Cetuximab promotes SN38 sensitivity via suppression of heat shock protein 27 in colorectal cancer cells with wild-type *RAS*

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Received February 12, 2017; Accepted June 6, 2017

DOI: 10.3892/or.2017.5734

Abstract. Combination treatment with cetuximab and CPT-11 produces beneficial and synergistic effects in wild-type *RAS* metastatic colorectal cancer (mCRC) patients. However, the mechanism underlying this synergism is not yet understood. We examined whether cetuximab had a synergistic effect with CPT-11 and its active metabolite, SN38, and examined the molecular mechanism of the synergism between cetuximab and SN38 in CRC cells with various mutational status. We hypothesized that cetuximab promotes sensitivity to SN38 via suppression of heat shock protein 27 (HSP27), a protein involved in multidrug resistance through blocking the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway, which is associated with chemosensitivity. Four human CRC cell lines with different *RAS* and *BRAF* mutational status were used. Expression levels of HSP27 protein correlated with SN38 sensitivity in these cell lines (R=0.841, P=0.159). Exposure to cetuximab and various concentration of AG490, an inhibitor of JAK2, STAT3 and HSP27 protein levels, except in the *KRAS* G12V mutant line, SW620. A synergistic effect of cetuximab in combination with SN38 was observed in *RAS* and *BRAF* wild-type cells (here, Caco2), but not in the three other *RAS*- or *BRAF*-mutated cell lines. These results indicate that cetuximab may promote sensitivity to SN38 via suppression of HSP27 through blocking the JAK/STAT pathway in Caco2 cells. The mutational status of numerous downstream effectors, such as *RAS* and *BRAF*, is important in mono- or combination therapy with cetuximab. In conclusion, cetuximab may promote SN38 sensitivity via suppression of HSP27, through blocking the JAK/STAT signaling pathway, and shows synergistic effects when combined with SN38 in wild-type *RAS* CRC cells.

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Key words: heat shock protein 27, SN38, cetuximab, STAT3, colorectal cancer

Introduction

Colorectal cancer (CRC) is one of the most serious types of cancer worldwide, in terms of both morbidity and mortality. Despite improvements in medical therapies, the efficacy of chemotherapy has reached a plateau and the 5-year survival rate of patients with untreated metastatic CRC (mCRC) is still below 10%, and the underlying molecular basis remains to be clearly defined (1). Cancer cells often exhibit intrinsic resistance to chemotherapeutic agents, or develop resistance over time with treatment. The lack of responsiveness to chemotherapy is an important problem that needs to be resolved.

Cetuximab, a chimeric monoclonal antibody targeting the epidermal growth factor receptor (EGFR), has markedly improved the prognosis in patients with metastatic CRC (mCRC) who harbor wild-type *KRAS* and *BRAF* in their tumors (1-4). Furthermore, new findings regarding *RAS* (*NRAS*) mutations can further assist in predicting the therapeutic effects of anti-EGFR antibody therapy (5-7). CPT-11, one of the major cytotoxic agents in mCRC, is converted into an active metabolite, SN38, which then acts as a topoisomerase I inhibitor. Several studies have shown synergism between cetuximab and DNA-damaging agents, such as CPT-11, *in vitro* and *in vivo* (8,9). Although cetuximab may enhance the tumor response to some chemotherapeutic agents, including CPT-11 (10-12), the mechanism underlying this synergism remains unclear.

Mammalian heat shock proteins (HSPs) are known to be molecular chaperones in protein-protein interactions, acting as anti-apoptotic proteins and contribute to cell survival (13). HSPs have been classified into four major families, based on their molecular weights: HSP90, HSP70, HSP60 and 'small' HSPs (15-30 kDa), including HSP27 (14). Their expression can contribute to the malignant properties of cancer cells, including tumorigenicity, treatment resistance and apoptosis inhibition (14-18). Recently, HSP27 has been identified as a treatment target for several cancers, and clinical trials using an antisense oligonucleotide, OGX427, which inhibits HSP27 expression, have been performed in patients with prostate, bladder, ovarian, breast and non-small cell lung cancer, but not CRC (19). The therapy has been reported to be feasible and effective. In previous studies, we showed that HSP27 might

Table I. Mutation status of colon cancer cell lines.

	Caco2	WiDR	SW480	SW620
RAS	Wild-type	Wild-type	*KRAS* mutation (Codon G13D)	*KRAS* mutation (Codon G12V)
BRAF	Wild-type	Exon 15 (V600E)	Wild-type	Wild-type

Authentication of all cell lines was performed by investigating RAS mutation status of each cell line using PCR method. The mutational status of BRAF for a subset of the cell lines was obtained from previous reports (34,35).

contribute to resistance to 5-fluorouracil in CRC, *in vitro* and *in vivo* (20-22). It has also been reported that HSP27 expression is involved in resistance to CPT-11 and doxorubicin *in vitro* (23,24). HSP27 is also believed to be involved in multidrug resistance (25-27). Thus, we considered that cetuximab might promote sensitivity to CPT-11 and SN38 via suppression of HSP27.

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway and, in particular, STAT3, which is a transcription factor, are known to have oncogenic potential (28,29). In CRC cells, activated STAT3 causes resistance to CPT-11 and inhibition of STAT3 strongly enhances the cytotoxic action of CPT-11 (30,31). STAT3 activation in CRC patients is also associated with adverse clinical outcomes, supporting its potential roles as a prognostic biomarker and/or a therapeutic target (32). Furthermore, STAT3 has been reported to regulate HSP27 in breast epithelial cells (33).

We hypothesized that cetuximab might promote sensitivity to CPT-11 and SN38, via suppression of HSP27 through blocking of the JAK/STAT signaling pathway. The aim of the present study was to assess whether cetuximab promoted SN38 sensitivity, and to elucidate the molecular mechanism of the change in SN38 sensitivity caused by cetuximab in CRC cells with various mutational statuses.

Materials and methods

Drugs, colorectal cancer cell lines and cell culture conditions. Cetuximab (2 mg/ml) and SN38, the active metabolite of CPT-11, were kindly provided in powder form by Merck Laboratories (Darmstadt, Germany) and Yakult Honsha Co., Ltd. (Tokyo, Japan), respectively. SN38 was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 10 mM. For *in vitro* experiments, stock solutions of drugs were diluted in phosphate-buffered saline (PBS).

We used four human CRC cell lines: Caco2, WiDR, SW480 and SW620. They were cultured in Dulbecco's modified Eagle's medium ((DMEM; Gibco, Charlestown, MA, USA), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were cultured at 37˚C in 5% CO_2. Authentication of all cell lines was confirmed by investigating the *RAS* mutation status of each cell line using a polymerase chain reaction (PCR)-based method (conducted by SRL, Co., Tokyo, Japan). The mutational status of *BRAF* for cell lines was obtained from previous reports (34,35). The results are summarized in Table I.

Proliferation and cytotoxicity assays. We conducted two experiments. One was to determine cetuximab sensitivity, while the other investigated the combined effects of SN38 and cetuximab or SN38 and AG490, an inhibitor of JAK2, instead of cetuximab. Here, $5x10^3$ cells/well were seeded in 96-well plates. Then, tumor cells were treated with 1, 10 and 100 μg/ml cetuximab or a fixed cetuximab concentration of 10 μg/ml with SN38 or a fixed AG490 concentration of 40 μM with SN38 concentrations ranging from 0.01 to 30 μg/ml for 48 h, followed by overnight incubation in serum-free medium. For cetuximab/SN38 or AG490/SN38 co-treatment experiments, cells were pretreated with cetuximab or AG490 for 15 min before the SN38 addition. Cell viability was evaluated by the reduction of methylthiazol tetrazolium to formazan (0.5 mg/ml). The absorbance of each well was measured at 540 and 600 nm using a microplate spectrophotometer (Immuno Reader; Nalgen Nunc International, Rochester, NY, USA). Viability was assessed as the percentage of viable cells compared with untreated cells (100% viable). The assessment was based on three independent experiments.

Western blot analysis. Whole-cell extracts (20 μg/lane) were electrophoresed through 7.5% sodium dodecyl sulfate polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA) and were transferred to an Immuno-Blot polyvinylidene fluoride membrane (Bio-Rad Laboratories). The membranes were blocked for 1 h in PBS (Gibco-BRL) with 0.5% Tween-20 (PBS-T) and 5% non-fat dry milk at room temperature, then incubated at 4˚C overnight with anti-human HSP27 mouse monoclonal antibody (1:2,000; G3.1; Lab Vision Corp., Fremont, CA, USA), anti-human β-actin mouse monoclonal antibody (1:5,000; AC74; Sigma-Aldrich, St. Louis, MO, USA), and anti-human STAT3 mouse monoclonal antibody (1:200; C-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were incubated for 30 min with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) (1:2,500; Promega Corp., Madison, WI, USA). Proteins were detected using the ECL-Plus reagent (GE Healthcare Life Sciences, Little Chalfont, UK) according to the manufacturer's protocol. Each experiment was performed in triplicate. AG490 (Abcam, Cambridge, UK), JAK/STAT and a JAK2 inhibitor were reconstituted in DMSO and stored at -20˚C until used.

Statistical analysis. Data are reported as means ± standard deviation. Statistical analyses were performed using the Student's t-test or the Mann-Whitney U test. $P<0.05$ were considered to indicate a statistically significant difference.

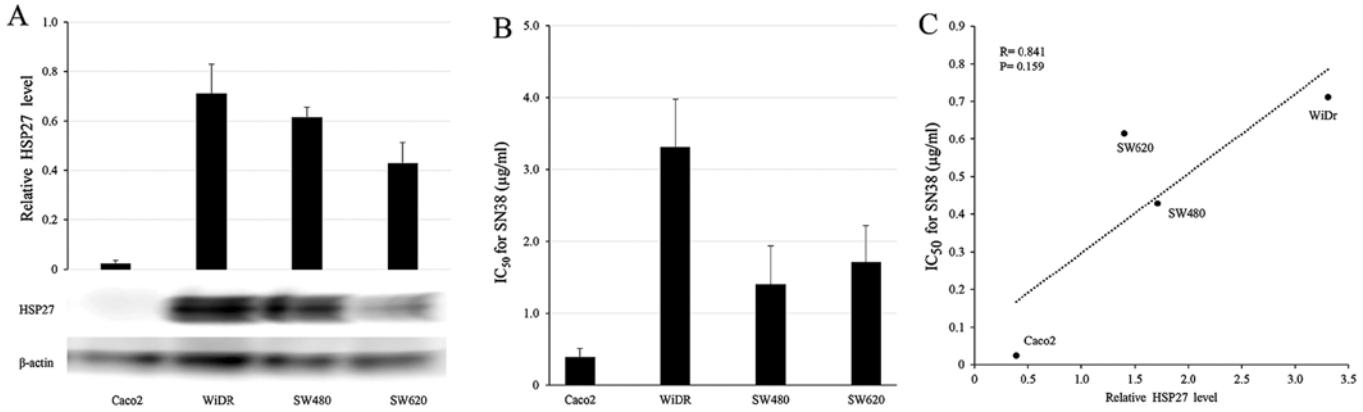

Figure 1. Heat shock protein 27 (HSP27) levels and SN38 sensitivity (IC_{50}) in colorectal cancer cell lines. (A) HSP27 protein levels in the cell lines were estimated by densitometry of western blots, standardized with β-actin levels. (B) SN38 sensitivity was estimated by the drug concentrations that caused 50% growth inhibition vs. the control (IC_{50}). (C) Correlations between HSP27 protein levels and IC_{50} values for SN38 were evaluated in colorectal cancer cell lines by regression analysis.

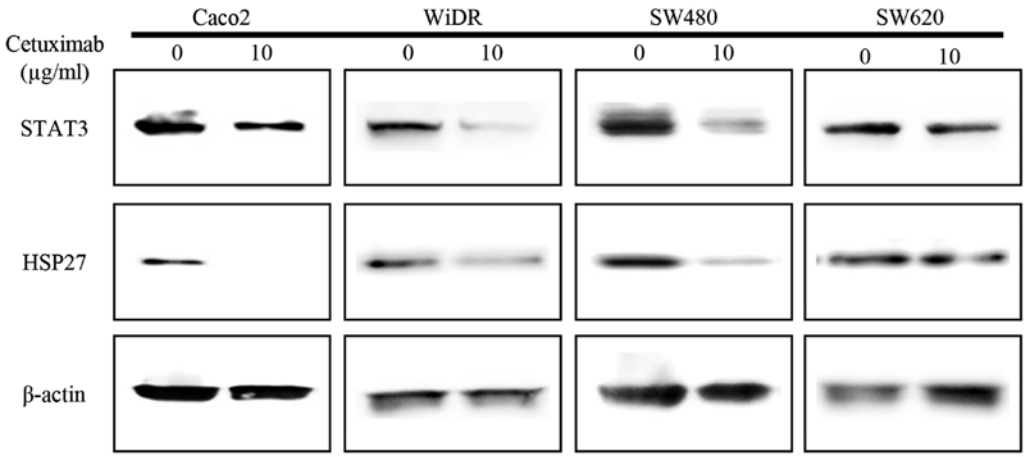

Figure 2. HSP27 downregulation with cetuximab exposure. Western blot analysis of the four colorectal cancer (CRC) cells treated with 10 μg/ml cetuximab for 24 h. STAT3 protein was expressed constitutively in the four CRC cells with no cetuximab exposure. Levels of HSP27 and STAT3 were markedly downregulated in SW480 and Caco2 cells.

Results

RAS and BRAF mutation status. The mutation status of each CRC cell line is shown in Table I. *KRAS* and *NRAS* mutations, which predict the efficacy of cetuximab treatment, were not detected in Caco2 or WiDR. However, the G13D and G12V *KRAS* mutations were detected in SW480 and SW620, respectively. The exon 15 mutation in *BRAF* was detected only in WiDR. From these results, Caco2 was selected as the 'positive' control because it had no mutation in *KRAS*, *NRAS*, or *BRAF*. WiDR, SW480 and SW620 were used as 'negative' controls because of *KRAS* or *BRAF* mutations.

Expression of HSP27 in CRC cell lines. Western blot analysis was conducted to determine HSP27 protein levels in the four CRC cell lines (Fig. 1A). WiDR, SW480 and SW620 cells showed high HSP27 protein levels and relative resistance to SN38, whereas Caco2 showed a low level of HSP27 protein and high sensitivity to SN38 (Fig. 1B). The HSP27 protein levels tended to correlate with the concentration of SN38, resulting in a 50% growth inhibition, but the results were not significant (IC_{50}; Fig. 1C; R=0.841, P=0.159).

HSP27 downregulation by exposure to cetuximab. HSP27 and STAT3 levels with exposure to cetuximab in the four CRC cells were evaluated by western blotting. STAT3 protein was expressed constitutively in the four CRC cell lines with no exposure to cetuximab. Levels of HSP27 and STAT3 were greatly downregulated in Caco2, WiDR and SW480 cells with exposure to 10 μg/ml cetuximab for 24 h vs. untreated cells. However, SW620 cells showed no significant difference in the expression of HSP27 or STAT3 with cetuximab exposure (Fig. 2).

Proliferation assay using cetuximab in CRC cell lines. Cell proliferation was assessed 48 h after exposure to cetuximab alone using the MTT assay. As shown in Fig. 3, little cytotoxic effect of cetuximab treatment (concentrations from 1 to 100 μg/ml) was observed in WiDR, SW480 or SW620 cells. However, the proliferation of Caco2 cells was suppressed according to cetuximab concentration.

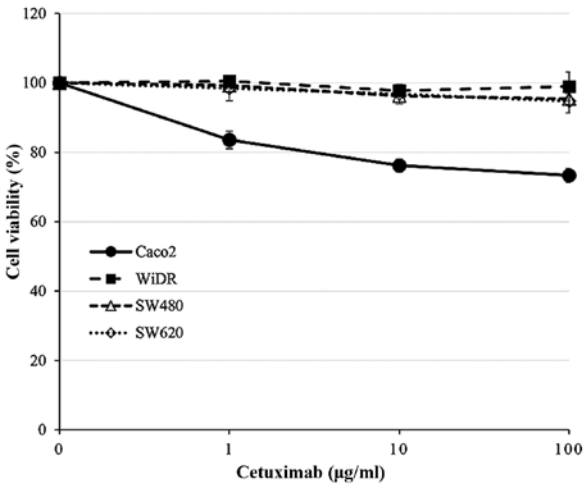

Figure 3. Cytotoxic effect of cetuximab treatment. Colorectal cancer cells were treated with various concentrations of cetuximab for 48 h. Cytotoxicity was determined using the MTT assay, assessed by the percentage of living cells.

Cytotoxic effect of cetuximab in combination with SN38 in CRC cell lines. The cytotoxic effects of cetuximab in combination with SN38 were examined. CRC cells were treated with serial concentrations of SN38 (from 0.01 to 30 μg/ml) in the presence or absence of cetuximab. Combined drug effects were evaluated in the four CRC cell lines. Fig. 4 shows representative effects of the cetuximab and SN38 combination. Treatment of Caco2 cells with 10 μg/ml cetuximab plus various concentrations of SN38 enhanced the cytotoxic effects significantly, depending on the SN38 concentration, compared with treatment with SN38 alone (Fig. 4A; P=0.04). However, there was no significant combination effect in the three other cell lines (Fig. 4B-D). The addition of cetuximab (at a concentration causing ~20% inhibition) reduced the IC_{50} value of SN38 from 0.52 to 0.31 μg/ml (40% reduction) in Caco2. These results suggest a synergistic effect of cetuximab and SN38 in Caco2 cells.

HSP27 downregulation by exposure to AG490. Western blot analyses showed a concentration-dependent decrease in the levels of STAT3 and HSP27 at 24 h after AG490 (JAK2 inhibitor) exposure in Caco2, SW480 and WiDR cells. STAT3 and HSP27 were almost undetectable with 160 μM AG490 in these three cell lines. However, no significant decrease in the levels of STAT3 or HSP27 was observed in SW620 cells (Fig. 5).

Cytotoxic effect of AG490 in combination with SN38 in Caco2 cells. The cytotoxic effects of AG490, in combination with SN38, were examined. Cell proliferation was assessed 48 h after exposure to AG490 alone using the MTT assay. Fig. 6A shows that the proliferation of Caco2 cells was suppressed based upon the AG490 concentration. Caco2 cells were treated with serial concentrations of SN38 (from 0.3 to 3.0 μg/ml) in the presence or absence of a fixed AG490 concentration (40 μM) for 48 h. Fig. 6B shows the representative effects of the AG490 and SN38 combination. Treatment of Caco2 cells with 40 μM AG490 plus the various concentrations of SN38 enhanced the cytotoxic effects comparable to that the effect with cetuximab plus SN38; however, the result was not significantly better than that obtained with treatment with SN38 alone (Fig. 6B; P=0.11).

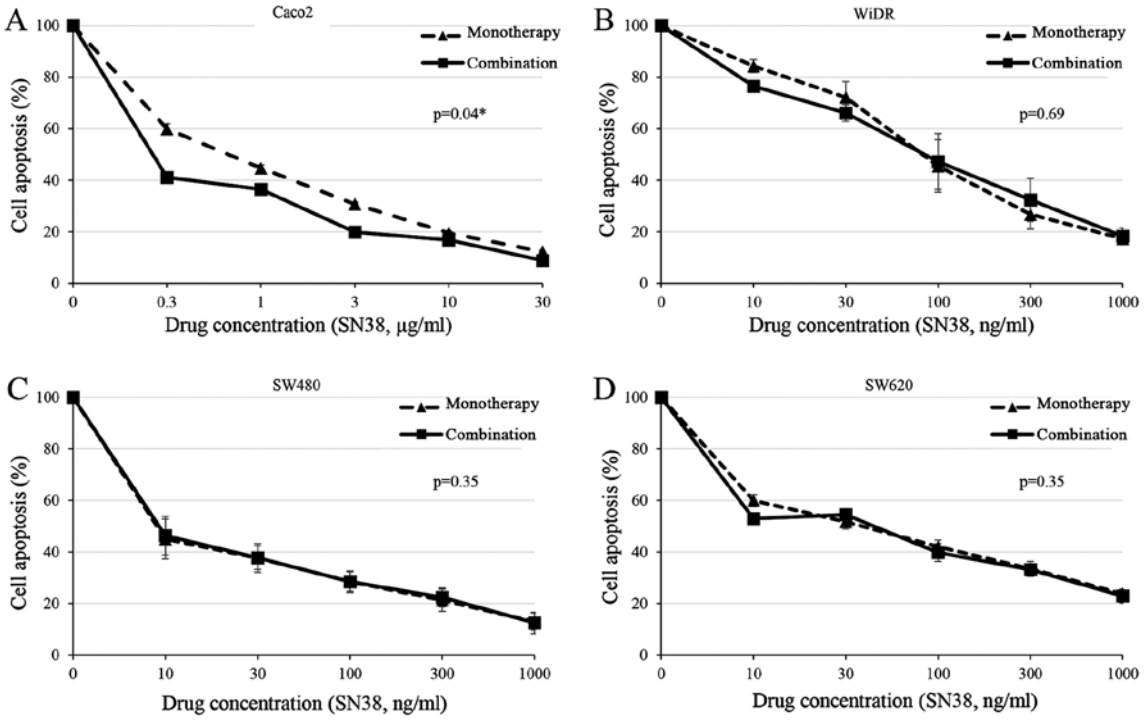

Figure 4. Combined effects of cetuximab and SN38 in CRC cell lines. (A) Caco2, (B) WiDR, (C) SW480 and (D) SW620 were incubated with various concentrations of SN38 in the presence or absence of 10 μg/ml cetuximab for 48 h. Cell viability was then determined with the MTT assay. Data are expressed as percent change (means ± SD) vs. the control. *P<0.05, vs. SN38 alone-treated cells.

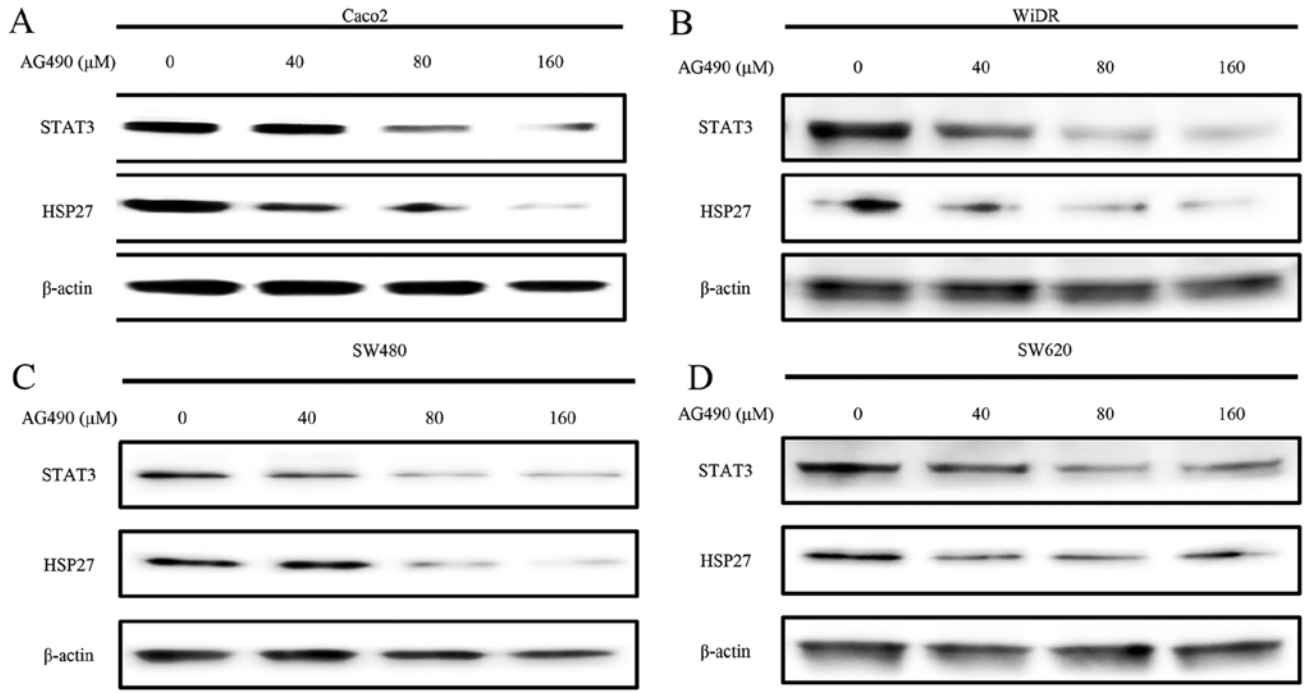

Figure 5. HSP27 downregulation by exposure to AG490. Western blot analysis of (A) Caco2, (B) WiDR, (C) SW480 and (D) SW620 cells treated with various concentrations of AG490 for 24 h. AG490 induced concentration-dependent decrease in HSP27 and STAT3 protein levels, respectively.

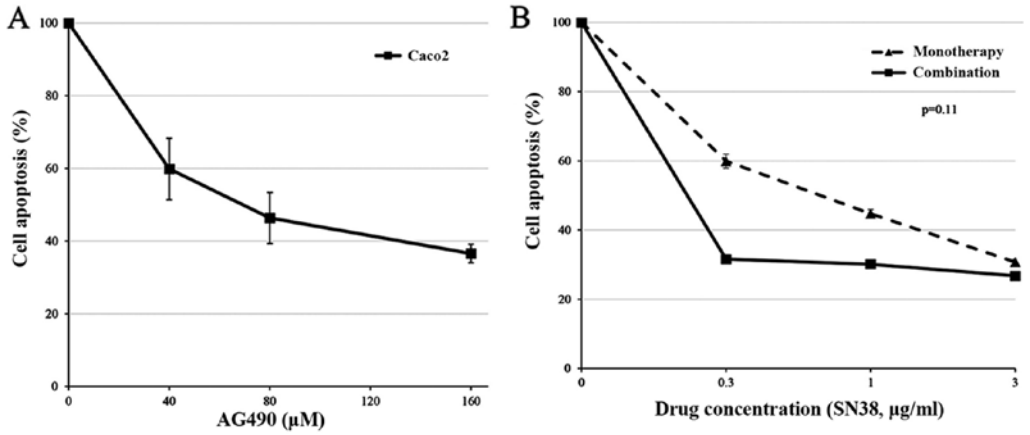

Figure 6. Cytotoxic effect of AG490 alone (A) and the combined effect of AG490 and SN38 (B) for 48 h in Caco2 cells. (A) AG490 induced a concentration-dependent decrease in proliferation. (B) Caco2 cells were incubated with various concentrations of SN38 in the presence or absence of 40 μM AG490 for 48 h. Data are expressed as percent change (means ± SD) vs. the control. P-value vs. SN38 alone-treated cells.

Discussion

Cetuximab is a human-mouse chimeric immunoglobulin (IgG1) monoclonal antibody for EGFR that is approved for use in patients with mCRC and an absence of mutations in *KRAS*, *NRAS* and *BRAF* (4-7). Some studies have shown synergistic effects of cetuximab and CPT-11 in wild-type *RAS* and *BRAF* mCRC patients (4,8,9). It is believed that cetuximab may enhance the tumor response to CPT-11 and its active metabolite, SN38, in addition to its 'original' antitumor effects. However, the molecular mechanism of this synergistic effect has not yet been fully investigated (10-12).

HSP27 is known to be a stress-activated, adenosine triphosphate-independent cytoprotective chaperone with many notable properties, including resistance to chemotherapy in several cancers. In CRC cells, HSP27 has been reported to be a resistance factor for CPT-11, as well as a prognostic factor (24-27).

The JAK/STAT signaling pathway, and especially the transcription factor STAT3, have been implicated in the regulation of drug sensitivity (28,29). In CRC cells, activated STAT3 causes resistance to CPT-11 and inhibition of STAT3 strongly enhances the cytotoxic action of CPT-11 (30,31). STAT3 activation in CRC patients is associated with adverse clinical outcomes, consistent with its potential roles as a prognostic biomarker and a chemoprevention and/or therapeutic target (32). Furthermore, STAT3 has been reported to regulate HSP27 in breast epithelial cells (33). Thus, we considered that cetuximab might promote sensitivity to CPT-11 and its active metabolite,

SN38, via suppression of the HSP27 through blocking the JAK/STAT signaling pathway.

Consistent with previous reports, HSP27 protein levels and SN38 sensitivity had a tendency to be correlated in the CRC cell lines (Fig. 1C; R=0.841, P=0.159) (24). Exposure to 10 μg/ml cetuximab and various concentrations of AG490, an inhibitor of *JAK2*, showed suppression of STAT3 and HSP27 protein levels except in the *KRAS* G12V mutant cell line, SW620. Synergistic effects of cetuximab in combination with SN38 have been confirmed only in *RAS* wild-type and *BRAF* wild-type cells (Caco2), and not in the three other *RAS*- or *BRAF*-mutated cells. Furthermore, the combined effects of AG490 plus SN38 exhibited a similar tendency to that of cetuximab plus SN38, but these results were not significant in Caco2 cells. These results indicate that cetuximab may promote sensitivity to SN38 via suppression of HSP27, through blocking of the JAK/STAT signaling pathway in Caco2 cells. A synergistic effect was only seen at lower concentrations of SN38. The reason is uncertain; however, higher concentrations of SN38 have a strong cytotoxicity as a single agent in Caco2 cells. Therefore, the synergism with cetuximab may be masked by the strong effect of SN38.

The present study revealed two novel and important findings. First, cetuximab may cause suppression of HSP27 through blocking the JAK/STAT signaling pathway in CRC cells, because AG490 caused a concentration-dependent decrease in the level of HSP27, similar to cetuximab. This suggests that HSP27 may be a downstream mediator of the JAK/STAT signaling pathway in CRC cells. Second, suppression of HSP27 caused by cetuximab was observed even in *RAS*- or *BRAF*-mutated cells (WiDR and SW480). Based on this result, it is expected that cetuximab promotes sensitivity to SN38, even in *RAS*- or *BRAF*-mutated cells. However, no combination effect of cetuximab and SN38 was observed in RAS- or BRAF-mutated cells. The reason for this may be that suppression of HSP27 alone, through blocking of the JAK/STAT signaling pathway, did not overcome the power of the RAS/RAF signaling pathway (also known as the MAPK pathway), accelerated by the presence of *RAS* or *BRAF* mutations. To the best of our knowledge, there are no reports focused on the power balance of the RAS/RAF and JAK/STAT signaling pathway in CRC. We speculate that the RAS/RAF signaling pathway has a stronger potential than the JAK/STAT signaling pathway because the RAS mutation is the only proven predictive marker for anti-EGFR antibody treatments. There are no reports on similar mutations in the JAK/STAT signaling pathway in CRC (5-7,36). Furthermore, no suppression of HSP27 and STAT3 by cetuximab or AG490 was observed in SW620 cells with the *KRAS* G12V mutation. The reason for this is uncertain; however, a previous study reported that mutant *KRAS* could activate STAT3 in SW620 cells (37). Thus, SW620 may be a unique CRC cell line that does not respond to stimulation by cetuximab or AG490 in the JAK/STAT signaling pathway.

We acknowledge several limitations of the present study. First, there is the absence of *in vivo* data. Secondly, we used only wild-type CRC cell lines and not CPT-11-refractory CRC cell lines. However, we used four CRC cell lines with different RAS and BRAF mutational statuses, which could affect the combination effect with cetuximab and SN38. Therefore, we believe that our data is clinically relevant. Further research is required to strengthen our hypothesis. Third, the changes in the protein levels of HSP27 and STAT3 by exposure to cetuximab or AG490 suggest that phosphorylation of HSP27 and STAT3 are also likely to gather a positive response. However, after 48 h of exposure to cetuximab, no changes in the phosphorylation of HSP27 and STAT3 were observed (data not shown). The reason could be that peak STAT3 phosphorylation occurs within 15-60 min in response to various stimuli, and even in the presence of a continuous cytokine, STAT3 activation decreases over several hours (38). Furthermore, HSP27 phosphorylation does not last more than several hours (39). Therefore, suppression of phosphorylated HSP27 and STAT3 was not confirmed after a 48-h exposure to cetuximab. Molecular mechanisms of the synergism between cetuximab and SN38 may be related not only to HSP27 suppression but also other factors, such as immunological effects. Recently, STAT3 inhibition can mediate anticancer effects by multiple mechanisms, including cell-autonomous effects and immunological effects (40). Cetuximab, when used in combination with FOLFIRI [one of standard chemotherapy regimens for mCRC that does no induce immunogenic cell death (ICD)], induces ICD *in vitro* and *in vivo* (41). Therefore, the molecular mechanisms of the synergism between cetuximab and SN38 potentially involve multiple mechanisms, including ICD induced by cetuximab via STAT3 inhibition, in addition to our hypothesis. A better understanding of these mechanisms will lead to novel treatments for CRC.

In conclusion, cetuximab may promote sensitivity to SN38 via suppression of HSP27 through blocking the JAK/STAT signaling pathway in RAS wild-type CRC cells.

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