NEOPLASIA
www.neoplasia.com

Volume 20 Number 7 July 2018 pp. 668–677 668

Biologic Response of Colorectal Cancer Xenograft Tumors to Sequential Treatment with Panitumumab and Bevacizumab

Hiroya Taniguchi[*,1], Yuji Baba[†,1], Yoji Sagiya[‡], Masamitsu Gotou[†], Kazuhide Nakamura[†], Hiroshi Sawada[†], Kazunori Yamanaka[†], Yukiko Sakakibara[‡], Ikuo Mori[‡], Yukiko Hikichi[§], Junpei Soeda[‡] and Hideo Baba[‖]

[*]Department of Clinical Oncology, Aichi Cancer Center Hospital, Nagoya 464-8681, Japan.; [†]Pharmaceutical Research Division, Takeda Pharmaceutical Company Limited, Fujisawa, Kanagawa 251-8555, Japan; [‡]Japan Medical Affairs, Japan Oncology Business Unit, Takeda Pharmaceutical Company Limited, Tokyo 103-8668, Japan; [§]Product Information Group, Japan Oncology Business Unit, Takeda Pharmaceutical Company Limited, Tokyo 103-8668, Japan; [‖]Department of Gastroenterological Surgery, Kumamoto University, Kumamoto 860-8556, Japan

Abstract

Recent studies in *RAS* wild-type (WT) metastatic colorectal cancer (mCRC) suggest that the survival benefits of therapy using anti-epidermal growth factor receptor (anti-EGFR) and anti-vascular endothelial growth factor (anti-VEGF) antibodies combined with chemotherapy are maximized when the anti-EGFR antibody is given as first-line, followed by subsequent anti-VEGF antibody therapy. We report reverse-translational research using LIM1215 xenografts of *RAS* WT mCRC to elucidate the biologic mechanisms underlying this clinical observation. Sequential administration of panitumumab then bevacizumab (PB) demonstrated a stronger tendency to inhibit tumor growth than bevacizumab then panitumumab (BP). Cell proliferation was reduced significantly with PB ($P < .01$) but not with BP based on Ki-67 index. Phosphoproteomic analysis demonstrated reduced phosphorylation of EGFR and EPHA2 with PB and BP compared with control. Western blotting showed reduced EPHA2 expression and S897-phosphorylation with PB; RSK phosphorylation was largely unaffected by PB but increased significantly with BP. In quantitative real-time PCR analyses, PB significantly reduced the expression of both lipogenic (*FASN, MVD*) and hypoxia-related (*CA9, TGFBI*) genes versus control. These results suggest that numerous mechanisms at the levels of gene expression, protein expression, and protein phosphorylation may explain the improved clinical activity of PB over BP in patients with *RAS* WT mCRC.

Neoplasia (2018) 20, 668–677

Abbreviations: BB, bevacizumab-bevacizumab; BP, bevacizumab followed by panitumumab; CA9, carbonic anhydrase 9; CRC, colorectal cancer; DEGs, differentially expressed genes; EGFR, epidermal growth factor receptor; EPHA2, ephrin type-A receptor 2; FASN, fatty acid synthase; FOLFIRI, fluorouracil, leucovorin, and irinotecan; FOLFOX, fluorouracil, leucovorin, and oxaliplatin; GR, growth rate; GTPase, guanosine triphosphate hydrolase; HIF, hypoxia-inducible factor; HMGCR, HMG-CoA reductase; HR, hazard ratio; IGF2R, insulin-like growth factor 2 receptor; LSS, lanosterol synthase; MAPK, mitogen-activated protein kinase; mCRC, metastatic colorectal cancer; MVD, mevalonate disphosphate decarboxylase; OS, overall survival; PB, panitumumab followed by bevacizumab; pEPHA2, phosphorylated EPHA2; PFS, progression-free survival; pRSK, phosphorylated RSK; qRT-PCR, quantitative real-time polymerase chain reaction; RSK, ribosomal S6 kinase; SCID, C.B17/Icr-scid/scid Jcl; SDS, sodium dodecyl sulfate; TGFBI, transforming growth factor-β induced protein; VEGF, vascular endothelial growth factor; WT, wild-type

Address all correspondence to: Junpei Soeda, MD, PhD, Japan Medical Affairs, Japan Oncology Business Unit, Takeda Pharmaceutical Company Limited, 12-10 Nihonbashi 2-chome, Chuo-ku, Tokyo 103-8668, Japan.
E-mail: jumpei.soeda@takeda.com
[1]These authors have contributed equally to the work.
Received 27 March 2018; Revised 24 April 2018; Accepted 24 April 2018

1476-5586/18
https://doi.org/10.1016/j.neo.2018.04.006

Introduction

Colorectal cancer (CRC) is the third most common cancer in men and the second most common in women, accounting for approximately 1.36 million new cases and 694,000 deaths worldwide each year [1]. Approximately 25% of CRC patients present metastatic disease (mCRC) at diagnosis, and almost half will subsequently develop metastases [2]. The current standard of care for mCRC involves a backbone of cytotoxic chemotherapy, using regimens such as FOLFIRI (fluorouracil, leucovorin, and irinotecan) and FOLFOX (fluorouracil, leucovorin, and oxaliplatin) combined with targeted agents [2,3]. Such regimens involving an anti-epidermal growth factor receptor (EGFR) antibody, such as panitumumab and cetuximab, or the anti-vascular endothelial growth factor (VEGF) antibody, bevacizumab, in combination with chemotherapy, improve survival compared with chemotherapy alone [4–9].

RAS is a small guanosine triphosphate hydrolase that is constitutively activated by mutation in ~20% of human cancers [10]. *KRAS* is the predominantly mutated isoform in CRC [10]; 55.9% of patients with CRC harbor a *RAS* (*KRAS/NRAS*) mutation [11]. Constitutive RAS activation facilitates oncogenesis through the up-regulation of signaling pathways such as mitogen-activated protein kinase (MAPK) and Akt [12,13].

Potential benefit with anti-EGFR antibodies appears to be limited to patients with *RAS* wild-type (WT) mCRC [14–16]; such patients had improved clinical outcomes when treated with an anti-EGFR antibody and chemotherapy as first-line therapy than when compared with an anti-VEGF antibody and chemotherapy [17]. *Post-hoc* analysis of the FIRE-3 study, in which patients with *RAS* WT mCRC received treatment with FOLFIRI plus cetuximab or bevacizumab, highlighted a durable overall survival (OS) advantage for the group of patients that received FOLFIRI and cetuximab as first-line therapy compared with FOLFIRI and bevacizumab (median 33.1 vs. 25.0 months; hazard ratio [HR] 0.70; $P = .0059$) [17]. There have also been indications that first-line therapy in *RAS* WT mCRC can determine the efficacy of subsequent treatments and affect outcomes [18–20]. Furthermore, an exploratory analysis of data from three randomized studies of mCRC suggested a trend towards improved OS with a first-line anti-EGFR antibody plus chemotherapy followed by a second-line anti-VEGF antibody compared with the opposite sequence [21].

While the use of an anti-EGFR antibody in first-line treatment can increase the efficacy of second-line anti-VEGF antibodies [22,23], initial treatment with an anti-VEGF antibody may decrease the efficacy of subsequent anti-EGFR antibodies [24–26]; a sufficient anti-VEGF antibody-free period prior to treatment with second-line anti-EGFR antibodies is necessary to limit this reduced efficacy [27]. The biologic rationale for this finding remains unknown, but mechanisms have been suggested that may contribute [19,20]. *RAS* WT mCRC tumor cells that develop resistance to an anti-EGFR antibody may retain sensitivity to an anti-VEGF antibody, but resistance to an anti-VEGF antibody can lead to the development of resistance to anti-EGFR antibodies [19,20]. Indirect evidence for this comes from the finding that resected liver metastases from Japanese patients with mCRC treated with bevacizumab demonstrated significantly increased tumoral *VEGFA* mRNA expression [28], while in pre-clinical models of CRC, overexpression of *VEGFA* or treatment with exogenous VEGF-A ligand conferred resistance to cetuximab [24,29]. Taken together, these findings highlight a potential mechanism of acquired resistance to anti-EGFR antibodies in mCRC that potentiates tumor angiogenic ability. Nevertheless, many details regarding the biologic mechanisms underlying the efficacy of the two different treatment sequences in the clinic are yet to be explored.

Here, we present the results of reverse-translational research using xenograft models of human CRC to evaluate the biologic reasons for the improved outcomes seen with sequential use of an anti-EGFR antibody (panitumumab) followed by an anti-VEGF antibody (bevacizumab) compared with the opposite sequence in patients with *RAS* WT mCRC. We performed quantitative phosphoproteomic and transcriptome analyses of xenograft tumors to identify biological changes with sequential treatment that may provide some explanation for the survival benefits previously demonstrated in clinical settings.

Materials and Methods

Cells and Reagents

The human colon cancer cell line LIM1215 was obtained from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). LIM1215 cells were cultured in conditions recommended by the ECACC. Panitumumab was provided by Amgen (Thousand Oaks, CA, USA). Bevacizumab was purchased from Roche (Basel, Switzerland).

Xenograft Construction and Study Treatment

All in vivo experimental protocols complied with the Guide for the Care and Use of Laboratory Animals (8th Edition), and were approved by the Institutional Animal Care and Use Committee of the Shonan Research Center (#00011823), Takeda Pharmaceutical Company Limited, or Shanghai Medicilon Inc. (Shanghai, China).

LIM1215 cells were selected because they have WT *RAS (*WT *KRAS* and *NRAS)* and WT *BRAF*, and panitumumab and bevacizumab have previously shown anti-tumor effects in xenografts of LIM1215 tumors [30]. Six- to seven-week-old female C.B17/Icr-scid/scid Jcl (SCID) mice (from CLEA, Tokyo, Japan, or Beijing Vital River Animal Technology, Beijing, China) maintained under specific pathogen-free conditions were injected subcutaneously in the right flank with 5 million LIM1215 cells mixed with Matrigel (Corning, NY, USA). In vivo LIM1215 xenografts were constructed at two different sites, LIM1215(A) and LIM1215(B). Once tumor volume reached 50–200 mm^3, mice were randomized to each treatment group. All treatment was intraperitoneal. The vehicle control group received saline twice-weekly for 2 weeks or 4 weeks. Panitumumab and bevacizumab were given twice-weekly at 3 mg/kg and 10 mg/kg, respectively. The panitumumab-bevacizumab (PB) group received panitumumab for 2 weeks followed by bevacizumab for 2 weeks; the bevacizumab-panitumumab (BP) group received the reverse sequence. One group received bevacizumab (BB) for 4 weeks and other groups received monotherapy with panitumumab (P group) or bevacizumab (B group) for 2 weeks (Supplementary Figure S1).

Tumor volumes (length × $width^2$ × 0.5) were measured twice-weekly with Vernier calipers and antitumor activity was evaluated by percentage of relative growth rate (GR) calculated using the following equation: %GR = (mean growth rate of treated tumor/mean growth rate of vehicle control group) × 100. Following final tumor volume measurements, mice were anesthetized 24 hours after final drug administration and euthanized by cervical dislocation, and tumor samples were collected. Samples from the LIM1215(A) xenografts were used for the transcriptome and phosphoproteome analyses, and quantitative real-time PCR (qRT-PCR). The LIM1215(B) xenograft samples were used for qRT-PCR, western blotting, and histology analyses.

Tumor Tissue Analyses

Formalin-fixed paraffin-embedded sections (4 μm) of resected tumor tissue were used for histologic analysis. Hematoxylin and eosin staining