

Interleukin-4 receptor α -based hybrid peptide effectively induces antitumor activity in head and neck squamous cell carcinoma

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Abstract. Interleukin-4 receptor α (IL-4R α) is highly expressed on the surface of various human solid tumors including head and neck squamous cell carcinoma (HNSCC). We designed a novel IL-4R α -lytic hybrid peptide composed of a binding peptide to IL-4R α and a cell-lytic peptide. In the present study, we evaluated the antitumor activity of the IL-4R α -lytic hybrid peptide as a novel molecular-targeted therapy in HNSCC. Immunoblot analysis revealed that IL-4R α was expressed in all tested HNSCC cell lines (HSC-2, HSC-3, HSC-4, Ca9-22 and OSC-19), but not in a human normal keratinocyte (HaCaT) cell line. Immunohistochemical expression levels of IL-4R α in HNSCC tissues were higher compared to those in normal epithelial tissue. The IL-4R α -lytic hybrid peptide showed cytotoxic activity in all five cancer cell lines with a concentration that killed 50% of all cells (IC₅₀) as low as 10 μ M. HaCaT cells were less sensitive to this peptide with an IC₅₀ of >30 μ M. In addition, intratumoral administration of IL-4R α -lytic hybrid peptide significantly inhibited tumor growth in a xenograft model of human HNSCC *in vivo*. These results indicate that the IL-4R α -lytic hybrid peptide may serve as a potent agent to provide a novel therapy for patients with HNSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC) which includes cancers of the oral cavity, oropharynx, larynx and

hypopharynx is the sixth most common cancer worldwide and has an incidence of approximately 600,000 cases per year (1). The current management and treatment of HNSCC involves multi-modality approaches of surgery, chemotherapy and radiotherapy (2). Despite recent advances in early detection, diagnosis and treatment, the 5-year survival for patients with HNSCC has remained at 50% for the past 30 years (3). Molecular-targeted therapy, based on molecular findings of the last 50 years, is one of the most promising gateways to the development of new strategies in oncology (4). Cetuximab, a monoclonal antibody to the epidermal growth factor receptor (EGFR), is the only molecular-targeted therapy to be routinely used in clinical practice for the treatment of recurrent and metastatic HNSCC (5). Based on a limited number of phase II and III trials that have investigated the efficacy of cetuximab in addition to cisplatin in patients who were refractory to platinum-based therapy, the combination appears to confer further benefit over anti-EGFR agents alone (6,7).

The first generation of immunotoxins developed 35 years ago, which heralded targeted therapy, employed chemical conjugations of antibodies and either intact toxins or toxins with attenuated cell-binding properties. We previously reported that recombinant fusion protein IL4 (38-37)-PE38KDEL (also termed IL4-PE), consisting of circularly permuted interleukin (IL)-4 and a mutated form of *Pseudomonas* exotoxin (PE), induced significant regression of established biliary tract tumors and significantly improved the survival of animals with disseminated tumors (8,9). In addition, IL4-PE was reported to be highly and specifically cytotoxic to glioma cell lines *in vitro*, and caused partial or complete regression of established human glioblastoma multiforme tumors in nude mice (10). IL-4 receptor α (IL-4R α)-targeted protein-based immunotoxin was tested in the clinic for the treatment of human solid tumors (11,12). However, its clinical application faced many challenges, including non-specific toxicities and immunogenicity (13).

To overcome these issues, we previously developed a 'hybrid peptide', composed of target-binding and cytotoxic

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Table I. Clinicopathological features of the HNSCC patients.

No.	Age (years)	Gender	Primary site	TNM classification	Differentiation of SCC
1	57	Male	Tongue	T1N0M0	Well
2	40	Male	Tongue	T2N0M0	Moderate
3	47	Female	Tongue	T2N0M0	Well
4	78	Male	Gingiva	T3N2bM0	Well
5	61	Male	Gingiva	T4aN2bM0	Moderate

SCC, squamous cell carcinoma.

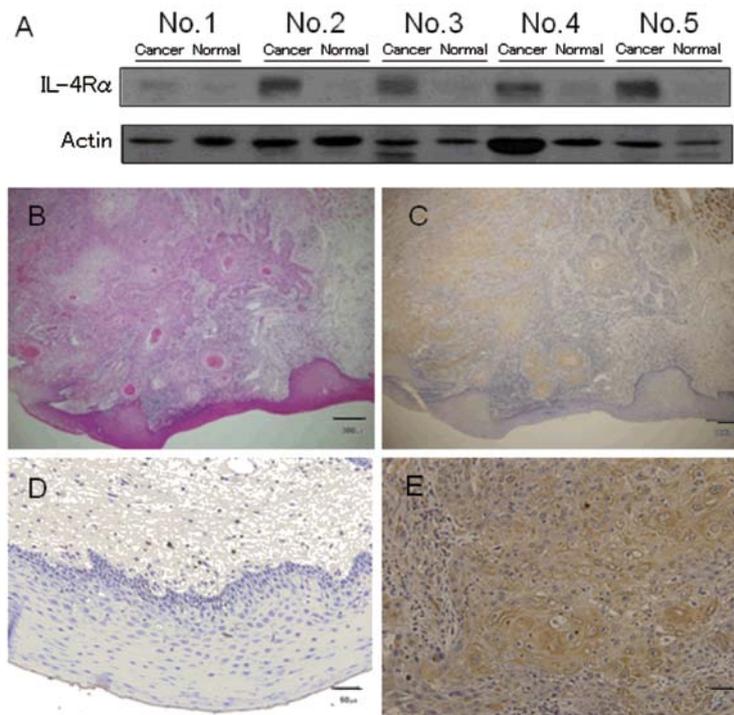


Figure 1. IL-4R α expression in HNSCC tissue specimens. (A) IL-4R α protein levels in HNSCC tissues. Tissue sections were prepared from 5 patients who underwent radical resection of oral cancer. Cancerous (cancer) and normal (normal) tissue regions were obtained as described in Materials and methods. (B-E) Immunohistochemical analysis of HNSCC samples of patient no. 2. (B) H&E staining. (C-E) Anti-IL-4R α antibody reactivity in normal area (D) and cancer area (E). Scale bars indicate 300 μ m (B and C) and 50 μ m (D and E).

volume (in mm³) was calculated using the following formula: Length x width² x 0.5. All values are expressed as means \pm SD.

Results

Expression of IL-4R α in HNSCC tissue specimens. We first analyzed the expression levels of IL-4R α in HNSCC tissue specimens. Patient characteristics including age, gender, primary site of tumor, TNM classification and differentiation are shown in Table I. Immunoblot analysis and immunohistochemistry were performed to investigate the expression levels of IL-4R α in tissue specimens from HNSCC patients. Immunoblot analysis showed that IL-4R α was expressed in all HNSCC specimens (tongue and gingival carcinomas) but not in the normal tissue specimens from the same patients (Fig. 1A). Similarly, immunohistochemical analysis using anti-IL-4R α

antibody showed IL-4R α immunopositivity in the HNSCC cancerous epithelium but not in the normal epithelium in all patients. Fig. 1B-E shows the staining pattern for patient no. 2.

Expression of IL-4R α in cultured HNSCC cell lines. We next investigated the expression levels of IL-4R α in HNSCC cell lines by immunoblot and real-time PCR analyses. Immunoblot and real-time PCR analyses demonstrated that all HNSCC cell lines expressed IL-4R α but HaCaT did not (Fig. 2A). We also examined mRNA expression levels of IL-4R α by real-time PCR analysis (Fig. 2B). Relative expression levels of IL-4R α in HSC-2 and HSC-3 cells were ~4-fold higher than that of HaCaT cells. The lowest expression level of IL-4R α in HNSCC cell lines was found in OSC-19, however, this level was still 2-fold higher than that of HaCaT (Fig. 2B).

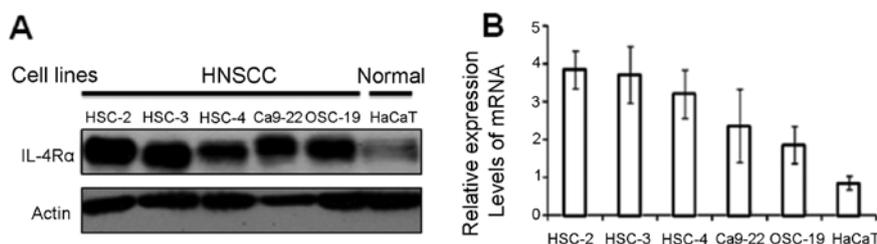


Figure 2. IL-4R α expression in cultured HNSCC cell lines. (A) Protein levels of IL-4R α in HNSCC cell lines and HaCaT normal keratinocytes. Specimens were prepared from five HNSCC cell lines: HSC-2, HSC-3, HSC-4, OSC-19 (tongue carcinoma), Ca9-22 (gingival carcinoma) and HaCaT cells. Actin was used as a loading control. (B) Expression levels of IL-4R α mRNA in HNSCC and HaCaT cell lines. Real-time PCR was performed as described in Materials and methods using IL-4R α -specific primers, and GAPDH served as an internal control.

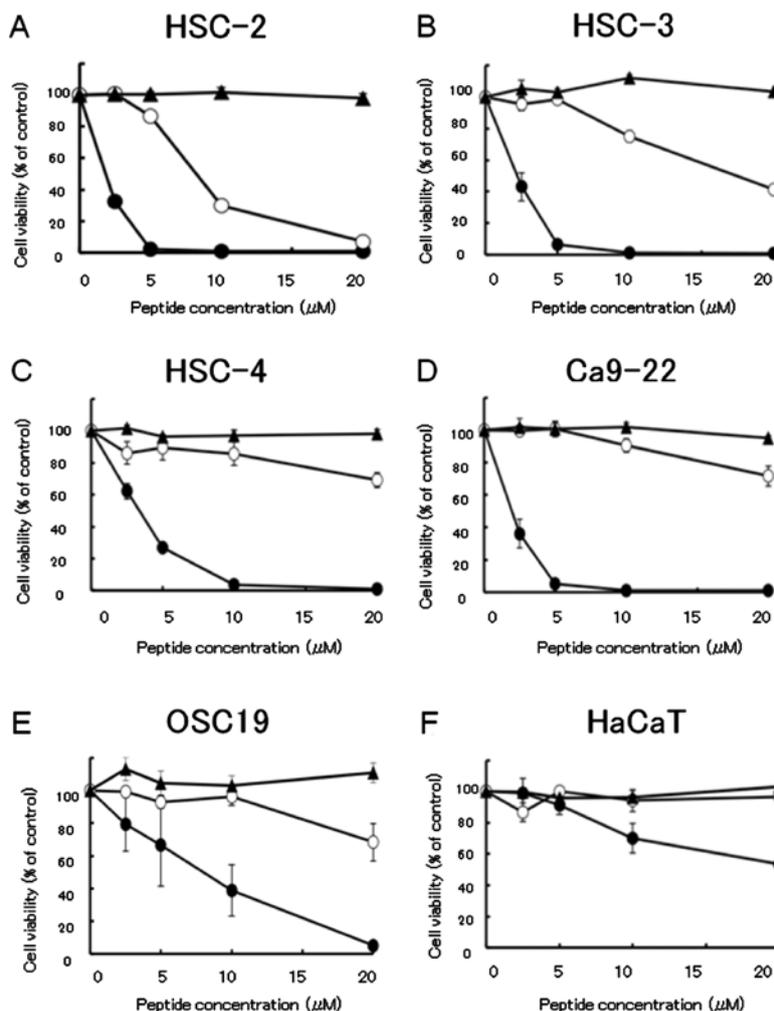


Figure 3. Cytotoxic activity of the IL-4R α -lytic hybrid peptide in HNSCC and HaCaT cell lines *in vitro*. The five HNSCC cell lines (HSC-2, HSC-3, HSC-4, Ca9-22 and OSC-19) and HaCaT cells (3×10^5 cells) were cultured with the indicated concentrations of peptides for 72 h, and cytotoxic activity was assessed using WST-8 reagent (●, IL-4R α -lytic hybrid peptide; ○, lytic peptide; ▲, IL-4 binding peptide). Data represent means \pm SD (bars) of triplicate determinations.

Cytotoxic activity of the IL-4R α -lytic hybrid peptide in HNSCC cell lines. To assess the *in vitro* cytotoxic activity of the IL-4R α -lytic hybrid peptide in HNSCC and HaCaT cells, the WST assay was performed using HNSCC cell lines treated with the IL-4R α -lytic hybrid peptide, lytic peptide or IL-4 binding peptide. HSC-2, HSC-3, HSC-4 and Ca9-22 cells were sensitive to the IL-4R α -lytic hybrid peptide; the concentration that killed 50% of all cells (IC₅₀) was <5 μ M. The OSC-19

cell line was also sensitive to the IL-4R α -lytic hybrid peptide with an IC₅₀ of <10 μ M. In contrast, optimal cell killing was not induced in HaCaT cells by either the lytic peptide or IL-4 binding peptide or IL-4R α -lytic hybrid peptide (Fig. 3). The cytotoxic activity of the hybrid peptide was strongly enhanced when compared with that of the lytic peptide. The cytotoxic activity of the IL-4R α -lytic hybrid peptide increased 4.0- to 13.2-fold when compared with that of the lytic peptide in the

Table II. Cytotoxic activity of each peptide in HNSCC and HaCaT cell lines.

Cell lines	IC ₅₀ (μM)		IC ₅₀ ratio
	IL-4Rα-lytic hybrid peptide	Lytic peptide	Lytic/IL-4Rα-lytic hybrid peptide
HNSCC cells			
HSC-2	1.9±0.1	8.2±0.2	4.4
HSC-3	2.3±0.6	17.4±1.2	7.4
HSC-4	2.8±0.8	33.2±6.1	11.9
Ca9-22	2.2±0.7	29.6±5.8	13.2
OSC-19	8.2±5.4	33.3±13.1	4.0
Normal cells			
HaCaT	37.6±15.3	43.4±13.6	1.2

IC₅₀ values (peptide concentration inducing 50% inhibition of control cell growth) are represented as means ± SD from triplicate determinations.

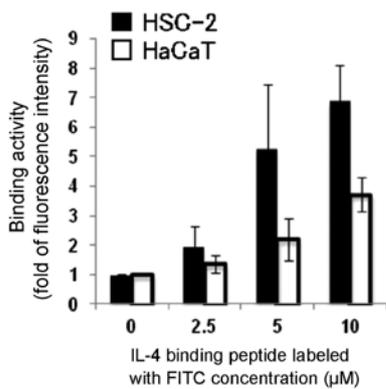


Figure 4. Binding assay of HSC-2 and HaCaT cells treated with the FITC-labeled IL-4 binding peptide. Quantification of the binding activity of this peptide to HSC-2 and HaCaT cells treated with various concentrations for 30 min. IL-4 binding peptide at 0 μM represents untreated cells used as a control. Fold-change in fluorescence intensity is the extent of binding of the FITC-labeled IL-4 binding peptide to each cell line, relative to the fluorescence intensity values. Data are presented as means ± SD (error bars) from triplicate determinations.

cancer cell lines (Table II). We examined the binding activity of the IL-4 binding peptide labeled with FITC to both HSC-2 and HaCaT cells by flow cytometry, and then found that exposure of HSC-2 to this peptide resulted in the increased binding activity of this peptide in a concentration-dependent manner (Fig. 4). These results suggest that the IL-4Rα-lytic hybrid peptide selectively kills cancer cells expressing IL-4Rα.

In vivo antitumor activity of the IL-4Rα-lytic hybrid peptide in a human HNSCC xenograft mouse model. Following the observation that the IL-4Rα-lytic hybrid peptide exhibits a marked cytotoxic effect on HNSCC cells *in vitro* (Fig. 3), the antitumor activity of the hybrid peptide was assessed in a xenograft model of human HNSCC. HSC-2 cells were inoculated

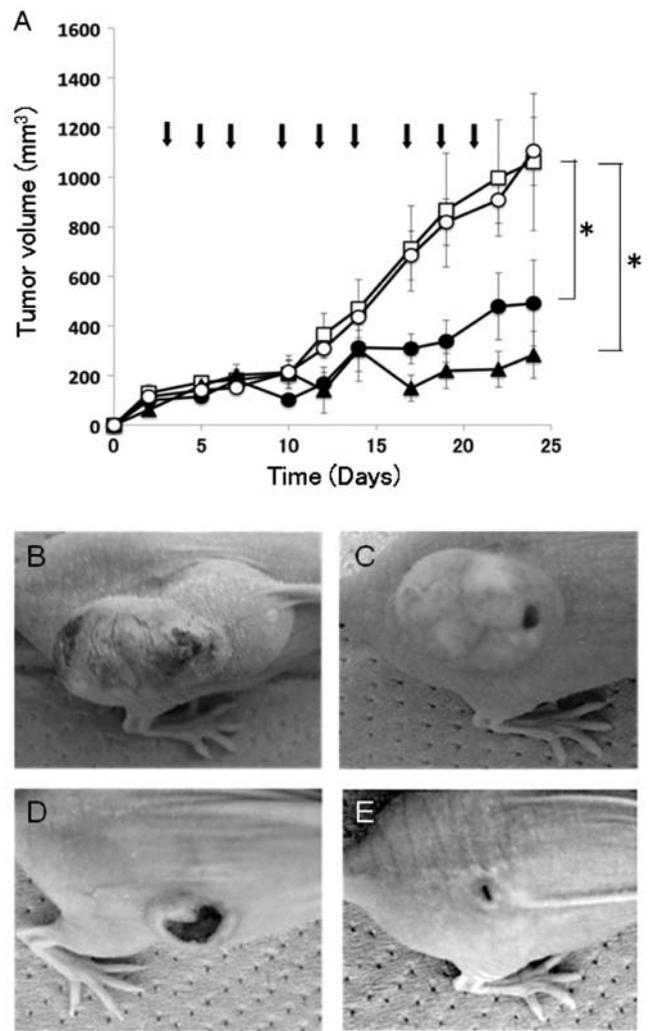


Figure 5. Antitumor activity of the IL-4Rα-lytic hybrid peptide in a xenograft model. (A) HSC-2 cells were inoculated subcutaneously into nude Balb/c mice. Animals received an intratumoral peptide injection or saline alone [□, control; lytic peptide (○, 5 mg/kg); IL-4Rα-lytic hybrid peptide (●, 5 mg/kg or ▲, 10 mg/kg)] three times per week from day 3 as indicated by the arrows. Data are expressed as mean ± SD (bars), n=6 animals per group. *P<0.01. (B-E) Images show tumor growth on day 24 after tumor inoculation: (B) saline alone; (C) lytic peptide; (D) IL-4Rα-lytic hybrid peptide (5 mg/kg); (E) IL-4Rα-lytic hybrid peptide (10 mg/kg).

subcutaneously into athymic nude mice, and the animals were subsequently treated with the IL-4Rα-lytic hybrid peptide by intratumoral injection. As shown in Fig. 5, tumors grew aggressively in the control mice injected with saline alone, reaching a volume of >1000 mm³ by day 24. In contrast, mice treated with the IL-4Rα-lytic hybrid peptide showed significant tumor regression at both dosages: mean tumor volumes were 491 mm³ (5 mg/kg) and 283 mm³ (10 mg/kg) on day 24. Moreover, tumors in mice injected with the lytic peptide grew rapidly similar to tumor growth in the control mice with saline alone, reaching a volume of 1104 mm³ (Fig. 5). No other abnormalities, such as loss of appetite and body weight, were observed in mice injected with the IL-4Rα-lytic hybrid peptide (data not shown). Histological analysis also showed no side effects in tissues from the major organs, including the liver and kidney, which were obtained from mice treated with

intratumoral administration of the IL-4R α -lytic hybrid peptide (data not shown). These results demonstrated that the IL-4R α -lytic hybrid peptide exhibited effective antitumor activity in a mouse xenograft model of HNSCC.

Discussion

The main treatment options for patients with HNSCC currently involve surgery, radiotherapy and chemotherapy, alone or in combination. Despite significant advances in HNSCC treatment, survival rates and prognosis have improved only moderately over the years (16). Systemic chemotherapy remains the only effective treatment option, but it is associated with significant rates of toxicity in HNSCC patients, who usually have a high prevalence of co-morbidities and problematic lifestyle habits (17). Ideally, future therapies should act over the short term, to minimize damage to healthy cells and target tumor compartments that have the highest sensitivity.

The concept of a 'magic bullet' proposed by Paul Ehrlich over 100 years ago has led to the search for agents that can selectively target cancer cells (18). Immunotoxins are proteins used to treat cancer and are composed of an antibody fragment linked to a toxin (19). Several disadvantages of these conventional immunotoxins for clinical use include immunogenicity, undesirable toxicity, manufacturing difficulties, short half-lives and neutralizing antibody production (20,21). However, peptides can be produced affordably by chemical synthesis, with a cost comparable to that of producing protein drugs. Moreover, since peptides are easy to produce, a wide variety of candidate peptides combining moieties for targeting and for toxicity can be tested in preclinical settings. We previously linked two functional peptide domains to produce a novel chimerical peptide termed a 'hybrid peptide', which was designed as a bifunctional peptide that binds to receptors or proteins overexpressing in cancer cells and consequently disrupts the cancer membrane (14,22,23). In the present study, we focused on IL-4R α as recent evidence suggests that IL-4R α is preferentially expressed on the surface of a variety of solid tumors including HNSCC (24).

The high degree of antitumor activity of the IL-4R α -lytic hybrid peptide in HNSCC correlated with the expression of IL-4R α *in vitro*. All HNSCC tumor specimens showed specific immunohistochemical staining for IL-4R α , and western blot analysis revealed expression of IL-4R α . However, IL-4R α expression was not observed in normal tissue specimens from the same patients (Fig. 1). These data are consistent with previous reports that HNSCC cells express IL-4R α on their cell surface and confirm that IL-4R α is expressed *in situ* (25,26). These results also indicate that this receptor may be an attractive target for the treatment of HNSCC.

Previous results suggest that IL-4 receptor-targeted cytotoxin may provide an effective therapeutic option for HNSCC (24,26). In the present study, the *in vitro* cytotoxicity of the IL-4R α -lytic hybrid peptide was examined in five HNSCC cell lines (Fig. 3). HSC-2 cells, which showed the highest level of IL-4R α expression in western blot analysis, also showed the highest sensitivity to the IL-4R α -lytic hybrid peptide (Figs. 2 and 3). Normal HaCaT cells with low IL-4R α expression were not sensitive to the IL-4R α -lytic hybrid peptide (Fig. 3). These results suggest that the cytotoxic effect

of the IL-4R α -lytic hybrid peptide correlates well with the level of IL-4R α expression.

In the present study, although the growth rate of HSC-2 was rapid, it was found that intratumoral administration of the hybrid peptide at 10 mg/kg, dramatically inhibited the growth of HSC-2 tumors *in vivo* (Fig. 5). Histological analysis also showed no abnormal changes in the tissues of major organs obtained from the mice injected with the hybrid peptide (data not shown). For clinical use, local injection may be effective for tumors such as HNSCC, and combination with prior chemoradiotherapy should be developed. These observations indicate that abundant IL-4R α expression in HNSCC tumors would facilitate efficient targeting by the IL-4R α -lytic hybrid peptide.

In conclusion, IL-4R α was overexpressed in both tumor specimens from patients with HNSCC and in HNSCC cell lines *in vitro*. The overexpressed IL-4R α on HNSCC cells could be successfully targeted with the IL-4R α -lytic hybrid peptide *in vitro* and *in vivo*. Future investigations using cancer progenitor cells isolated from primary malignant tissues at different stages during cancer progression and metastatic disease may help to identify new biomarkers for the development of more effective diagnostic and prognostic methods and targeted therapies (27). Additional studies should be performed to reveal the antitumor activity of the IL-4R α -hybrid peptide in animal models, and perhaps a phase I clinical trial should be undertaken to study its antitumor activity.

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