ANTICANCER RESEARCH *41*: 4907-4916 (2021)
doi:10.21873/anticanres.15304

Antibody to Interleukin-6 Receptor Inhibits *In Vivo* Growth of Human Colorectal Carcinoma Cell Xenografts

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Abstract. *Background: Interleukin-6 receptor antibody (IL6R) inhibits colony formation and invasion by colorectal carcinoma (CRC) in vitro. We examined the effect of IL6R antibody on tumor growth of CRC xenografts in vivo. Materials and Methods: SW480 cells inoculated subcutaneously into NU/NU mice were treated with anti-IL6R and tumor histology and growth-related signaling were subsequently estimated by hematoxylin and eosin and immunohistochemical staining. Results: Tumor growth was inhibited by anti-IL6R treatment at dosages of both 0.1 and 1.0 mg/kg. Tumor cells had invaded into surrounding tissues in untreated mice, while there was no invasion of tumors in the IL6R antibody-treated mice. The expression of Ki-67, signal transducer and activator of transcription protein 3 (STAT3) and phosphor-extracellular signal-regulated kinase 1 and 2 (ERK1/2) were suppressed in anti-IL6R-treated tumors. Conclusion: IL6R antibody inhibited tumor growth and invasiveness in vivo by suppressing the expression of Ki-67, STAT3 and phosphor-ERK1/2. The results imply that the anti-IL6R may be a promising targeted drug for CRC.*

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Key Words: Anti-interleukin-6 receptor, colorectal carcinoma, xenograft, tumor growth.

Interleukin-6 (IL6) is a pleiotropic cytokine secreted by immune cells and many tumor cells as an autocrine growth factor (1-5). Clinical studies by our research team and others have shown serum and tumor tissue IL6 concentrations to be closely associated with disease status, metastasis and patient prognosis in colorectal cancer (CRC) (6-13). Recent studies further revealed that the IL6 expression in a tumor and its microenvironment were strongly associated with resistance of CRC to chemotherapy and immunotherapy (14, 15). IL6 signaling has been implicated as a possible target for treating CRC (16). Some pilot studies have been performed in the 21st century using peptides or antibodies to interfere with the action of IL6 and its receptor (17, 18). A study performed by our research team showed that IL6 stimulated SW480 cells to form more colonies in soft agar than the untreated control, and this increase in colonies was compromised by the addition of IL6 receptor (IL6R) antibody (19). Subsequently, another report indicated that IL6 regulates the Janus kinase (JAK)–signal transducer and activator of transcription (STAT3), phosphatidylinositol 3-kinase (PI3K)–protein kinase B (AKT) and MAPK/ERK kinase (MEK)–extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathways and promoted malignant behavior, including anchorage-independent growth and invasiveness of SW480 CRC cells. The addition of anti-IL6R was sufficient to reverse the effect of IL6, which suggested that anti-IL6R may be a potential agent for the suppression of CRC progression (20). However, there is no evidence to demonstrate whether anti-IL6R can inhibit tumor growth and suppress the invasiveness of CRC *in vivo*. In this study, we used tumor xenografts of SW480 CRC cells in NU/NU mice

to assess the effects of anti-IL6R on tumor growth and invasiveness *in vivo*, and possible mechanisms involved.

Materials and Methods

Materials. Leibovitz 15 medium (L-15), fetal bovine serum, L-glutamine, trypsin and antibiotics were purchased from Gibco Ltd. (Paisley, UK). Tween-20 and matrix gel were from Sigma (St. Louis, MO, USA). Mouse monoclonal anti-ERK1/2 and rabbit anti-phospho-ERK1/2 (The202/Tyr204) antibodies were from Zymed (San Francisco, CA, USA). Sheep polyclonal anti-STAT3 and anti-phosphor-STAT3 (S727) antibodies were from R&D Systems (Minneapolis, MN, USA). Rabbit polyclonal anti-Ki-67 was from Abcam (Cambridge, MA, USA). Anti-mouse and anti-rabbit secondary antibodies conjugated with poly-peroxidase and the chromogen 3,3'-diaminobenzidine were from GeneTex (Taipei, Taiwan, ROC). Colon carcinoma cell line SW480 was purchased from the Bioresource Collection and Research Center, Taiwan, and cultured in 90% L-15 supplemented with 10% heat-inactivated fetal bovine serum, 25 U/ml penicillin and 25 μg/ml streptomycin at 37˚C in room air and a water-saturated atmosphere. All experiments were carried out on cell lines passaged 5-20 times.

Animal study. The protocol of our animal study complied with the Guide for the Care and Use of Laboratory Animals (8th Edition) (21), and was reviewed and approved by the Institutional Animal Care and Use Committee of China Medical University, Taichung, Taiwan (No. 2016-371). Male nude mice (6- to 8-week-old) (strain NU/NU) purchased from BioLASCO Taiwan Co., Ltd., Taiwan, ROC, were housed under specific pathogen-free conditions. SW480 cells were washed with phosphate-buffered saline (PBS) 2-3 times and then suspended in PBS at a density of 5×10^7 cells/ml. Each nude mouse was inoculated subcutaneously with 0.1 ml of suspended cells using a 1-ml syringe and a 27-gauge needle. After the tumors had reached approximately 50 mm^3 in size, the mice were divided into three groups, each containing five animals, and underwent peritoneal injection with 0.1 mg/kg or 1.0 mg/kg IL6R antibody (Tocilizumab; F. Hoffmann-La Roche Ltd., Basel, Switzerland) in 0.1 ml PBS twice a week. The untreated group were injected with an equal volume of PBS as a replacement for the IL6R antibody. The body weight of the nude mice and tumor length, width and height were measured once a week, and the tumor volume was calculated (V=length × width × height/2). After 3 weeks, the mice were sacrificed, and the tumors and other organs such as the liver, lungs, spleen, heart and kidneys were removed surgically and fixed in 3% formalin/PBS.

Tissue processing and hematoxylin and eosin (H&E) staining. The formalin-fixed tissues were placed in plastic tissue support racks and then moved into an automatic dehydration machine, with sequential immersing of tissues at 2-hour intervals in 50%, 70%, 85%, 95% and absolute ethanol, and then xylene. The dehydrated tissues were embedded in molten paraffin wax. The paraffin-embedded tissues were then sliced with a microtome (2-3-μm-thick) (Leica RM2125 RTS; Leica Microsystems Inc., Buffalo Grove, IL, USA) and the slices were fixed on glass slides by heating at 60˚C overnight. For H&E staining, the slices were placed in an oven at 65˚C oven for at least 30 minutes to remove the embedded wax, and the residual paraffin was then washed with xylene. After the hydration process (100% alcohol, 95% alcohol, 80% alcohol, water

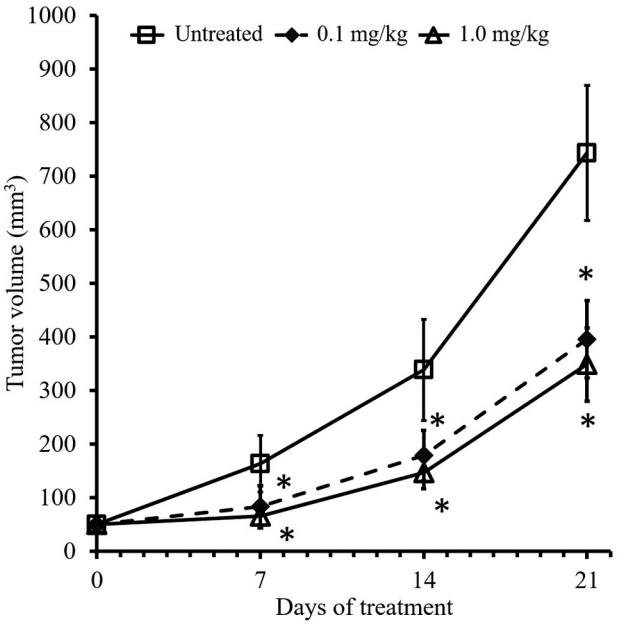

Figure 1. *Anti-interleukin-6 receptor (IL6R) inhibited growth of colorectal cancer xenografts. SW480 cells (5×10^6) were inoculated subcutaneously into NU/NU mice and anti-IL6R was administered by peritoneal injection at the indicated concentration twice a week. Tumor volume was measured once a week, as described in the Materials and Methods. Data of each group were averaged from tumor volumes of five mice and are presented as means±SEM. *Significantly different at $p<0.05$.*

washing), the slices were immersed in Mayer hematoxylin liquid for 3-5 minutes. They were then rinsed with tap water for 5-10 minutes, and stained with 1% eosin for 1-2 min. The stained slices were washed and then underwent the dehydration process as described above, and were subsequently mounted with mounting glue. The stained slices were scanned using a digital pathology scanner (MoticEasyScan; Motic Inc., Xiamen, Fujian, PR China) using 40× standard mode, and the digital microscopy images were viewed on a computer using the image program DSAssistant (Motic Inc.).

Immunohistochemical staining. The paraffin-embedded tumor slices were first de-waxed in an oven at 65˚C for at least 30 min and then soaked in xylene for 10 min to remove residual paraffin. The de-waxed slices were rehydrated by immersion in a solution series as follows: Absolute alcohol, 95% alcohol and 80% alcohol, and finally immersed in Tris-buffered saline for 10 minutes to complete hydration. The hydrated slices were subjected to antigen retrieval in 1 mM citrate buffer, being heated to 95˚C in water for 40 min. Endogenous peroxidases in the slices were further de-activated with 3% H_2O_2, followed by incubation with anti-Ki-67 (growth indicator), ERK1/2, phosphorylated ERK1/2, STAT3, phosphorylated STAT3 (IL6 signaling pathway) antibodies at the recommended dilutions. The slices were washed three times with Tris-buffered saline and the location and intensity of the antigens were visualized using the labeled streptavidin–biotin staining system. The slices were stained with hematoxylin as the counterstain, following which the stained slices were dehydrated using the process described above and images were obtained using a digital

Table I. *Body and organ weights (g) of interleukin-6 receptor (IL6R) antibody-treated nude mice. Data of each group were averaged from five mice and are presented as the means±SEM.*

	Body weight	Heart	Liver	Spleen	Lung	Kidney
Control	27.92±1.69	0.18±0.04	1.57±0.12	0.18±0.09	0.26±0.03	0.64±0.05
0.1 mg/kg anti-IL6R	26.03±1.34	0.20±0.05	1.72±0.42	0.16±0.06	0.25±0.03	0.60±0.05
1.0 mg/kg anti-IL6R	26.11±2.94	0.16±0.05	1.63±0.18	0.17±0.04	0.24±0.02	0.58±0.10

pathology scanner. The immunological activities of ERK1/2, phosphorylated ERK1/2, STAT3 and phosphorylated STAT3 were calculated by semi-quantitative analysis as follows. The percentage score was divided into five levels: Level 1: fewer than 10% of cells were stained, level 2: 11-25% of cells were stained, level 3: 26-50% of cells were stained, level 4: 51-75% of cells were stained, and level 5: greater than 75% of cells were stained. Intensity scores were defined as follows: 0: no staining, 1: light brown, 2: medium brown, 3: dark brown. The overall score was obtained by multiplication of the percentage score by the intensity score. The Ki-67 percentage score was defined as the percentage of positively-stained tumor cells among the total number of malignant cells assessed (22).

Statistical analysis. Data were analyzed by the Kruskal–Wallis test, which is a nonparametric test, to evaluate significant differences between the control group and treatment groups. When significant differences existed, a multiple comparison test was then used to identify which groups were different. Differences among group means were considered significant at $p<0.05$.

Results

Anti-IL6R inhibited tumor growth in vivo. The tumor size was increased from a volume of 50 mm^3 to approximately 700 mm^3 after 21 days in the untreated group. In contrast to the untreated group, growth appeared very slow in the groups treated with either 0.1 mg/kg or 1.0 mg/kg anti-IL6R during the first 7 days. The tumor volume was increased 7 days later, but was lower than 400 mm^3 in the 0.1 mg/kg group and 300 mm^3 in the 1.0 mg/kg group at the end of the experiment (Figure 1). Although the average tumor volume in the 0.1 mg/kg group was higher than that in the 1.0 mg/kg group, the difference between the groups was not significant. The tumor volume of the untreated group was significantly larger than both treatment groups at all measurements. After sacrifice, the whole bodies and organs of the mice were weighed in order to evaluate the toxicity of the treatment. As shown in Table I, the weights of the body, heart, lungs, liver, kidneys and spleen were similar in the untreated and treated mice. The organs were sliced and stained with H&E. There were no differences in organ morphology between the treatment groups and the untreated control group according to microscopy examination (data not shown).

Table II. *Verification of results regarding differences in protein expressions between untreated controls and treatment groups.*

Variable	Chi-square	Degrees of freedom	Asymptotic difference (*p*-value)
Ki-67	21.601	2	**<0.001**
Phosphor-ERK1/2	6.309	2	**0.043**
STAT3	21.674	2	**<0.001**

ERK1/2: Extracellular signal-regulated kinase 1 and 2; STAT3: signal transducer and activator of transcription 3. Statistically significant *p*-values are shown in bold.

Anti-IL6R suppressed tumor invasiveness. Tumors from the nude mice were sliced and stained with H&E to evaluate the morphological features. Most of the tumors were surrounded by fibrous tissue, which formed an obvious divide between the tumor cells and normal tissues such as skin and fat. However, the tumors of the untreated group showed an unclear border near the abdominal cavity, with invasion into the surrounding tissues, and even into muscle (Figure 2A). In contrast to the untreated group, it was difficult to find any tumor cells invading or infiltrating into the surrounding tissues in the tumors of both the 0.1 mg/kg and 1.0 mg/kg IL6R antibody-treated mice (Figure 2B and C).

Anti-IL6R inhibited tumor cell proliferation. To evaluate the proliferative potential of the tumor cells, Ki-67 was used to stain tumor sections for immunohistochemical evaluation. As shown in Figure 3A, the Ki-67-positive cells were dark brown to light brown, and approximately one-third of the cells in the control group tumor showed moderate to strong staining. The fraction of positive cells decreased to approximately 25% in tumors from 0.1 mg/kg anti-IL6R-treated mice, which showed brown to light brown staining (Figure 3B). There were only a few positive cells found in tumors from mice treated with 1.0 mg/kg anti-IL6R, represented by a light brown color (Figure 3C). Scoring of the Ki-67-positive cells by immunostaining in the control group tumor cells was 34.92%, whereas it was 19.07% in 0.1 mg/kg-treated tumor cells and 13.56% in 1.0 mg/kg-treated tumor cells (Figure 3D). The differences

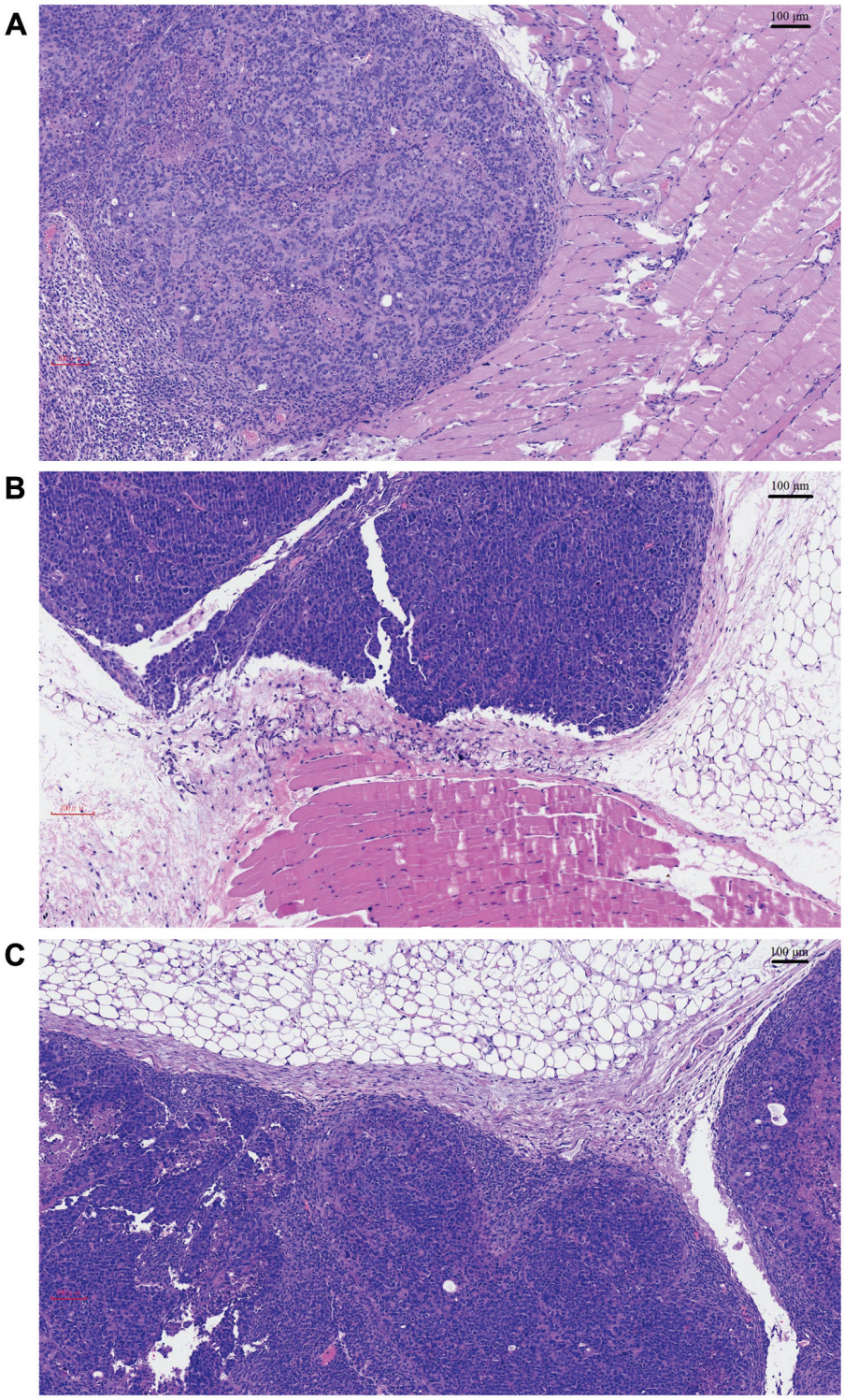

Figure 2. *Hematoxylin and eosin (H&E) staining of tumor sections for morphological examination of colorectal cancer xenografts. At the end of treatment, tumors from sacrificed nude mice were resected, fixed in paraformaldehyde, dehydrated with ethanol and xylene, then embedded in paraffin, followed by slicing into 3-μm-thick slices. The tumor slices were then stained with H&E and the images scanned using a microscanner, as described in the Materials and Methods. Representative images showing the border between tumor and normal tissues were captured. The borders of tumors from untreated control mice were not smooth, and some tumor mass had invaded into the muscle layer of the mouse abdomen (A). Borders of tumors from mice treated with 0.1 mg/kg (B) and 1.0 mg/kg (C) anti-interleukin-6 receptor were surrounded by fibrotic tissues.*

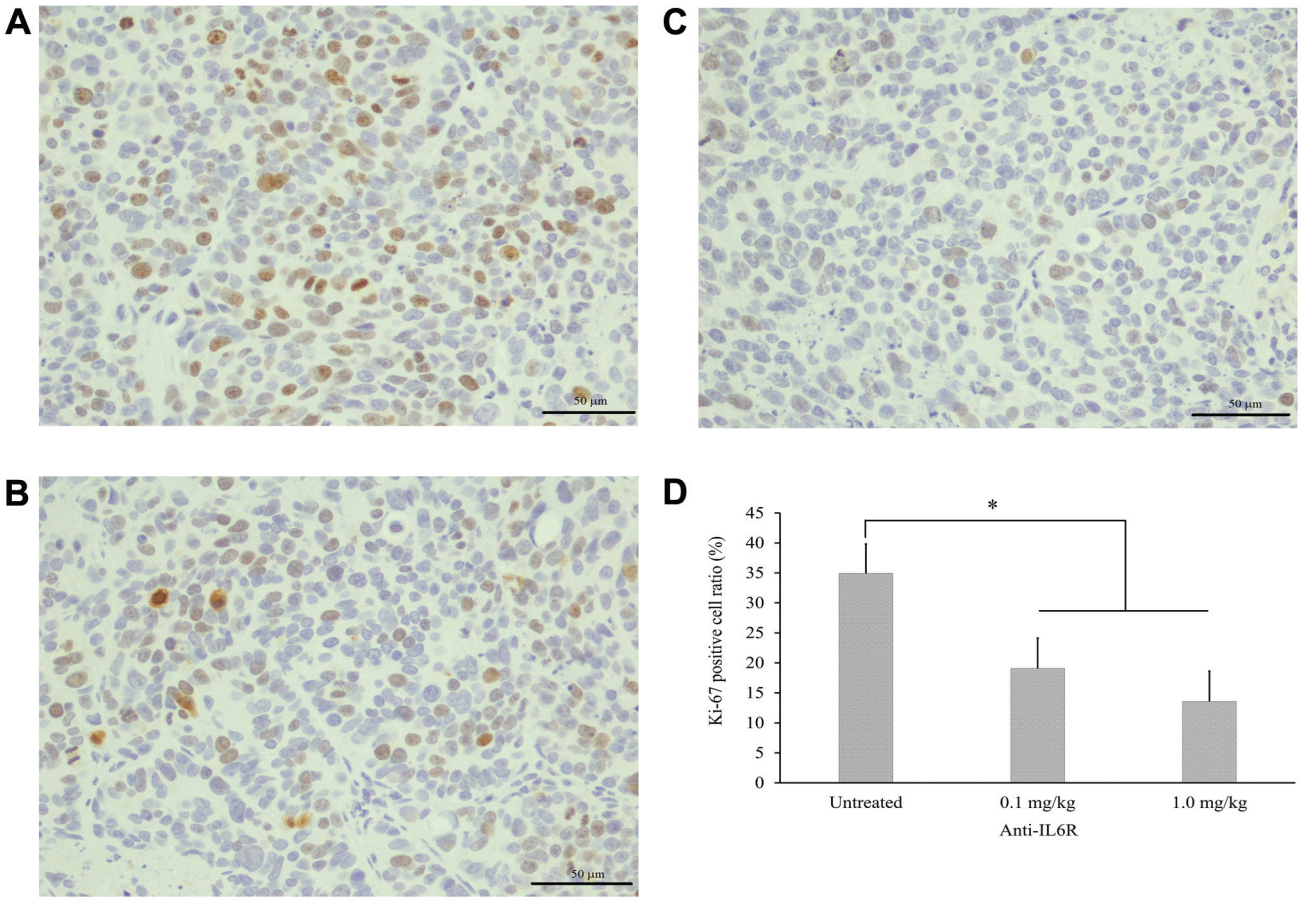

Figure 3. *Anti-interleukin-6 receptor (IL6R) inhibited Ki-67 expression in colorectal cancer xenografts. Tumor slices were stained with anti-Ki-67 for immunohistochemical evaluation and the images were scanned using a microscanner, as described in Materials and Methods. Representative images were captured of tumor tissues from the untreated control (A), and mice treated with 0.1 mg/kg (B) and 1.0 mg/kg (C) anti-IL6R. The Ki-67-positive cells were counted and calculated as a percentage of the total tumor cells in the tumor area of the image (D). *Significantly different at p<0.05 between the control and treatment groups.*

between the control and treatment groups were calculated using the Kruskal–Wallis test. As shown in Table II, the *p*-value of the Kruskal–Wallis test was lower than 0.05, showing that at least one median value differed from the others. Multiple comparisons were then performed in order to determine where differences lay between groups. As shown in Table III, Ki-67 positivity was significantly lower in both treatment groups than the control group, with an adjusted significance lower than 0.05 (Table III). However, there were no significant differences between the 0.1 mg/kg- and 1 mg/kg-treated groups. These results revealed that IL6R antibody treatment inhibited the proliferation of CRC cells *in vivo*.

Anti-IL6R suppressed growth signaling. Tumor sections from xenografts were subjected to immunochemical staining with antibodies for STAT3, ERK1/2 and phosphor-ERK1/2. STAT3 staining was almost wholly positive in the tumor cells of the control group (Figure 4A), and dose-dependently decreased in the 0.1 mg/kg- and 1.0 mg/kg-treated groups (Figure 4B and C). Semi-quantitative analysis showed that the score for STAT3 expression of control-group tumor cells was 36.00 compared with 22.00 in tumors from 0.1 mg/kg-treated and 13.33 in 1.0 mg/kg-treated mice (Figure 4D). The results of the Kruskal–Wallis test showed that there were statistically significant differences in the median STAT3 value between the control group and treatment groups (Table II). Multiple comparisons of these groups were performed, as presented in Table IV, and showed STAT3 expression in both treatment groups was significantly lower than that of the control group, while there was no significant difference between the 0.1 mg/kg- and 1 mg/kg-treated groups.

Strong staining of ERK1/2 was observed in all tested tumor sections, and there were no differences between the untreated control and treatment groups (data not shown). However,

Table III. *Multiple comparisons to examine significant differences in tumor Ki-67 expression level between treatment and control groups.*

Sample 1 *vs.* 2	Test statistic	Standard error	Standard verification statistics	*p*-Value	Adjusted *p*-value
1.0 *vs.* 0.1 mg/kg anti-IL6R	5.509	4.489	1.227	0.220	0.659
1.0 mg/kg anti-IL6R *vs.* control	21.354	4.685	4.558	**<0.001**	**<0.001**
0.1 mg/kg anti-IL6R *vs.* control	15.8845	4.826	3.283	**0.001**	**0.003**

Statistically significant *p*-values are shown in bold.

Table IV. *Multiple comparisons to examine significant differences in the level of tumor expression of signal transducer and activator of transcription (STAT3) between treatment and control groups.*

Sample 1 *vs.* 2	Test statistic	Standard error	Standard verification statistics	*p*-Value	Adjusted *p*-value
1.0 *vs.* 0.1 mg/kg anti-IL6R	8.667	4.928	1.759	0.079	0.236
1.0 mg/kg anti-IL6R *vs.* control	22.667	4.928	4.600	**<0.001**	**<0.001**
0.1 mg/kg anti-IL6R *vs.* control	14.000	4.848	2.888	**0.004**	**0.012**

Statistically significant p-values are shown in bold.

Table V. *Multiple comparisons to examine significant differences in the level of tumor expression of phosphor- extracellular signal-regulated kinase 1 and 2 (ERK1/2) between treatment and control groups.*

Sample 1 *vs.* 2	Test statistic	Standard error	Standard verification statistics	*p*-Value	Adjusted *p*-value
1.0 *vs.* 0.1 mg/kg anti-IL6R	3.625	4.845	0.748	0.454	>0.99
1.0 mg/kg anti-IL6R *vs.* control	12.094	4.925	2.456	**0.014**	**0.042**
0.1 mg/kg anti-IL6R *vs.* control	8.469	4.925	1.720	0.086	0.257

Statistically significant *p*-values are shown in bold.

phosphor-ERK1/2 staining of the tumor cells gradually decreased from the untreated control (Figure 5A) to the groups treated with 0.1 mg/kg (Figure 5B) and 1.0 mg/kg (Figure 5C) anti-IL6R. Semi-quantitative analysis showed that the phosphor-ERK1/2 expression of the control group tumor cells was 31.00, whereas it was 22.53 and 18.91 in tumors from the 0.1 mg/kg-treated and 1.0 mg/kg-treated mice (Figure 5D). The results of the Kruskal–Wallis test showed there were statistically significant differences in the median phosphor-ERK1/2 expression score between the control group and treatment groups, with $p=0.043$ (Table II). Multiple comparisons of these groups were performed, as shown in Table V, and phosphor-ERK1/2 expression of 1.0 mg/kg-treated group was significantly lower than that of the control group, while no other significant differences were observed.

Discussion

Previous studies revealed that IL6/IL6R-glycoprotein 130 inflammation signaling plays a crucial rule in the promotion of carcinogenesis in CRC with IL6R overexpression (23, 24). Treatment of IL6R-overexpressing CRC cells *in vitro* using the anti-IL6R was capable of inhibiting clonogenic growth in soft agar and invasion by disruption of MEK/ERK1/2, JAK/STAT3 and PI3K/AKT phosphorylation/activation (19, 20). In the present study, we examined the efficacy of IL6R antibody in suppressing tumor growth *in vivo*. The results demonstrated that the antibody effectively inhibited tumor growth at concentrations of 0.1 mg/kg and 1.0 mg/kg in a mouse study of SW480 cells. Both proliferation of and invasion by tumor cells were suppressed. The expression of proliferation-associated protein Ki-67 was reduced, indicating that suppression of tumor growth in the anti-IL6R-treated groups may have been due to inhibition of tumor cell proliferation. These effects are purportedly regulated by IL6/IL6R signaling, such as STAT3 and ERK1/2. The results clearly suggest that use of the anti-IL6R might be a potential treatment strategy for IL6R-expressing CRC.

In our previous study, activation of IL-6/IL6R signaling in SW480 CRC cells triggered the JAK, MEK or PI3K pathways, by which up-regulation of their effector proteins such as STAT3, AKT and ERK1/2 was observed. This activation was

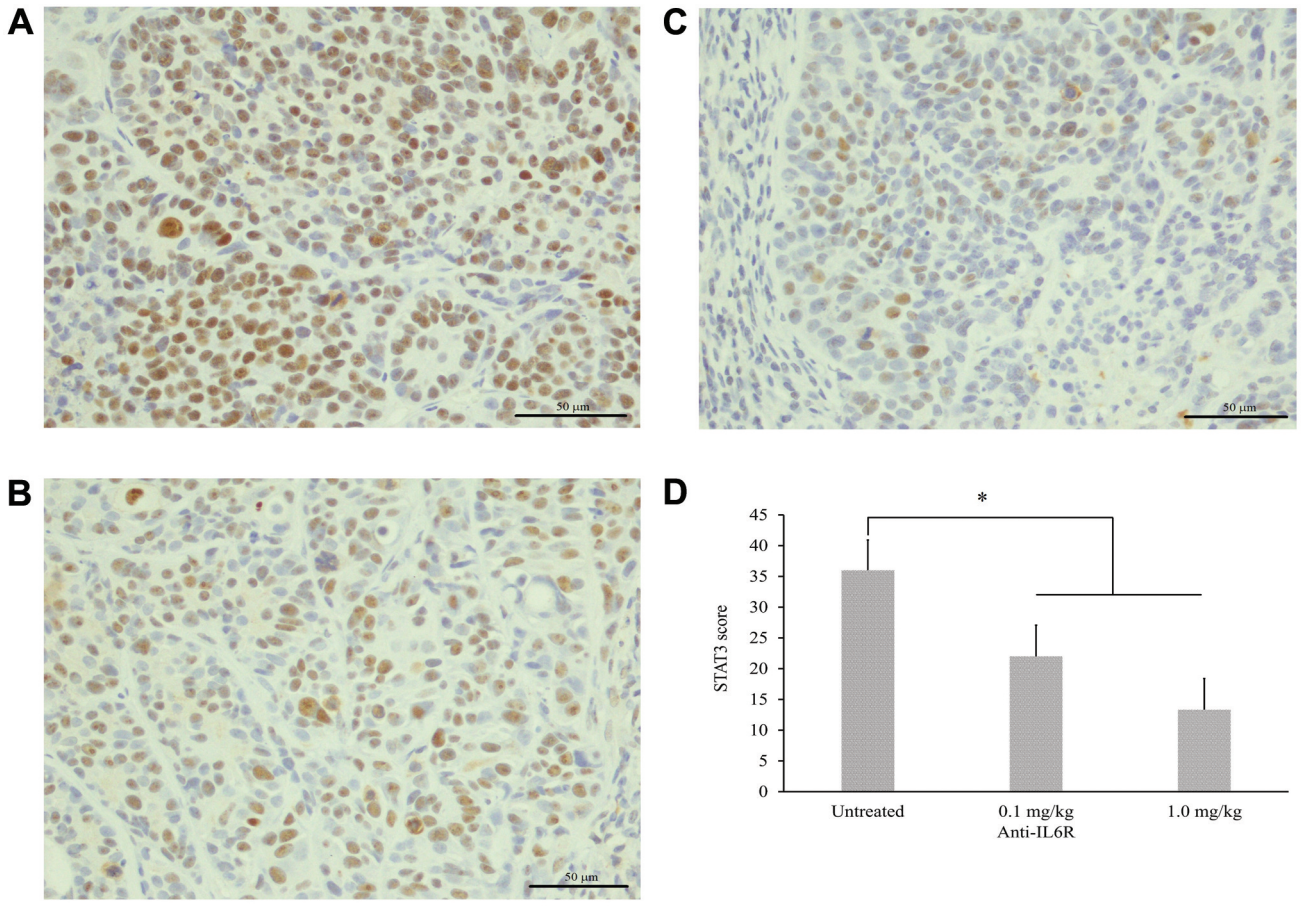

Figure 4. *Anti-interleukin-6 receptor (IL6R) inhibits expression of signal transducer and activator of transcription 3 (STAT3) in colorectal cancer xenografts. Tumor slices were stained with anti-STAT3 for immunohistochemical evaluation and the images were scanned using a microscanner, as described in the Materials and Methods. Representative images were captured of tumor tissues from the untreated control (A), mice treated with 0.1 mg/kg (B) and 1.0 mg/kg (C) IL6R. The expression of STAT3 is represented as the average of scores by calculating the percentage and intensity of 10 different areas of a 400× image for each tumor image examined (D). *Significantly different at $p<0.05$ between the control and treatment groups.*

strongly associated with anchorage-independent clonogenicity and invasiveness of SW480 cells, and was reversed by treatment with anti-IL6R (19, 20). These *in vitro* results were also partially apparent in the xenograft tumors of SW480 cells treated with IL6R antibody. Some similar phenomena were observed, including tumor growth inhibition with suppressed Ki-67 expression, apparently diminished invasive ability and reduced phosphorylation of ERK1/2 in the tumors of treated mice. The results presented here indicate that the IL6R antibody down-regulated the phosphorylation/activation of ERK1/2 and appeared to concomitantly suppress tumor cell invasion *in vivo*, which confirmed the findings of the previous *in vitro* study [20].

As demonstrated in a previous study, IL6/IL6R interaction induces dimerization of glycoprotein 130 chains, resulting in phosphorylation/activation of the associated JAK/STAT3 pathway and stimulation of tumor growth and other malignant changes of phenotype (23). Our previous study also demonstrated that anti-IL6R treatment of SW480 cells inhibited STAT3 phosphorylation *in vitro* (20). However, the results of the current study demonstrated suppression of the protein expression of STAT3 in xenografts of SW480 cells *in vivo*, which was inconsistent with the previous study. The reason for the suppression of the STAT3 protein expression in anti-IL6R-treated xenografts is unclear. The effect of IL6R antibody on the JAK/STAT3 pathway, not only in blockage of phosphorylation/activation, but also in suppression of the protein expression of STAT3, is a novel finding. This dual function of IL6R antibody on the JAK/STAT3 pathway is an intriguing issue for further investigation.

IL6 has been found to be involved in cancer development, epithelial to mesenchymal transition, invasiveness, metastasis,

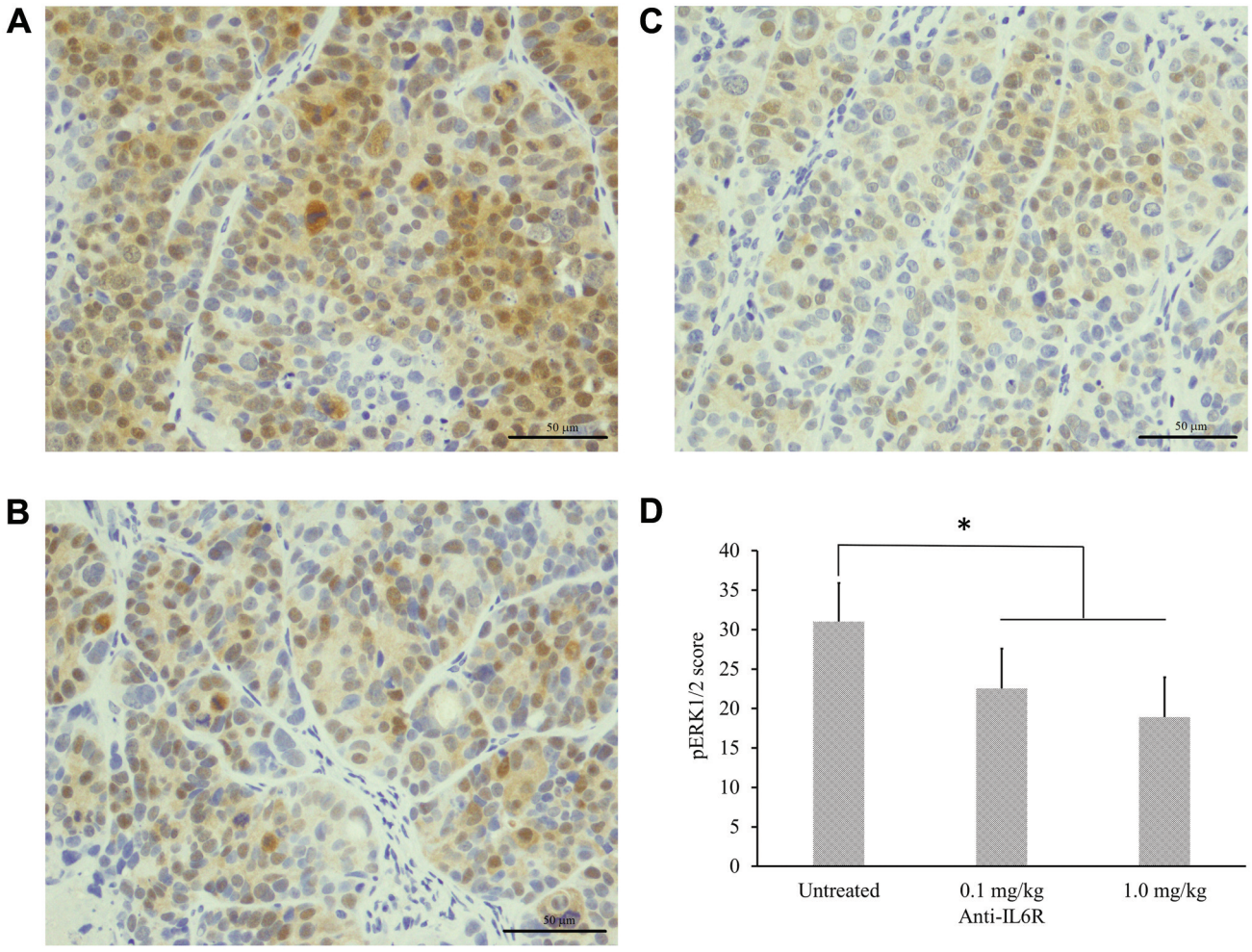

Figure 5. *Anti-interleukin-6 receptor (IL6R) disrupted the phosphorylation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) in colorectal cancer xenografts. Tumor slices were stained with anti-phosphor-ERK1/2 for immunohistochemical staining and the images were scanned using a microscanner, as described in the Materials and Methods. Representative images were captured of tumor tissues from the untreated control (A), mice treated with 0.1 mg/kg (B) and 1.0 mg/kg (C) IL6R. The expression of phosphor-ERK1/2 is represented as the average of scores by calculating the percentage and intensity of 10 different areas of a 400× image from each tumor image examined (D). *Significantly different at $p<0.05$ between the control and treatment groups.*

chemoresistance, and stem cell behavior (26-29). The inflammatory pathway of IL6 is strongly implicated as a trigger stimulating cancer stem cells in several malignancies, such as breast (27, 28, 30, 31), ovarian (32), liver (33) and lung cancer (34, 35), and CRC (28, 29, 36, 37). Our previous *in vitro* studies using an anchorage-independent colony-forming assay revealed that IL6 increased colonies of tested CRC cells, which was reversed by anti-IL6R treatment (19). It should be noted that colony formation in soft agar is considered a pilot assay for *in vivo* tumorigenesis and is associated with cancer stem cell behavior (38, 39). In the present study, we further demonstrated the *in vivo* suppression of CRC tumor growth by treatment with anti-IL6R, which not only reflected the results of the *in vitro* study, but also implies a regulatory role of IL6R in CRC stem cells. Although Ki-67 is a well-recognized protein marker for proliferative characteristics of tumor tissues (40, 41), a recent study revealed that Ki-67 has a further role in maintenance of cancer stem cells (42). As IL6R antibody suppressed Ki-67 expression in CRC tumor cells, this implies that it may regulate cancer stem cells in CRC.

In conclusion, we demonstrated that the IL6R antibody inhibited tumor growth of CRC xenografts *in vivo* by suppression of Ki-67 and STAT3 expression and ERK1/2 phosphorylation. Treatment of xenograft-bearing mice with IL6R antibody did not result in obvious toxicity. The results suggest that the IL6R antibody might be a promising anti-colorectal cancer drug for further assessment in clinical trials.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Authors' Contributions

Yuan-Chiang Chung provided the grant and original concept and study design. Yung-Lung Ku prepared the draft of the article. Hua-Che Chiang applied for the animal study approvement and designed the animal study. Wei-Chun Liu, Ting-Yu Kao and Chiu-Chen Huang performed all experiments and collected all of the results and data analysis. Chien-Hui Yang performed the statistical analysis of the data and reviewed the draft of article. Chih-Ping Hsu, as the corresponding author, designed the scheme and all of the experiments, as well as correcting the draft of the article. All Authors read and approved the final article.

Acknowledgements

The Authors appreciate the technical support from Dr. Hsien-Neng Huang. This work was supported by the Ministry of Science and Technology#1 under grant MOST 107-2314-B-264-001 - and Cheng-Ching General Hospital#2 under grant CH10500191A.

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Received July 13, 2021
Revised August 10, 2021
Accepted August 25, 2021