Research Article

Targeting Interleukin-4 Receptor Alpha by Hybrid Peptide for Novel Biliary Tract Cancer Therapy

Kahori Seto,^{1,2} Junichi Shoda,³ Tomohisa Horibe,² Eiji Warabi,⁴ Masayuki Kohno,²
Toru Yanagawa,¹ Hiroki Bukawa,¹ Yasuni Nakanuma,⁵ and Koji Kawakami²

¹ Department of Oral and Maxillofacial Surgery, Clinical Sciences, University of Tsukuba, Ibaraki 305-8575, Japan

² Department of Pharmacoepidemiology, Graduate School of Medicine and Public Health, Kyoto University, Yoshida Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan

³ Medical Science, Faculty of Medicine, University of Tsukuba, Ibaraki 305-8575, Japan

⁴ Division of Biomedical Science, Faculty of Medicine, University of Tsukuba, Ibaraki 305-8575, Japan

⁵ Department of Human Pathology, Graduate School of Medical Science, Kanazawa University, Kanazawa 920-1192, Japan

Correspondence should be addressed to Koji Kawakami; kawakami.koji.4e@kyoto-u.ac.jp

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It is known that the interleukin-4 receptor α (IL-4R α) is highly expressed on the surface of various human solid tumors. We previously designed novel IL-4R α -lytic hybrid peptide composed of binding peptide to IL-4R α and cell-lytic peptide and reported that the designed IL-4R α -lytic hybrid peptide exhibited cytotoxic and antitumor activity both *in vitro* and *in vivo* against the human pancreatic cancer cells expressing IL-4R α . Here, we evaluated the antitumor activity of the IL-4R α -lytic hybrid peptide as a novel molecular targeted therapy for human biliary tract cancer (BTC). The IL-4R α -lytic hybrid peptide showed cytotoxic activity in six BTC cell lines with a concentration that killed 50% of all cells (IC₅₀) as low as 5 μ M. We also showed that IL-4R α -lytic hybrid peptide in combination with gemcitabine exhibited synergistic cytotoxic activity *in vitro*. In addition, intravenous administration of IL-4R α -lytic hybrid peptide significantly inhibited tumor growth in a xenograft model of human BTC *in vivo*. Taken together, these results indicated that the IL-4R α -lytic hybrid peptide is a potent agent that might provide a novel therapy for patients with BTC.

1. Introduction

Biliary tract cancer such as gallbladder cancer and extrahepatic bile duct cancer as well as intrahepatic bile duct cancer (one of the primary liver cancers) is likely to undergo metastasis to the peritoneum (peritoneal dissemination) or the liver at early stages and is often resistant to conventional chemotherapy and radiotherapy. These cancers have been thus viewed as intractable cancers unlikely to be cured completely. In Japan, the incidence of biliary tract cancer and intrahepatic bile duct cancer is about 10 out of every 100,000 people [1]. As for intrahepatic bile duct cancer, both the incidence and death rate have been rising in Japan in recent years, resembling the trend observed in western countries [2, 3]. In Japan, gemcitabine and S-1 have recently begun to be used for anticancer chemotherapy, and these drugs are expected

to prolong the survival period of patients as compared to existing anticancer drugs [4]. However, because of frequent adverse events of the hematological system arising from these drugs and because of compromised hepatic function often noted in patients with intrahepatic bile duct cancer due to accompanying liver cirrhosis and in those with extrahepatic bile duct cancer or gallbladder cancer due to accompanying obstructive cholestasis, treatment with these drugs has to be discontinued or stopped. To improve the outcome of treatment of these cancers, it is very important to identify the tumor biological factors involved in the progression (e.g., infiltration and metastasis) of the cancers and to develop new valid means of auxiliary treatment targeted at these factors.

Based on the immunohistochemical analysis showing that cultured BTC cell lines and cancerous epithelia in BTC tissue expressed receptors for interleukin-4 (IL-4) *in situ* at

TABLE 1: Cytotoxic activity of IL-4R α -lytic hybrid peptide against BTC.

Cell line	IC ₅₀ (μ M)		IC ₅₀ ratio (Lytic/IL-4R α -lytic)
	Lytic peptide alone	IL-4R α -lytic peptide	
TGBC-1-TKB	16.99 $\pm$ 1.28	2.17 $\pm$ 0.54	7.83
TGBC-44-TKB	21.37 $\pm$ 4.91	3.27 $\pm$ 0.51	6.53
CCKS-1	15.83 $\pm$ 0.02	2.73 $\pm$ 1.26	5.80
KKU-100	18.93 $\pm$ 3.85	2.99 $\pm$ 1.01	6.34
KMBC	18.50 $\pm$ 1.65	3.25 $\pm$ 1.36	5.69
SK-1	13.44 $\pm$ 0.42	4.47 $\pm$ 0.40	3.00

IC₅₀ values (peptide concentration inducing 50% inhibition of control cell growth) are represented as mean $\pm$ SD from triplicate determinations.

FIGURE 1: Expression levels of IL-4R α in BTC cells. Cell lysates were prepared from six BTC cell lines. β -Actin was used as an internal control.

3. Results

3.1. Expression of IL-4R α in BTC Cell Lines. Immunoblot analysis demonstrated that IL-4R α protein is expressed in all cultured BTC cell lines used in this study. As shown in Figure 1, the expression levels of IL-4R α protein were the highest in intrahepatic cholangiocarcinoma cell lines (CCKS-1 and KKU-100) among tested cell lines. On the other hand, it was also confirmed that gallbladder carcinoma cell lines (TGBC-1-TKB and TGBC-44-TKB) and extrahepatic cholangiocarcinoma cell lines (KMBC and Sk-ChA-1) expressed IL-4R α , although the expression levels were lower than those of CCKS-1 and KKU-100 cells.

3.2. Cytotoxic Activity of IL-4R α -Lytic Hybrid Peptide in BTC Cell Lines. To assess the *in vitro* cytotoxic activity of IL-4R α -lytic hybrid peptide to BTC cell lines, the WST assay was performed using BTC cell lines treated with IL-4R α -lytic hybrid peptide and lytic peptide (Figure 2). TGBC-1-TKB, TGBC-44-TKB, CCKS-1, KKU-100, and KMBC were sensitive to IL-4R α -lytic hybrid peptide; the concentration that killed 50% of all cells (IC₅₀) was less than 3.5 μ M. Sk-ChA-1 was also sensitive to IL-4R α -lytic hybrid peptide with an IC₅₀ of less than 4.5 μ M. In contrast, optimal cell killing was not induced in these cells by lytic peptide. The cytotoxic activity of the hybrid peptide was strongly enhanced when compared with that of the lytic peptide. The cytotoxic activity of IL-4R α -lytic hybrid peptide increased 3.0–7.8-fold stronger than that of lytic peptide in BTC cell lines (Table 1).

3.3. Synergistic Cytotoxicity of IL-4R α -Lytic Hybrid Peptide and Gemcitabine in BTC Cells. Gemcitabine alone mediated a dose-dependent cytotoxicity with IC₅₀ of 12 nM in Sk-ChA-1. When it combined with IL-4R α -lytic hybrid peptide,

TABLE 2: Combination therapy of hybrid peptide and GEM.

	IC ₅₀	CI
IL-4R hybrid peptide (μ M)	4.47	
Gemcitabine (nM)	12.2	
IL-4R hybrid peptide (μ M) + gemcitabine 0.3 nM	4.29	0.95
IL-4R hybrid peptide (μ M) + gemcitabine 3 nM	3.25	0.75

CI (combination index) < 1, CI = 1, and CI > 1 indicate synergistic, additive, and antagonistic effect, respectively.

cytotoxicity in Sk-ChA-1 was greatly enhanced (Figure 3). IC₅₀ of IL-4R α -lytic hybrid peptide was 4.29 and 3.25 μ M by adding 0.3 and 3 nM of gemcitabine, respectively (Table 2). The combination index was <1 at all concentrations of gemcitabine. The same phenomenon was also observed in TGBC-44-TKB (data not shown).

3.4. In Vivo Antitumor Activity by IL-4R α -Lytic Hybrid Peptide in a Human BTC Xenograft Model. Following the observation that IL-4R α -lytic hybrid peptide was remarkably cytotoxic to BTC cell lines *in vitro* (Figure 2), the antitumor activity of the hybrid peptide was assessed in a xenograft model of human BTC. TGBC-44-TKB cells were inoculated subcutaneously into athymic nude mice and then the animals were treated with IL-4R α -lytic hybrid peptide by intravenous injection. As shown in Figure 4, tumors grew aggressively in control mice injected with saline alone, reaching a volume of 1285 mm³ by day 22. In contrast, mice treated with IL-4R α -lytic hybrid peptide showed significant tumor regression at both dosages: mean tumor volumes were 1025 mm³ (2 mg/kg) and 594 mm³ (5 mg/kg) on day 22. The tumor volume was inhibited significantly at a dose of 5 mg/kg (P < 0.05). No other abnormalities, such as loss of appetite and body weight, were observed in mice injected with IL-4R α -lytic hybrid peptide (data not shown). The number of leukocytes (the mean ($\times 10^3$) and SD values) after the experiments is as follows: control group: 5.1 $\pm$ 2.1, 2 mg/kg group: 5.6 $\pm$ 4.0, and 5 mg/kg group: 6.6 $\pm$ 4.7, and there are no significant differences in each group. Histological analysis also showed no side effects in tissue from the major organs, including liver and kidney, which were obtained from mice treated with intravenous administration of IL-4R α -lytic hybrid peptide

FIGURE 2: Cytotoxic activity of IL-4R α -lytic hybrid peptide. Six BTC cell lines were cultured with various concentrations of IL-4R α -lytic hybrid peptide or lytic peptide (0–20 μ M) for 72 h, and cytotoxic activity was assessed using WST-8 reagent. The results are represented as mean $\pm$ SD (bars) of triplicate determinations, and the assay was repeated three times.

(data not shown). These results demonstrated that IL-4R α -lytic hybrid peptide induced an effective antitumor activity in a mouse xenograft model of BTC.

4. Discussion

Therapeutic options for BTC are unsatisfactory, and the survival outcome is therefore poor. Effective therapeutic approaches against this aggressive disease are urgently needed. We previously demonstrated that human BTC tissue specimens expressed high levels of IL-4 receptor compared

to normal gallbladders [5]. These observations were also confirmed in cultured human BTC cell lines that expressed IL-4R by immunoblot analysis. Immunohistochemical analysis showed overexpression of IL-4R in a large proportion (50–63%) of human BTC tissue specimens. Following the results of preclinical study for treatment of BTC with IL-4-PE [5], the efficacy of IL-4R α -lytic hybrid peptide against human BTC was evaluated in preclinical models in this study.

Using the approach of selective receptor targeting, we have tested the efficacy of IL-4R α -lytic hybrid peptide on

FIGURE 3: Cytotoxic activity by combination therapy of IL-4R α -lytic hybrid peptide with gemcitabine. SK-1 was incubated with various concentrations of IL-4R α -lytic hybrid peptide (0–20 μ M) and gemcitabine (0–30 nM). The results are represented as mean $\pm$ SD (bars) of triplicate determinations, and the assay was repeated three times.

BTC cells. The overexpression of IL-4R α in BTC cell lines sensitized them to the cytotoxic effect of this peptide, which correlated with the level of IL-4R α expression. The *in vitro* cytotoxicity of this peptide was assessed for 6 cultured BTC cell lines (Figure 1). KMBC and CCKS-1 cell lines showing higher levels of IL-4R α expression showed higher sensitivity (IC_{50} of IL-4R α -lytic hybrid peptide toward these cancer cells was approximately 2 μ M, shown in Table 1). However the degree of the effect of IL-4R α -lytic hybrid peptide against each BTC cell line is hardly different. These observations indicate that abundant IL-4R α expression in BTC tumors will facilitate efficient targeting by this hybrid peptide. We previously reported that IL-4R α -lytic had effective cytotoxic activity toward cancer cells expressing IL-4R α and that enhancement of the IL-4R α -lytic peptide-induced cytotoxicity correlates well with the expression levels of IL-4R α [17]. We also previously showed the specificity of this hybrid peptide between normal and cancer cells by binding assay [25]. Moreover, it was demonstrated that intravenous administration of this hybrid peptide at 5 mg/kg dramatically inhibited the growth of TGBC44-TKB tumors *in vivo* (Figure 4). Histologic analysis also showed no abnormal changes in the tissue of major organs obtained from mice treated with the hybrid peptide (data not shown).

In this study, IL-4R α -lytic hybrid peptide in combination with gemcitabine exhibited synergistic cytotoxic activity *in vitro* (Figure 3). Gemcitabine (Gemzar) is a widely accepted first-line therapy for advanced BTC, although the median survival time is not favorable [26]. Most studies using a single agent showed a low response rate and little effect on patient survival in advanced adenocarcinoma. It has recently been

FIGURE 4: Antitumor activity of IL-4R α -lytic hybrid peptide in tumor xenograft model *in vivo*. TGBC-44-TKB were implanted subcutaneously into athymic nude mice. Intravenous injection of saline and IL-4R α -lytic hybrid peptide (2 mg/kg or 5 mg/kg) is indicated by the arrows. Data are expressed as mean $\pm$ SD ($n = 6$ animals in each group).

reported that cisplatin plus gemcitabine is associated with a significant survival advantage as compared with gemcitabine alone [4]. Moreover, several clinical trials using a combined approach of molecular target therapy with gemcitabine and other drugs have been initiated [27, 28]. Previously, IL-4-PE was shown to exert a synergistic effect with gemcitabine against the *in vitro* and *in vivo* pancreatic cancer models [28]. The results of this study support our observations of gemcitabine synergizing with IL-4R α -lytic hybrid peptide on BTC cells, although the precise mechanism of the synergistic effect of gemcitabine with this hybrid peptide still remains obscure. It is known that gemcitabine inhibits DNA synthesis through inhibition of ribonucleotide reductase and depletion of deoxynucleotide pools [29]. It is suggested that the inhibition effect by gemcitabine in cancer cells might be exerted synergetically with the disintegration activity toward cancer cell membrane by IL-4R α -lytic hybrid peptide [12, 17]. Thus, it is possible that gemcitabine enhances cell death induced by IL-4R α -lytic hybrid peptide. Considering cancer cell death, the combination therapy of these drugs, which act through different mechanisms, may be a beneficial treatment option for patients with BTC.

5. Conclusions

Our current data describe that IL-4R α is overexpressed in BTC cell lines *in vitro*, and BTC cells which overexpress the receptor can be successfully targeted with IL-4R α -lytic hybrid peptide both *in vitro* and *in vivo*. IL-4R α -lytic hybrid peptide induces regression of the established mouse model of BTC tumor. Thus, the findings of this study will assist and are

available for elucidation of novel and effective BTC therapy targeting IL-4R α by IL-4R α -lytic hybrid peptide.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

- [1] "Japan VSo. Tokyo (Japan): Japanese Ministry of Health and Welfare: Statistics Assoc.," 1998.
- [2] T. Patel, "Increasing incidence and mortality of primary intrahepatic cholangiocarcinoma in the United States," *Hepatology*, vol. 33, no. 6, pp. 1353–1357, 2001.
- [3] S. D. Taylor-Robinson, M. B. Toledano, S. Arora et al., "Increase in mortality rates from intrahepatic cholangiocarcinoma in England and Wales 1968–1998," *Gut*, vol. 48, no. 6, pp. 816–820, 2001.
- [4] J. Valle, H. Wasan, D. H. Palmer et al., "Cisplatin plus gemcitabine versus gemcitabine for biliary tract cancer," *The New England Journal of Medicine*, vol. 362, no. 14, pp. 1273–1281, 2010.
- [5] K. Ishige, J. Shoda, T. Kawamoto et al., "Potent *in vitro* and *in vivo* antitumor activity of interleukin-4-conjugated *Pseudomonas* exotoxin against human biliary tract carcinoma," *International Journal of Cancer*, vol. 123, no. 12, pp. 2915–2922, 2008.
- [6] T. Shimamura, R. E. Royal, M. Kioi, A. Nakajima, S. R. Husain, and R. K. Puri, "Interleukin-4 cytotoxin therapy synergizes with gemcitabine in a mouse model of pancreatic ductal adenocarcinoma," *Cancer Research*, vol. 67, no. 20, pp. 9903–9912, 2007.
- [7] R. J. Kreitman, "Immunotoxins in cancer therapy," *Current Opinion in Immunology*, vol. 11, no. 5, pp. 570–578, 1999.
- [8] F. Weber, A. Asher, R. Bucholz et al., "Safety, tolerability, and tumor response of IL4-*Pseudomonas* exotoxin (NBI-3001) in patients with recurrent malignant glioma," *Journal of Neuro-Oncology*, vol. 64, no. 1–2, pp. 125–137, 2003.
- [9] P. Attia, D. J. Powell Jr., A. V. Maker, R. J. Kreitman, I. Pastan, and S. A. Rosenberg, "Selective elimination of human regulatory T lymphocytes *in vitro* with the recombinant immunotoxin LMB-2," *Journal of Immunotherapy*, vol. 29, no. 2, pp. 208–214, 2006.
- [10] R. J. Kreitman, "Immunotoxins in cancer therapy," *Current Opinion in Immunology*, vol. 11, no. 5, pp. 570–578, 1999.
- [11] S. Choudhary, M. Mathew, and R. S. Verma, "Therapeutic potential of anticancer immunotoxins," *Drug Discovery Today*, vol. 16, no. 11–12, pp. 495–503, 2011.
- [12] M. Kohno, T. Horibe, M. Haramoto et al., "A novel hybrid peptide targeting EGFR-expressing cancers," *European Journal of Cancer*, vol. 47, no. 5, pp. 773–783, 2011.
- [13] M. Kawamoto, T. Horibe, M. Kohno, and K. Kawakami, "A novel transferrin receptor-targeted hybrid peptide disintegrates cancer cell membrane to induce rapid killing of cancer cells," *BMC Cancer*, vol. 11, article 359, 2011.
- [14] T. Horibe, M. Kohno, M. Haramoto, K. Ohara, and K. Kawakami, "Designed hybrid TPR peptide targeting Hsp90 as a novel anticancer agent," *Journal of Translational Medicine*, vol. 9, article 8, 2011.
- [15] N. Tada, T. Horibe, M. Haramoto, K. Ohara, M. Kohno, and K. Kawakami, "A single replacement of histidine to arginine in EGFR-lytic hybrid peptide demonstrates the improved anticancer activity," *Biochemical and Biophysical Research Communications*, vol. 407, no. 2, pp. 383–388, 2011.
- [16] H. Ueyama, T. Horibe, O. Nakajima, K. Ohara, M. Kohno, and K. Kawakami, "Semaphorin 3A lytic hybrid peptide binding to neuropilin-1 as a novel anti-cancer agent in pancreatic cancer," *Biochemical and Biophysical Research Communications*, vol. 414, no. 1, pp. 60–66, 2011.
- [17] L. Yang, T. Horibe, M. Kohno et al., "Targeting interleukin-4 receptor α with hybrid peptide for effective cancer therapy," *Molecular Cancer Therapeutics*, vol. 11, no. 1, pp. 235–243, 2012.
- [18] H. Yano, M. Maruiwa, A. Iemura, A. Mizoguchi, and M. Kojiro, "Establishment and characterization of a new human extrahepatic bile duct carcinoma cell line (KMBC)," *Cancer*, vol. 69, no. 7, pp. 1664–1673, 1992.
- [19] A. Knuth, H. Gabbert, W. Dippold et al., "Biliary adenocarcinoma. Characterisation of three new human tumor cell lines," *Journal of Hepatology*, vol. 1, no. 6, pp. 579–596, 1985.
- [20] K. Saito, H. Minato, N. Kono, Y. Nakanuma, F. Ishida, and M. Kosugi, "Establishment of the human cholangiocellular carcinoma cell line (CCKS1)," *Kanzo*, vol. 34, no. 2, pp. 122–129, 1993 (Japanese).
- [21] B. Sripa, S. Leungwattananit, T. Nitta et al., "Establishment and characterization of an opisthorchiasis-associated cholangiocarcinoma cell line (KKU-100)," *World Journal of Gastroenterology*, vol. 11, no. 22, pp. 3392–3397, 2005.
- [22] N. Tepsiri, L. Chaturat, B. Sripa et al., "Drug sensitivity and drug resistance profiles of human intrahepatic cholangiocarcinoma cell lines," *World Journal of Gastroenterology*, vol. 11, no. 18, pp. 2748–2753, 2005.
- [23] N. Koike, T. Todoroki, T. Kawamoto et al., "The invasion potentials of human biliary tract carcinoma cell lines: correlation between invasiveness and morphologic characteristics," *International Journal of Oncology*, vol. 13, no. 6, pp. 1269–1274, 1998.
- [24] T. Fujisawa, H. Nakashima, A. Nakajima, B. H. Joshi, and R. K. Puri, "Targeting IL-13R α 2 in human pancreatic ductal adenocarcinoma with combination therapy of IL-13-PE and gemcitabine," *International Journal of Cancer*, vol. 128, no. 5, pp. 1221–1231, 2011.
- [25] K. Seto, J. Shoda, T. Horibe et al., "Interleukin-4 receptor -based hybrid peptide effectively induced antitumor activity in head and neck squamous cell carcinoma," *Oral Oncology*, vol. 29, pp. 2147–2153, 2013.
- [26] T. Okusaka, H. Ishii, A. Funakoshi et al., "Phase II study of single-agent gemcitabine in patients with advanced biliary tract cancer," *Cancer Chemotherapy and Pharmacology*, vol. 57, no. 5, pp. 647–653, 2006.

- [27] B. Gruenberger, J. Schueller, U. Heubrandtner et al., "Cetuximab, gemcitabine, and oxaliplatin in patients with unresectable advanced or metastatic biliary tract cancer: a phase 2 study," *The Lancet Oncology*, vol. 11, no. 12, pp. 1142–1148, 2010.
- [28] H. Safran, T. Miner, M. Resnick et al., "Lapatinib/gemcitabine and lapatinib/gemcitabine/oxaliplatin: a phase I study for advanced pancreaticobiliary cancer," *The American Journal of Clinical Oncology*, vol. 31, no. 2, pp. 140–144, 2008.
- [29] A. Morgan, "Recently approved antineoplastic agents," *Oncology Practice Highlights*, vol. 14, pp. 74–79, 1996.