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Original Articles

Broad RTK-targeted therapy overcomes molecular heterogeneity-driven resistance to cetuximab via vectored immunoprophylaxis in colorectal cancer

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ABSTRACT

The human epidermal growth factor receptor (EGFR) targeting chimeric monoclonal antibody, cetuximab (Erbitux®), is a widely used drug in the treatment of metastatic colorectal cancer. However, the activation of the extensive crosstalk among the EGFR family receptors as well as other tyrosine kinase receptors (RTKs) impairs the efficacy of the drug by fueling acquired resistance. To identify the responsible potential activation pathway underlying cetuximab resistance and generate novel treatment strategies, cetuximab-resistant colorectal cancer cell lines were generated and validated and a functional RNAi screen targeting human RTKs was used to identify extensive receptor tyrosine kinase signaling networks established in resistant cancer cells. MET, Axl, and IGF-1R were identified as contributors to the acquired resistance to cetuximab. Targeting vectored immunoprophylaxis (VIPs) to different RTKs were generated and characterized. Different VIP approaches were evaluated *in vivo* with parental and cetuximab-resistance xenografts and the RTKs in resistant cancer xenografts were inhibited with VIPs via re-sensitization to cetuximab treatment. Combination of VIPs was more broadly efficacious, mechanistically, due to co-blocking the EGFR/Axl/MET signaling pathway, which was cross-activated in the resistant cell lines. Moreover, a VIP-based procedural treatment strategy not only eliminated the tumor but also afforded long-lasting protection from tumor recurrence and resistance. Overall, EGFR-related RTK pathway-network activation represents a novel mechanism underlying cetuximab resistance. A broad VIP combination strategy and VIP-based procedural treatment strategy may be a recommended addition to cetuximab-based targeted therapy. Our results establish a new principle to achieve combined RTK inhibition and reverse drug resistance using a VIP approach.

Introduction

Colorectal cancer (CRC), as the third most common cancer in the western world, now affects 200,000 new cases worldwide every year [1]. The management of early-stage CRC is accomplished with screening, surgery, and medical therapies, though these methods are less efficacious in advanced stages. Precise medical strategies, such as inhibiting EGFR kinase in CRC driven by defined genomic alterations with targeted therapeutic options like monoclonal antibodies (mAb) cetuximab and panitumumab, have improved the overall survival of patients at the metastatic stage of this disease. However, acquired resistance inevitably develops, limiting the benefit of targeted therapies [2].

Cancer can display high levels of molecular heterogeneity and clonal evolution, which are generated from random mutations and clonal selection [3–5], representing one of the greatest challenges in cancer therapeutics. Indeed, exposure to certain

Precis: Results establish a new principle to overcome acquired resistance to cetuximab and achieve profound anti-tumor effect by VIP-based co-targeting treatment strategies.

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therapies may result in exerting selective pressures on sub-clonal cell populations, which enable cancer cell evolution [5–7]. An accumulating body of knowledge has indicated that more than half of the heterogeneous genetic alterations of colorectal cancers, which were reported to respond negatively to cetuximab and panitumumab, were genes related to the constitutive activation of signaling pathways downstream of EGFR. Moreover, the analysis of KRAS mutations as a test to restrict the use of cetuximab in combination with chemotherapy to first-line metastatic CRC patients with wild-type tumors has been widely approved.

Additional genetic mechanisms have been proposed to meditate drug resistance in the absence of molecular alterations affecting the EGFR–RAS pathway or its immediate downstream effectors. We [8] and others [9–11] have identified bypass receptor tyrosine kinase (RTK) pathway activation and signaling shift as key processes in resistance to targeted therapy, and combined inhibition strategies for co-blocking key RTK signaling networks may have clinical benefits. However, systematic studies of co-targeting the RTKs involved in the secondary resistance to cetuximab were seldom reported. Therefore, new insight into the molecular mechanisms of secondary resistance may help to develop novel effective therapeutic strategies or guide the usage of therapeutic combinations for patients who develop clinical cetuximab resistance. Notably, due to the molecular heterogeneity complexity of drug resistance and because multiple different RTKs may be involved in different patient sub-populations and/or different disease courses, it is costly and inefficient to evaluate multiple RTK co-inhibition treatment strategies via a combination of targeting therapeutic agents using high-cost manufacturing and sophisticated technology, especially associated with large-scale mammalian cell culture and column chromatography of the antibody.

Vector-mediated gene transfer could be used as an alternative to antibody treatment, as these approaches could be used to engineer the secretion of the existing anti-cancer antibodies into the circulation. Vectored immunoprophylaxis (VIP) is a practical implementation of this approach, which employs a single intramuscular injection to achieve lifelong expression of these monoclonal antibodies at high concentrations in mice, using a specialized adeno-associated virus (AAV) vector optimized for the production of full-length antibody from muscle tissue [12]. We therefore hypothesized that AAV gene transfer vectors could be designed to express multiple anti-cancer antibodies as a useful tool to study combined treatment strategies. In the present study, we employed an unbiased functional genetic screening approach, in which a siRNA library targeting most human RTKs was transfected into cetuximab-resistant cell models and the effects on cetuximab sensitivity were then analyzed. The candidate RTKs were validated, and VIPs expressing targeting antibodies were generated to have a broad RTK targeting treatment strategy. Different broad RTK targeting treatment strategies were carefully investigated *in vivo*, and the molecular basis of the bypass signaling shifts were characterized. Moreover, a VIP-based procedural treatment strategy exhibited significant efficacy *in vivo* with long-lasting protection against tumor cells allowing the mice to survive a subsequent resistant cancer cell re-challenge.

Materials and methods

Cell lines, antibodies, and animals

Cetuximab-sensitive colorectal carcinoma cells LIM1215, SW48 and DiFi were used in the current study. The LIM1215 colon cancer cell line was obtained from Cell Bank Australia (Westmead, NSW, Australia). SW48 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). DiFi colorectal carcinoma cells have been described previously [13] and were obtained from Kunming Cell Bank of the Chinese Academy of Sciences (Kunming, China). LIM1215, SW48, and DiFi cells were cultured in DMEM medium or F12 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) in 5% CO_2 at 37.8 °C in a humidified incubator. All the cell lines were authenticated twice by morphologic and isoenzyme analyses during the study period. Human HGF protein was purchased from Biorbyt. Cetuximab and dalotuzumab were purchased from Merck. Ficlatuzumab was purchased from Aveo Pharmaceuticals. An anti-Axl antibody 58C46 was cloned, expressed, and purified following reported procedures [14]. BALB/c nude mice were purchased from the Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China). All the animals were treated in accordance with guidelines of the Committee on Animals of the Chinese Academy of Sciences.

Construction and production of VIP vectors

The heavy and light chains of cetuximab, 58C46, ficlatuzumab and dalotuzumab were cloned into VIP expression vectors as previously described [15]. To ensure efficient transgene expression, we use a VIP expression vector containing an AAV2-derived plasmid backbone modularized muscle-optimized promoter, a splice donor and splice acceptor pair, a polyadenylation signal, a posttranscriptional enhancer and AAV2 inverted terminal repeats to permit vector genome encapsidation. Vectors were produced by co-transfection of different VIP expression vectors, helper plasmid pHELP, and a packaging pAAV2/8 plasmid with 293 cells followed by cesium chloride purification [16]. Virus was aliquoted and stored at –80 °C. For each viral preparation, viral genomes were determined by qPCR [17]. Briefly, frozen aliquots of AAV were thawed and diluted ten-fold in digestion buffer containing 10 units of DNase I (Roche) and incubated at 37 °C for 45 min. Virus was DNase-digested and serially diluted. Total reaction volume was 15 µl with PerfeCTa SYBR Green SuperMix, ROX (Quanta Biosciences) was used for 5 µl of each dilution with primers designed against the CMV enhancer (5′ CMV: AACGCCAATAGGGACTTTCC and 3′ CMV: GGGCGTACT TGGCATATGAT) or the luciferase transgene (5′ Luc: ACGTGCAAAAGAAGCTACCG and 3′ Luc: AATGGGAAGTCACGAAGGTG). The samples were run in duplicate on a 7500 Fast Real-Time PCR System (Applied Biosystems).

Construction, expression and protein purification

RTK-ECDs and the heavy and light chains of 58C46 were cloned as described previously [18,19] employing the pcDNA3.1(+) expressing vector (Invitrogen, Carlsbad, CA) and the FreeStyle 293 expression system (Invitrogen). Protein A affinity chromatography was used to purify the recombinant antibodies from the serum-free culture supernatant. Protein concentrations were determined by absorbance at 280 nm, and purity was confirmed by SDS-PAGE analysis. Bioactivity was measured in antibody affinity assays.

siRNA transfection

Knockdown of AXL, IGF1R, and MET was performed using specific single or pooled siRNAs, as indicated, targeting the indicated genes, purchased from Dharmacon RNAi Technologies (Thermo Scientific, Rockford, IL). Non-targeting siRNAs served as negative controls. The introduction of siRNA was performed with the Dharma-FECT4 reagent according to the manufacturer's instructions. The efficiency of the knockdown at different times or dose points was assessed by western blotting on cell lysates.

Immunoblotting

Cells were incubated with the indicated antibodies in serum-free medium for 1 hour at 36.5 °C, then treated with RTK ligands or not for 15 minutes. After washing, the cells were lysed, and the lysates were subjected to SDS-PAGE and immunoblotted with antibodies against EGFR, phospho-EGFR1068, MET, phospho-MET (all from Cell Signaling Technology), AXL and phospho-AXL (R&D Systems, Minneapolis, MN). For signaling complex detection and crosstalk experiments, recombinant human EGF, HGF, IGF1 and GAS6 (R&D Systems) were added at a final concentration of 0.1 nmol/l. All the assays were performed independently in triplicate.

Cell proliferation assay

In total, 3000–4000 cells/well were plated in 96-well plates. The next day, the cells were treated with the indicated treatment in 5% serum-containing medium. Viable cell numbers were determined using CellTiter-Glo or MTS assay kits, as indicated in the figure legends, according to the manufacturer's protocols (Promega, Madison, WI, USA). All the assays were performed independently in triplicate.

AAV intramuscular injection and quantification of antibody production

Aliquots of viruses were thawed slowly on ice and diluted to achieve the predetermined dose in a 50 µl volume. Mice were anaesthetized by isoflurane inhalation, and a single 50 µl injection was administered into the gastrocnemius muscle with a 28 G insulin syringe. Serum concentrations of the antibodies were measured by competitive ELISAs. Briefly, serial dilutions of serum samples were incubated with a subsaturating concentration of the indicated antibodies-biotin on RTK-ECD-coated ELISA plates at 37 °C for 1 h. The detection was performed with alkaline phosphatase-conjugated avidin.