage was classified according to the Union for International Cancer Control (UICC) system. The tumor histological grade was determined according to the criteria recommended by the World Health Organization. All patients were treated with surgery with curative intent and exhibited negative resection margins. Patients with T3 or T4 stage of disease and lymph node metastasis were further treated with radiation with or without chemotherapy after surgery. Tissue samples were collected, immediately snap frozen in liquid nitrogen and stored at –80 °C until further analysis. Written informed consent from all patients and approval of the Hospital Ethic Review Committees was obtained.

The detailed methods were described in the Supplementary Materials, including cell culture, chemical compound and cytokine preparation, immunohistochemistry, siRNA and shRNA transfection, cell proliferation assay, colony formation assay, cell migration and invasion assays, apoptosis assay, cell cycle analysis, Western blot analysis, and animal experiments.

Results

Upregulated RET is correlated with poor prognosis in HNSCC patients

To investigate RET gene expression in HNSCC, we first accessed information regarding RET mutations, deletions, amplifications and expression level provided by the Cancer Genome Atlas (TCGA) (Fig. 1A,B). RET mutation or gene amplification was observed in a small proportion of patients, and the RET gene was expressed at high levels compared with normal tissues. We analyzed HNSCC tissue samples to further evaluate RET expression levels. As shown in Fig. 1C, RET protein levels were significantly elevated in 6/8 (75%) specimens of human primary HNSCC tissue compared with matched adjacent non-tumor tissues. To assess the prognostic value of RET in HNSCC patients, we analyzed RET protein expression by conducting immunohistochemistry (IHC) assays of paraffin sections from HNSCC patients who underwent surgery at the Shanghai Ninth People's Hospital between September 2007 and June 2009. Representative images of negative, moderate and strong RET staining are presented in Fig. 1D. The correlations of RET expression with clinical and pathological parameters are shown in Supplementary Table S1. Positive RET expression was detected in 79 of the 145 cases and was significantly correlated with larger tumor size (greater than 2 cm; pT2–pT4; $p = 0.029$) and advanced tumor stage ($p = 0.019$). RET expression levels were not significantly associated with the other parameters evaluated, including age, gender, histology and lymph node status. Moreover, Kaplan–Meier analyses and COX regression analysis revealed that RET expression was significantly correlated with decreased overall survival in HNSCC patients and was an independent predictor of overall survival in HNSCC (Fig. 1E and Supplementary Table S2). These data suggest that RET is an independent biomarker and prognostic indicator of clinical outcomes in HNSCC patients.

RET is required for the maintenance of HNSCC cell proliferation and invasion

To further explore the role of RET in HNSCC, we analyzed RET protein expression in seven HNSCC cell lines (HN4, HN6, HN12, HN13, HN30, Cal27 and SCC4). Relatively high levels of RET were observed in HN4, HN6, and HN13 cells, whereas moderate to low levels were observed in Cal27, SCC4 and HN30 cells. RET expression was not detected in HN12 cells (Supplementary Fig. S1A). We conducted RET knockdown experiments using specific siRNAs in HN6 and HN13 cells, which expressed high levels of RET.As shown in Fig. 2A, two RET siRNAs (siRET1 and siRET2) efficiently suppressed endogenous RET expression in both HNSCC cell lines. RET knockdown markedly inhibited proliferation and viability in both HN6 and HN13 cells (Fig. 2B and Supplementary Fig. S1B). The number of HN6 and HN13 cells significantly decreased after transfection with siRET1 and siRET2 compared with the negative controls ($p < 0.001$). RET knockdown also significantly inhibited colony formation in HN6 and HN13 cells ($p < 0.001$) (Fig. 2C). Consistent with these findings, we observed cell cycle arrest and apoptotic cells in both RET knockdown cell lines (Fig. 2D,E), indicating that RET is associated with the proliferation of HNSCC cells.

To further evaluate the effect of RET in HNSCC metastasis, we conducted transwell migration and invasion assays. The RET knockdown cells exhibited a significant decrease in migration and invasion capability ($p < 0.001$ in both assays) (Fig. 2F,G). Therefore, we investigated the phosphorylation status of AKT and ERK in response to GDNF stimulation in RET-knockdown and negative control cells. Western blot analysis revealed that p-ERK and p-AKT levels increased in response to GDNF stimulation in both the RET-knockdown and negative control cells. By contrast, the negative control cells exhibited markedly increased levels of p-ERK and p-AKT compared with RET-knockdown cells (Fig. 2H).

In order to examine the influence of RET overexpression on HNSCC, we selected HN30 cells with relatively lower expression of RET than HN6 and HN13 cells (Supplementary Fig. S1A) in the following experiment. As shown in Supplementary Fig. S2A, ectopic expression of RET (RET_{OE}) was found in HN30 cells. Accordingly, RET overexpression has significantly enhanced the proliferation (Supplementary Fig. S2B) and invasion of HN30 (Supplementary Fig. S2C).

These results suggest that RET stimulates HNSCC cell proliferation, migration and invasion primarily by activating ERK and AKT signaling.

RET knockdown suppresses tumor growth in HNSCC cells in vivo

To confirm that RET knockdown inhibits cell growth *in vivo*, we established a xenograft model in nude mice. HN6 shRET cells and HN6 shNC cells were injected subcutaneously in the flank in the nude mice, and tumor growth was recorded over 20 days. Consistent with the *in vitro* findings, tumors derived from HN6-shRET cells grew at