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A novel hybrid peptide targeting EGFR-expressing cancers

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ABSTRACT

Several potential molecular-targeted anticancer drugs focus on the inhibition of receptor tyrosine kinase and tumour growth, but these tyrosine kinase inhibitors (TKI) have been reported that the mutations of kinase-related signal molecule genes in cancer cells lead to the drug resistance. To overcome this issue, we have designed a novel targeting anticancer 'hybrid-peptide' EGFR-lytic peptide, in which epidermal growth factor receptor (EGFR) binding peptide is conjugated with a newly designed lytic-type peptide containing cationic-rich amino acids that disintegrates the cell membrane to kill cancer cells. In this report, cytotoxic activity of EGFR-lytic peptide was investigated in various human cancer and normal cell lines. It was found that the resulting conformational change in the novel lytic peptide enabled it to bind selectively to the membrane of cancer cells, and due to its acquired synergistic action, hybrid peptide demonstrated selective destruction of cancer cells as swiftly as 10 min after exposure. Treatment with EGFR-lytic peptide exerted a sufficient *in vitro* cytotoxic activity against TKI-resistant cancer cells with K-ras mutations. Moreover, *in vivo* analyses revealed that this peptide displayed significant antitumour activity in mouse xenograft models of both human K-ras mutation negative and positive cancers. Thus, hybrid peptide can be a unique and powerful tool for a new cancer-targeted therapy.

1. Introduction

Several potential molecular-targeted anticancer drugs on the market inhibit receptor tyrosine kinase and tumour growth. In some cases, mutations of kinase-related signal molecule genes in cancer cells result in the resistance to these tyrosine kinase inhibitors (TKIs). Recently, it is revealed that K-ras mutations are significantly associated with a lack of response not only to epidermal growth factor receptor (EGFR) TKIs but also to EGFR antibody drugs like cetuximab in patients with non-small-cell lung cancer and advanced colorectal cancer.[1] To overcome this critical issue, we have designed a novel molecular-targeted anticancer drug named hybrid peptide that directly kills cancer cells superior to signal pathway blockers.

Immunotoxins, monoclonal antibodies or ligands against overexpressed proteins on the surface of cancer cells conjugated to plant or bacterial toxins, have been extensively investigated for their possible use as anticancer agents.[2] A number of immunotoxins have been tested in preclinical and clinical trials, and interleukin-2-diphteria toxin fusion protein (IL2-DT; Ontak™) has been approved for the treatment of cutaneous T-cell lymphoma.[3,4] In addition, *Pseudomonas* exotoxin-based immunotoxins including interleukin-4-*Pseudomonas*

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exotoxin [IL4(38-37)-PE38KDEL] and interleukin-13-*Pseudomonas* exotoxin (IL13-PE38QQR) fusion proteins have been tested in clinical trials.[5,6] Both Diphtheria toxin and *Pseudomonas* exotoxin act by catalytically inactivating the elongation factor 2 in the ribosome complex, after taken into lysosomes, activated and translocated into the cytosol. This mechanism of action enables the immunotoxins to effectively destroy dormant, non-replicating tumour cells.

Although the targeting approach towards cancer utilising bacterial toxin-based immunotoxin is fascinating, its limitation lies in the liver toxicity due to the bacterial toxin and immunogenicity caused by the toxic proteins.[3,5,7] In addition, molecular size of immunotoxins is generally larger compared to chemical compounds or fragment antibody drugs, and this might prevent drugs from efficiently penetrating into tumour mass in the human body. To overcome these issues, new generation immunotoxins with evolutional approach are needed.

EGFR has been an important tumour-specific target for drug therapies for many years.[8,9] EGFR plays important roles in cell growth, differentiation and migration. Its positive signalling is found to cause increased proliferation, decreased apoptosis and enhanced tumour cell motility and angiogenesis.[10] EGFR overexpression is frequently found in a wide spectrum of human tumours of epithelial origin, including breast, lung, gastric, colorectal, prostate, pancreatic and ovarian cancers.[11] All these findings have brought EGFR as an important target for receptor-mediated delivery system of drugs. Recently, several studies reported successful identification of peptide ligands of EGFR by screening phage display libraries, implicating possible drug delivery by targeting to EGFR.[12,13]

Therapeutic peptides are increasingly gaining popularity as therapeutic agents for a variety of applications[14], including tumour vaccine,[15] antimicrobial therapy[16] and nucleic acid delivery.[17] In addition, research and development of new cancer therapeutics involving peptide-based drug has been widely undertaken.[18,19] It is also known that peptide drugs are relatively easily synthesised using either recombinant or solid-phase chemical synthesis techniques and the production costs are generally affordable when compared to antibody-based therapeutics. Recently, Papo and Shai reported that a new lytic-type peptide (D-K_6L_9) composed of 15-amino acids diastereomeric sequence containing D- and L-forms of leucine and lysine disrupts the plasma membrane.[20,21] This peptide kills tumour cells better than normal cells, and disintegrates the cell membrane in a detergent-like manner. In addition, the peptide's diastereomeric sequence preserves its anti-tumour activity in serum and in the presence of proteolytic enzymes. On the other hand, it is suggested that the peptide's selectivity to the cancer cells is probably determined predominantly by an increase in the level of acidic components or phosphatidylserine on the cancer cell membrane.[20] Even though this lytic-type peptide has selective cytotoxicity between normal and cancer cells, this peptide still kills normal cells in lower concentration, and thus, it is not considered suitable for the combination with targeting moiety.

Using aforementioned and recent identification of peptide sequences binding to EGFR and lytic-type peptide sequence, we have developed a new hybrid peptide targeting EGFR-overexpressed cancer cells. This hybrid peptide, termed EGFR-lytic peptide, is composed of an EGFR-binding moiety and a novel designed lytic moiety that is stable when combined with targeting peptide with less toxic effect to normal cell lines compared to D-K_6L_9 peptide, with three glycine spacer. In this study, we demonstrated both *in vitro* cytotoxic activity and selectivity of cell death induced by EGFR-lytic peptide in seven human cancer cell lines derived from breast, pancreas, lung, prostate and brain cancer. In addition, we investigated the interaction of EGFR-lytic peptide with the cancer cell surface and the mode of action of peptide-induced cancer cell death. *In vivo* experiments also revealed that this novel hybrid peptide displayed significant antitumour activity.

2. Materials and methods

2.1. Materials

Gefinitib and Erlotinib were purchased from Toronto Research Chemicals (Ontario, Canada). Anti-EGFR mouse monoclonal antibody (Clone 225) and PD153035 were purchased from Calbiochem (La Jolla, CA).

2.2. Cell culture

Human breast cancer (BT-20 and MDA-MB-231), lung cancer (H322 and H460), pancreatic cancer (SU.86.86), prostate cancer (LNCaP), glioma (U251) and lung fibroblast (MRC-5 and WI-38) cell lines were purchased from the American Type Culture Collection (Manassas, VA). Human pancreatic cancer (BXPC-3) and colon cancer (HCT116 and DLD-1) cell lines were purchased from the European Collection of Cell Cultures (ECACC; Salisbury, Wiltshire, UK). Human embryonic kidney cell line (HEK293) was purchased from RIKEN Cell Bank (Tsukuba, Japan). Human colon cancer cell line (SW837) was purchased from HSRRB (Osaka, Japan). Cells were cultured in RPMI1640 (BT-20, MDA-MB-231, H322, H460, SU.86.86, LNCap, U251, BXPC-3, DLD-1 and SW837), MEM (MRC-5 and WI-38), McCoy's 5a (HCT116) or D-MEM (HEK293) containing 10% FBS v/v (BioWest, Miami, FL), 100 μg/ml penicillin and 100 μg/ml streptomycin (Nacalai Tesque, Kyoto, Japan).

2.3. Peptides

The following peptides were purchased from Invitrogen (Carlsbad, CA):

1. Designed lytic-peptide: K**L**LLK**L**L**KK**LLK**L**LKKK-OH (bold letters are D-amino acids.)
2. EGFR binding + designed lytic hybrid peptide (EGFR-lytic peptide): YHWYGYTPQNVIGGGK**L**LLK**L**L**KK**LLK**L**LKKK-OH
3. D-K_6L_9 peptide: LK**L**LK**K**L**L**K**K**LL**K**LL-NH_2
4. EGFR binding + D-K_6L_9 peptide: YHWYGYTPQNVIGGG LK**L**LK**K**L**L**K**K**LL**K**LL-NH_2

Nos. 3 and 4 peptides were used for Supplementary Figs. S1 and S2 only.

All peptides were synthesised by use of solid-phase chemistry, purified to homogeneity (i.e. >90% purity) by reversed-phase high-pressure liquid chromatography and assessed by mass spectrometry. Peptides were dissolved in water and

buffered to pH 7.4. Peptide solutions were prepared freshly every time immediately prior to use and were not stored.

2.4. Visualisation of membrane permeabilisation

Cell membrane permeabilisation assay by hybrid peptide was performed as described previously.[22] Briefly, a soluble fluorescent molecule, calcein was added to MDA-MB-231 cells in a glass-bottomed dish at a final concentration of 2 μM. Small aliquots of labelled-peptides, EGFR-lytic-TAMRA-OH or lytic-TAMRA-OH (Invitrogen) (15 μl) were added directly into the dish at a final concentration of 10 μM. Confocal images were taken using an Olympus FV1000 (Olympus) confocal laser scanning microscope.

2.5. Cell viability assay

A total of 3×10^3 cells per well were seeded into 96-well plates and incubated for 24 h in medium containing 10% FBS v/v. The cells were then incubated with increasing concentrations of lytic peptide or the hybrid peptide in 100 μl of medium for 48 or 72 h at 37 °C. Cell viability was measured using WST-8 solution (Cell Count Reagent SF; Nacalai Tesque).

2.6. Immunofluorescence staining

EGFR expression was determined using flow cytometry by incubating of 1×10^5 cells with a FITC-conjugated human monoclonal antibody to EGFR at 1:50 dilution (Santa Cruz Biotechnology, Santa Cruz, CA). Staining was performed at room temperature for 40 min. The cell fluorescence was measured by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA). The mean fluorescence intensity (MFI) of EGFR-positive cells was determined using the CellQuest software (Becton Dickinson).

2.7. Annexin V and caspase analysis

BT-20 cells were treated for 2 h at 37 °C with or without the EGFR-lytic peptide at 5 μM. For determination of caspase activation and apoptosis, peptide-treated cultures were simultaneously analysed for caspase activity and propidium iodide (PI) staining using a carboxyfluorescein FLICA caspase-3&7 assay (Immunochemistry Technologies, Bloomington, MN), or, alternatively, for Annexin V labelling and PI staining using Annexin V-Fluorescein Staining Kit (Wako Chemicals, Osaka, Japan), by multiparametric flow cytometry.

2.8. Clonogenic assay

The *in vitro* cytotoxic activity of the lytic peptide and EGFR-lytic peptide against H322 cells were determined by a colony-forming assay. The cells were plated in triplicate in 6 cm Petri dishes with 3 ml of RPMI-1640 containing 10% FBS v/v and were allowed to attach for 24 h. The number of cells per plate was chosen such that >100 colonies were obtained in the control group. The cells were exposed to different concentrations of the lytic peptide or the EGFR-lytic peptide (0–22.5 μM) for 10 days at 37 °C in a humidified incubator. The cells were washed, fixed and stained with crystal violet (0.25% w/v in 25% alcohol v/v). Colonies consisting of >50 cells were scored. The percentage of colony survival was determined from the number of colonies formed in the control and treatment groups.

2.9. TUNEL assay

Terminal deoxynucleotidyl transferase-mediated UTP nick end labelling (TUNEL) assay was performed by MEBSTAIN Apoptosis Kit Direct (MBL, Nagoya, Japan) using confocal laser scanning microscope according to the manufacturer's instructions.

2.10. Antitumour activity of EGFR-lytic peptide in tumour xenografts in vivo

Animal experiments were carried out in accordance with the guidelines of Kyoto University School of Medicine. Cells of the pancreas cancer cell line BXPC-3 or the breast cancer cell line MDA-MB-231 (5×10^6 cells), resuspended in 150 μl of PBS, were transplanted subcutaneously into the flank region of 6–9-week-old athymic female nude mice weighing 17–21 g. When tumours reached 20–60 mm^3 in volume, animals were randomised into three or four groups, and saline (control) or EGFR-lytic peptide (2, 5 or 10 mg/kg) was injected intravenously (50 μl/injection) three times a week for a total of nine doses. Tumours were measured with a caliper and the tumour volume (in mm^3) was calculated using the following formula: $\text{length} \times \text{width}^2 \times 0.5$. All values are expressed as the mean ± SD and statistical analysis was calculated by a one-way ANOVA with Dunnett test. Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Design of novel hybrid peptide with selective cytotoxicity against EGFR-overexpressed cancer cells

To create a novel molecular-targeted anticancer drug with a novel mechanism, we have particularly focused on peptides disintegrating the cancer cell membrane, and newly modified and designed a novel lytic-type peptide, which is suitable for the combination with EGFR binding peptide, based on its amphiphilicity in secondary structure. As shown in Supplementary Fig. S1, the location of cluster of positively charged amino acids (Lys) in novel designed lytic peptide was still retained after the combination with EGFR binding peptide compared with that of EGFR-D-K_6L_9 peptide (Supplementary Fig. S1A and B). CD spectra analysis demonstrated that EGFR-novel designed lytic (EGFR-lytic) peptide weakly bound to small unilamellar vesicles (SUVs) composed of phosphatidylcholine (PC), which is the dominant lipid component on the surface of normal cell membranes, and was not well structured with this PC–SUV, however, this novel designed EGFR-lytic peptide bound to SUVs containing phosphatidylserine (PS), which is exposed specifically on cancer cell membranes, and conformed to a partial helical structure as characterised by double minima at 209–210 and 222 nm (Supplementary Fig. S1D). On the other hand, EGFR-D-K_6L_9 peptide strongly bound to both PC and PC/PS SUVs and conformed to

helices (Supplementary Fig. S1C). These results indicate that novel designed EGFR-lytic peptide in this study has a selectivity to PS-containing membranes, conforming to helices, which are supposed to be necessary for making a pore on the cell surface.[20]

3.2. *Treatment with EGFR-lytic peptide exerts a sufficient cytotoxicity against TKI-resistant cancer cells*

TKIs have shown less anticancer activity to tumours with genomic mutations. We compared the cytotoxic activity of EGFR-lytic peptide and TKIs in cancer cells with or without K-ras mutation. K-ras wild-type (WT) cancer cell lines (H322 and BT-20) were sensitive to erlotinib and anti-EGFR antibody, however, K-ras mutant cancer cell lines (MDA-MB-231, HCT116, SW837, and DLD-1) were resistant to them (Fig. 1A). Treatment of three TKIs, erlotinib, gefitinib and PD153035 resulted in a dose-dependent growth inhibition but insufficient cytotoxicity in K-ras WT cancer cell lines (H322, BT-20, and BXPC-3). On the other hand, treatment of EGFR-lytic peptide resulted in a sufficient cytotoxicity against these cancer cell lines but not in lung normal cell line MRC-5 (Fig. 1B). Although K-ras mutant cancer cell lines (MDA-MB-231, HCT116, SW837 and DLD-1) were resistant to erlotinib, gefitinib and PD153035, treatment with EGFR-lytic peptide exerted a sufficient cytotoxicity against TKI-resistant cancer cells with K-ras mutation (Fig. 1C).

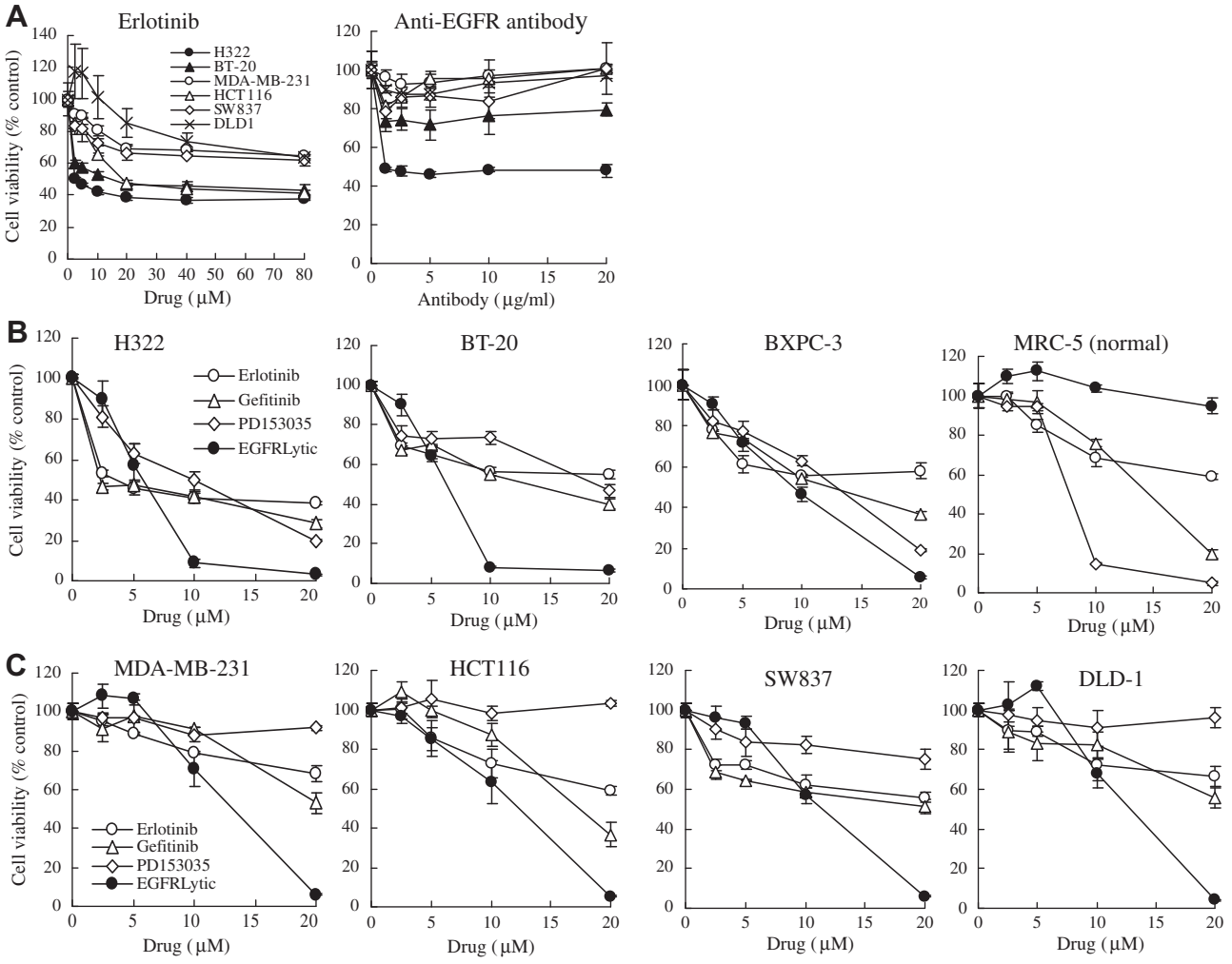

Fig. 1 – Treatment with EGFR-lytic peptide exerts a sufficient cytotoxic activity against TKI-resistant cancer cell lines. (A) Resistance to TKIs against K-ras mutant cancer cell lines. K-ras WT cell lines (H322 and BT-20) and K-ras mutant cell lines (MDA-MB-231, HCT116, SW837 and DLD-1) were cultured with various concentrations of erlotinib (0–80 μM) or anti-EGFR antibody (0–20 μg/ml) for 72 h, and cytotoxic activity was assessed using WST-8 reagent. (B) Comparison of cytotoxicity between TKIs and EGFR-lytic peptide against K-ras wild type cell lines. Cancer cell lines (H322, BT-20 and BXPC-3) and lung normal cell line MRC-5 were cultured with various concentrations of TKIs (erlotinib, gefitinib and PD153035; 0–20 μM) or EGFR-lytic peptide (0–20 μM) for 72 h, and cytotoxic activity was assessed using WST-8 reagent. (C) Treatment with EGFR-lytic peptide exerts a sufficient cytotoxicity against TKI-resistant cancer cells with K-ras mutations. Four K-ras mutant cancer cell lines were cultured with various concentrations of three TKIs or EGFR-lytic peptide (0–20 μM) for 72 h, and cytotoxic activity was assessed using WST-8 reagent. The results are represented as means ± SD (bars) of triplicate determinations, and the assay was repeated three times.

3.3. *EGFR binding moiety enhances the cytotoxic efficacy of the novel designed lytic peptide dependent on the expression levels of EGFR on the cell surface*

We then compared the cytotoxic activity of the novel designed lytic peptide with EGFR-lytic peptide against seven EGFR expressing cancer cell lines. As shown in Fig. 2A, treatment with the lytic peptide alone and EGFR-lytic peptide resulted in a dose-dependent cytotoxicity in all cancer cell lines tested. EGFR-lytic peptide demonstrated considerable enhanced cytotoxic activity to cancer cells, when compared with the lytic peptide alone. A 15–20 μM solution of the EGFR-lytic peptide was sufficient to induce more than 80% of cell death in all the cell lines. In contrast, the same concentration of lytic peptide alone did not induce sufficient cell killing of cancer cells. As shown in Table 1, conjugation of EGFR binding moiety to lytic peptide enhanced the IC_{50} (the peptide concentration inducing 50% inhibition of control cell growth) for cancer cells by 1.6- to 3.1-fold, suggesting that cancer cells are more susceptible to EGFR-lytic peptide than to lytic peptide alone. We then assessed the cytotoxic activity of EGFR-lytic peptide and lytic peptide in three normal cell lines. As shown in Fig. 2A, three normal cell lines MRC-5, WI-38 and HEK293 were less sensitive to lytic peptide, demonstrating less cytotoxicity compared to cancer cell lines. The IC_{50} of EGFR-lytic peptide for normal cells was 3.6–7.5-fold higher than for cancer cells (Table 1). The targeting to EGFR by combination of lytic peptide with EGFR binding moiety increased the cytotoxic activity to both normal and cancer cells with EGFR expression and the IC_{50} for normal cells were higher in comparison with that for cancer cells (Fig. 2A and Table 1). These findings suggest that lytic peptide induces cytotoxic activity in cancer cells rather than in normal cells, and EGFR-lytic peptide has superior cytotoxic activity to EGFR expressing cancer cells. Interestingly, the combination of EGFR binding moiety with D-K_6L_9 peptide did not show the enhancement of cytotoxic activity, and moreover, EGFR-D-K_6L_9 peptide killed even normal cells in lower concentration, indicating that D-K_6L_9 peptide was not suitable for the chimerisation with EGFR binding peptide. It was also confirmed that

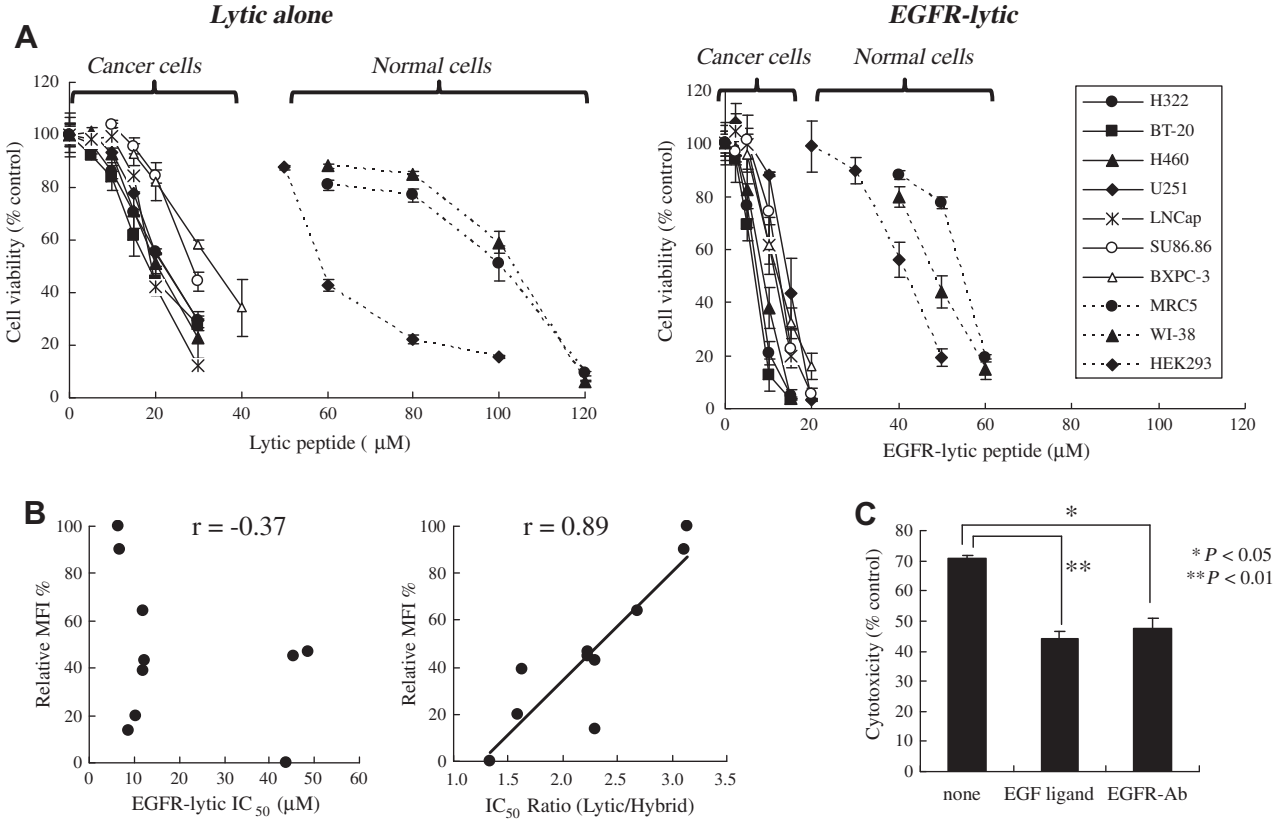

Fig. 2 – Designed lytic peptide is suitable for the chimera peptide to increase the cytotoxic activity for cancer cells. (A) Cancer and normal cell lines, H322, BT20, H460, U251, LNCap, SU.86.86, BXPC3, MRC5, WI38, and HEK293 were cultured with various concentrations of lytic peptide alone (left panel) or EGFR-lytic peptide (right panel) for 72 h, and cytotoxic activity was assessed using WST-8 reagent. (B) Enhancement of cytotoxicity by addition of EGFR binding moiety to the lytic peptide is dependent on EGFR expression on the cell surface. Correlation between the IC_{50} of EGFR-lytic peptide (left panel) or the IC_{50} ratio of lytic/hybrid peptide (right panel) and the relative mean fluorescence intensity (MFI) of EGFR antibody binding for seven cancer and three normal cell lines was plotted, and correlation index was calculated. (C) Inhibition of cytotoxicity of EGFR-lytic peptide to BXPC-3 cells was evaluated by the addition of a polyclonal anti-EGFR antibody or recombinant EGF ligand 1 h before peptide treatment. The results are represented as means ± SD (bars) of triplicate determinations, and the assay was repeated three times. $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 1 – Cytotoxicity of peptides to various cell lines and EGFR expression.

Cell line	IC_{50} (μM)		IC_{50} ratio lytic/EGFR-lytic	Relative MFI[a] (anti-EGFR antibody, %)
	Lytic peptide alone Mean ± SD	EGFR-lytic peptide Mean ± SD		Mean ± SD
Cancer cells				
H322	21 ± 3.2	6.8 ± 0.5	3.1	90 ± 23
BT-20	20 ± 2.9	6.5 ± 0.7	3.1	100
U251	20 ± 2.5	12 ± 2.0	1.6	39 ± 5.6
H460	20 ± 1.6	8.9 ± 1.6	2.3	13 ± 3.6
BXPC-3	32 ± 1.6	12 ± 0.9	2.7	64 ± 17
SU.86.86	28 ± 0.5	12 ± 2.3	2.3	43 ± 4.6
LNCap	16 ± 2.5	10 ± 1.3	1.6	20 ± 7.0
Normal cells				
WI-38	100 ± 3.1	46 ± 2.7	2.2	45 ± 4.6
MRC-5	110 ± 8.1	49 ± 5.8	2.3	47 ± 13
HEK293	58 ± 0.3	44 ± 2.8	1.3	0

a The relative MFI (mean fluorescence intensity) is the extent of binding of the FITC-conjugated anti-EGFR polyclonal antibody to cells, where the mean MFI values for BT-20 and HEK293 cells are set at 100% and 0%, respectively.

hybrid EGFR-lytic peptide had lower cytotoxicity than D-K6L9 peptide to normal cells and this EGFR-lytic peptide had almost similar cytotoxic activity with D-K6L9 peptide to cancer cells (Supplementary Fig. S2). We next examined whether the increase in cytotoxicity of the EGFR-lytic peptide was correlated with the expression levels of EGFR on the cell surface. The expression levels of EGFR for seven cancer cell lines and three normal cell lines were assessed by flow cytometry using an FITC-conjugated anti-EGFR monoclonal antibody. The expression levels of EGFR did not correlate with IC_{50} of EGFR-lytic peptide or lytic peptide ($r = -0.37$ for EGFR-lytic peptide (Fig. 2B, left) and $r = -0.14$ for lytic peptide (data not shown)), because IC_{50} values of lytic peptide were in wide range. On the other hand, it was correlated well with IC_{50} ratio of lytic peptide to EGFR-lytic peptide, suggesting that the increase in cytotoxicity that occurs when the EGFR binding moiety is combined to lytic peptide is dependent on the EGFR expression levels on the cell surface ($r = 0.89$) (Fig. 2B, right). To further confirm the specificity of EGFR-lytic peptide to EGFR, anti-EGFR polyclonal antibody (Ab) or recombinant human EGF was added to the BXPC-3 culture 1 h prior to the exposure of EGFR-lytic peptide to assess the cytotoxic activity to cells. As shown in Fig. 2C, both 0.16 μM EGF protein and 0.25 μg/ml EGFR-Ab were able to block the cytotoxicity of 15 μM EGFR-lytic, demonstrating 27% inhibition by EGF ligand and 23% inhibition by EGFR-Ab. These results suggest that the binding of EGFR-lytic peptide to cells is dependent on the cell surface expression levels of EGFR.

3.4. *Interaction kinetics for the EGFR-lytic peptide binding to EGFR protein and cell surface membrane proteins*

To understand the binding property of peptides to EGFR, EGFR protein was immobilised on sensor chips and interaction kinetics with EGFR-lytic peptide or lytic peptide alone were analysed using Biacore. As shown in Supplementary Fig. S3A, the resonance signal intensity increased according to the concentrations of EGFR-lytic peptide, indicating that the amount of EGFR-lytic peptide bound to EGFR protein was proportional to the increase in the concentrations of this peptide. In contrast, the resonance signal intensity by lytic peptide minimally increased according to the concentrations. Next, to understand the binding property of peptides to cells, either EGFR-lytic peptide or lytic peptide alone was immobilised on sensor chips, and interaction kinetics with cell surface membrane proteins extracted from H322, BT-20 and MRC-5 cells was analysed using Biacore. As shown in Supplementary Fig. S3B and C, the resonance signal intensity increased according to the concentrations of cell membrane proteins, indicating that the amount of cell membrane proteins bound to the peptide was proportional to the increase in the concentrations of cell membrane proteins. Interaction of cell membrane proteins to lytic peptide alone demonstrated similar binding constants in each cell line with increased level of the peptide. On the other hand, binding constants of EGFR-lytic peptide to H322 and BT-20 cancer cell membrane proteins were 4.2-fold (H322) and 4.4-fold (BT-20) stronger than corresponding values for lytic peptide (Supplementary Fig. S3B–D). In contrast, the binding constant for EGFR-lytic peptide and MRC-5 normal cell membrane proteins did not differ significantly from the corresponding values for lytic peptide alone (Supplementary Fig. S3D). These results were consistent with the data obtained from the WST assays (Fig. 2A), indicating that the cytotoxic activity of EGFR-lytic peptide correlated well with the affinity to the cell membranes.

3.5. *Characterisation of EGFR-lytic peptide induced cancer cell death*

To explore the duration of EGFR-lytic peptide exposure necessary to kill cancer cells, H322 and BT-20 cells were each treated with either EGFR-lytic peptide or lytic peptide alone for 10 min, 30 min, 1 h or 48 h. As shown in Fig. 3A, treatment of H322 and BT-20 cells with lytic peptide resulted in time-dependent loss of viability. In contrast, a mere 10 min exposure of H322 and BT-20 cells to EGFR-lytic peptide (10 μM) sufficiently killed cancer cells and more than 70% of cells lost cell

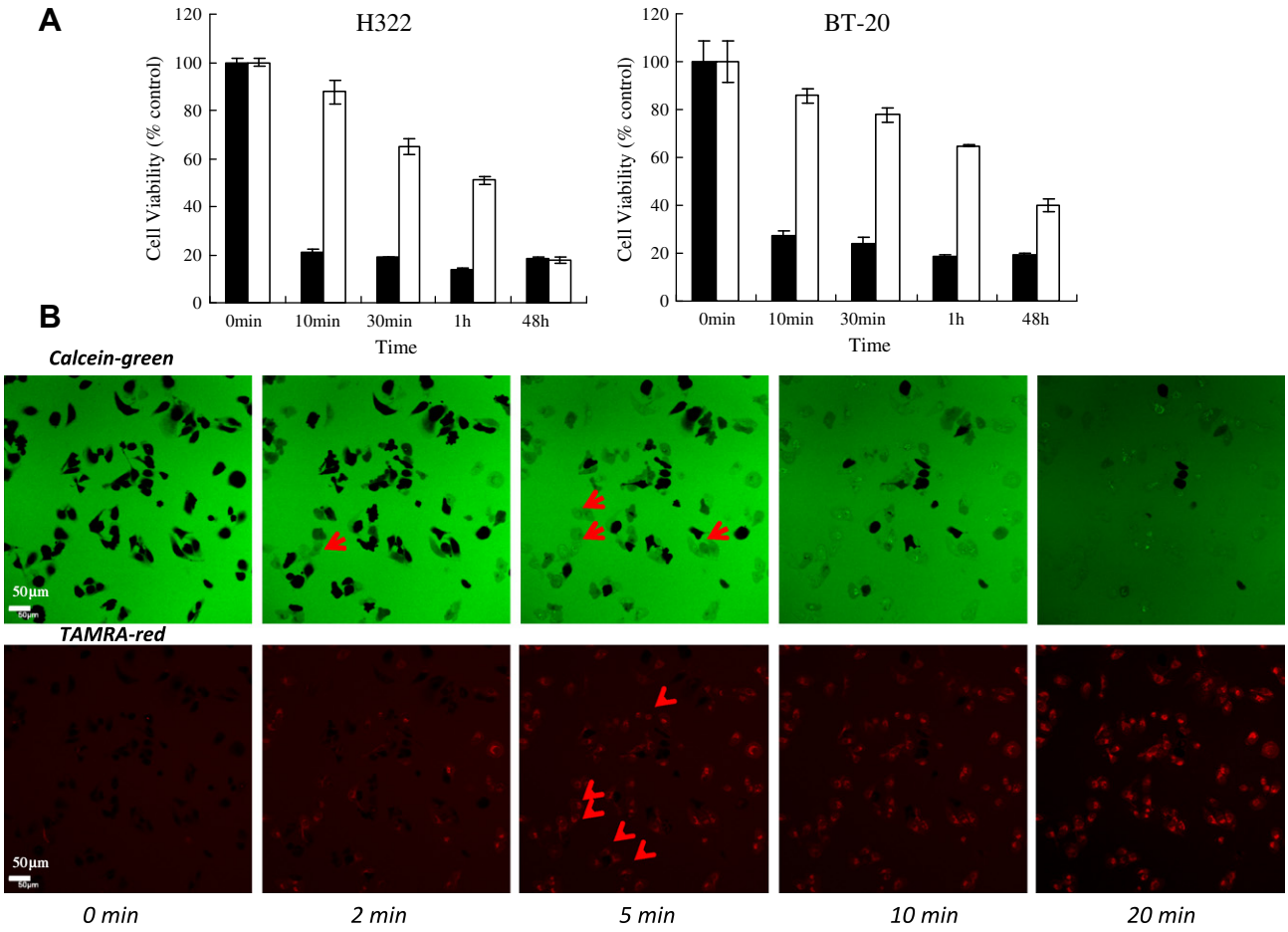

Fig. 3 – EGFR-lytic peptide penetrates the plasma membrane to induce rapid killing of cancer cells. (A) H322 and BT-20 cells were treated with EGFR-lytic peptide (*black columns*) or lytic peptide alone (*white columns*) for 10 min, 30 min, 1 h, or 48 h, and then the medium containing the peptides was replaced with fresh medium and cells were cultured for further 48 h. The cells were analysed for cell viability using WST-8. The results are represented as means ± SD (bars). (B) Permeabilisation of cell membranes by EGFR-lytic peptide. MDA-MB-231 breast cancer cells (3×10^4 cells/ml) in calcein solution were treated with EGFR-lytic peptide labelled with TAMRA at a final concentration of 10 μM for 0–20 min. Cells were then analysed by fluorescence (calcein-green or TAMRA-red). All images were taken using confocal microscopy as described in Section 2. Arrows and arrow heads indicate penetrated cells and membrane-permeabilizing peptides, respectively. Scale bars, 50 μm.

viability. Confocal microscope analysis also demonstrated that EGFR-lytic peptide penetrated the cell membrane to make a pore on the cancer cell surface, and the influx of calcein-labelled medium to cytosol of cancer cells was observed within 20 min (Fig. 3B), however, this rapid penetrating was not observed with lytic peptide (Supplementary Fig. S4). These results suggest that EGFR-lytic peptide kills cancer cells quite rapidly compared to lytic peptide. *In vitro* clonogenic assay also demonstrated that EGFR-lytic peptide inhibited the cell growth of H322 cancer cells in concentration dependent manner (Fig. 4A). These results next prompted us to investigate the mechanism of cell death caused by EGFR-lytic peptide. As assessed by flow cytometry analysis, Annexin V or caspase 3, 7 positive cells were found when EGFR-lytic peptide was added to breast cancer BT-20 cells (Fig. 4B). In addition, TUNEL assay also showed that there were several TUNEL positive cells after treatment with EGFR-lytic peptide (Fig. 4C). These results suggest that EGFR-lytic peptide induces cancer cell death by apoptotic mechanism.

3.6. *Antitumour activity of EGFR-lytic peptide in vivo*

To assess the antitumour effect of EGFR-lytic peptide in a xenograft model of human cancer, BXPC-3 pancreas or MDA-MB-231 breast cancer cells, which are K-ras mutation negative or positive cells, respectively, were implanted subcutaneously into athymic nude mice. Administration of EGFR-lytic peptide (2, 5 or 10 mg/kg, administered *i.v.* three times a week for a total of nine doses) suppressed the tumour growth remarkably (Fig. 5A and B). As shown in Fig. 5A, the tumour size of BXPC-3 on day 55 in the 2, 5 and 10 mg/kg dosage group were reduced to 34% (440 mm^3; $P < 0.05$), 30% (399 mm^3; $P < 0.05$) and 19% (244 mm^3; $P < 0.01$), respectively, of the control tumours in saline-treated animals (1310 mm^3). As shown in Fig. 5B, the tumour size of MDA-MB-231 on day 47 in the 2 and 5 mg/kg dosage group were reduced to 49% (933 mm^3; $P < 0.01$) and 22% (419 mm^3; $P < 0.01$), respectively, of the control tumours in saline-treated animals (1885 mm^3). In addition, histological examination of liver, kidney and spleen

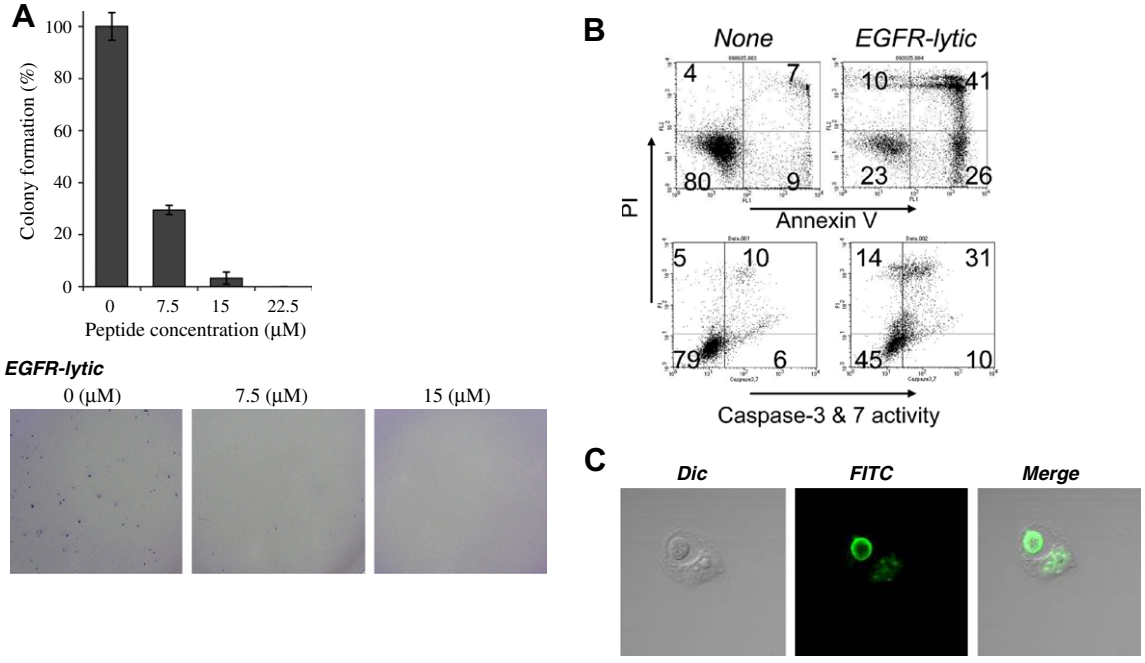

Fig. 4 – Characterisation of EGFR-lytic peptide induced cancer cell death. (A) EGFR-lytic peptide inhibits the cell growth. H322 cells were cultured for 10 days in medium containing various concentrations (0–22.5 μM) of EGFR-lytic peptide. Colonies consisting of at least 50 cells were scored after staining with crystal violet, and the results are expressed as a percentage relative to untreated cells, based on number of colonies. Untreated cells formed 117 ± 10 colonies. Data are the means of duplicate determinations; bars, SD. (B) BT-20 cells were incubated with EGFR-lytic peptide (5 μM) then analysed after 2 h by dual-colour flow cytometry for Annexin V labelling (upper panel), or for caspase 3 and 7 activity according to DEVDase activity (lower panel) in the green channel and PI staining in the red channel. The percentage of cells in each quadrant is indicated. (C) TUNEL positive cells treated with EGFR-lytic peptide. TUNEL assay was performed by fluorescence (FITC) phase-contrast (DIC) or merge image (TUNEL and DIC) after 6 h treatment with EGFR-lytic peptide. All images were taken using confocal laser scanning microscopy as described in Section 2.

was equally unremarkable in the saline or EGFR-lytic peptide-treated mice (Fig. 5C). A slight body weight loss was observed in the 10 mg/kg dose receiving mice during the treatment period, however, which recovered in all the mice after the treatment. In addition, all mice tolerated the treatment remaining healthy (data not shown). These results suggest that EGFR-lytic peptide successfully induces both K-ras mutation positive and negative tumours death *in vivo*.

4. Discussion

In this study, we have linked two functional domains of peptides to produce a novel chimeric peptide termed 'hybrid peptide', which is designed as a bifunctional peptide that binds to EGFR and disrupts the plasma membrane to target the EGFR-overexpressing cancer cells. First, we modified and developed the D-K_6L_9 peptide reported previously by Papo and Shai,[20] since this peptide was not considered suitable for the chimerisation with the targeting peptide (Supplementary Fig. S2). It was found that novel designed hybrid peptide (EGFR-lytic peptide) in this study had selectivity between normal and cancer membrane and was suitable for the combination with the targeting peptide. In addition, treatment with EGFR-lytic peptide exerted a sufficient cytotoxicity against TKI-resistant cancer cells with K-ras mutation. We found that this EGFR-lytic peptide killed cancer cells more rapidly and efficiently when compared with lytic peptide. On the other hand, normal cells were only minimally susceptible to both peptides.

In clinical, most patients initially respond well to chemotherapy, but later, chemoresistance is acquired. To solve this problem, the concept of 'cytotoxic' targeted therapy, e.g. immunotoxins has been developed. However, the toxic part of bacterial or plant toxin-based chimeric proteins elicit a high degree of humoral response in humans, and neutralisation antibody of immunotoxins limits their clinical applications.[23] To combat these problems, the concept of 'non-cytotoxic' molecular-targeted therapy with TKIs has been developed.[24] Recently, it is revealed that mutations in kinase-related signal molecule genes in cancer cells result in the resistance to TKIs.[25] These problems have prompted us to develop the concept of novel cytotoxic molecular-targeted therapy with hybrid peptide.

We propose the mechanism of action of EGFR-lytic peptide in cancer killing as follows. First, the EGFR binding moiety of the hybrid peptide binds to EGFR on the cell surface, and then, the lytic moiety of this peptide penetrates to make a pore and disrupts the cell membrane. Because of the binding of the EGFR binding moiety to EGFR, EGFR-lytic peptide penetrates and disrupts the cell membrane more rapidly than lytic peptide alone. The EGFR binding moiety binds specifically[12] and efficiently to EGFR as assessed by Biacore system (Supplementary Fig. S3), therefore, the affinity between the EGFR binding moiety and EGFR must be greater than the affinity

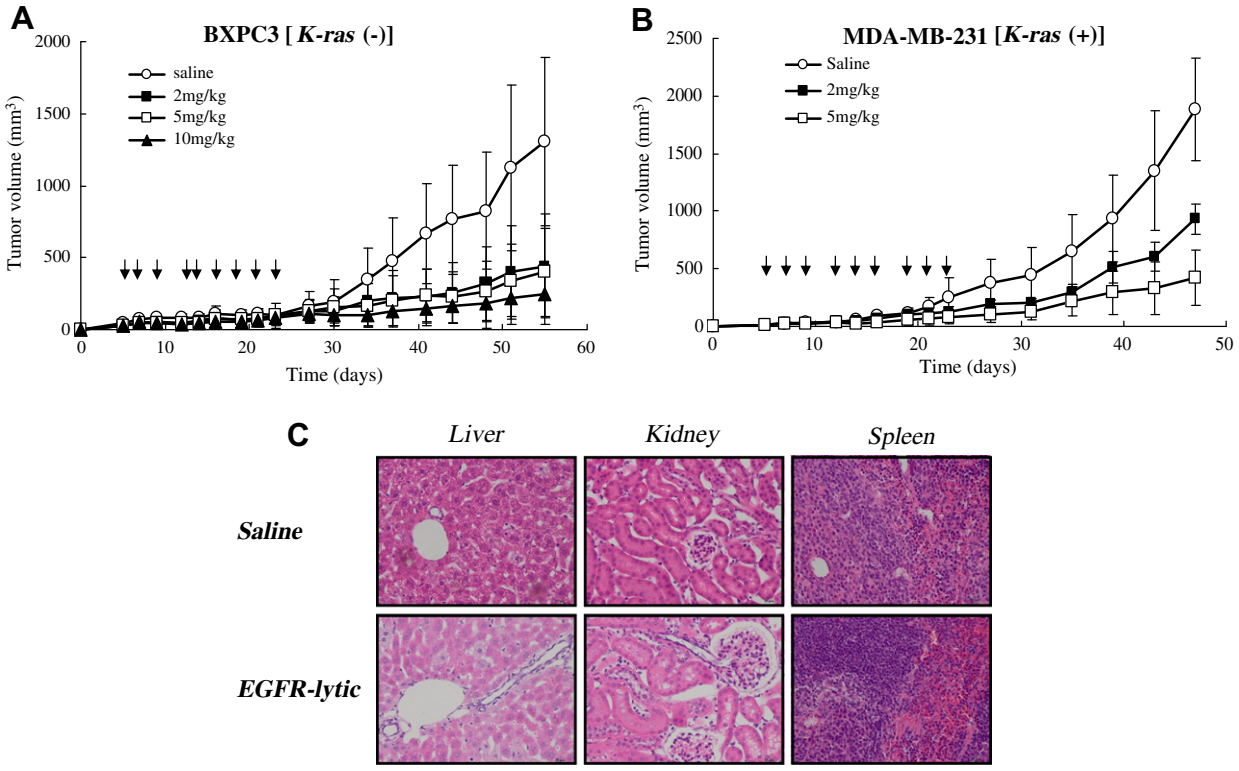

Fig. 5 – EGFR-lytic peptide has anti-tumour activity to both K-ras mutation positive and negative tumours. BXPC-3, K-ras mutation negative [K-ras (–)] pancreas cancer cell (A) and MDA-MB-231, K-ras mutation positive [K-ras (+)] breast cancer cell (B) were implanted subcutaneously into athymic nude mice. *I.v.* injection of either saline (control) or EGFR-lytic peptide (2, 5 or 10 mg/kg) was provided from day 5 as indicated by the arrows. Each group had six animals ($n = 6$), and experiments were repeated twice. Data are expressed as mean ± SD. (C) Histological examination after treatment with EGFR-lytic peptide. Images (×400 magnification) of liver, kidney and spleen from mice after treatment with saline (control) or EGFR-lytic peptide (5 mg/kg) nine times were obtained by staining with hemaetoxylin and eosin (H&E).

between lytic peptide and cell membrane. On the other hand, *Pseudomonas* exotoxin-based immunotoxins including interleukin-13-*Pseudomonas* exotoxin (IL13-PE38QQR) induces apoptosis partially, and mere 10–30% of head and neck cancer cells undergo apoptotic cell death.[26] The cancer cells treated with EGFR-lytic peptide were Annexin V and caspase 3, 7 positive as assessed by flow cytometry and TUNEL positive cells were found. In addition, this peptide also caused rapid cancer cell death (Fig. 3 and 4). From the above observations, it is suggested that this hybrid peptide has an advantage in rapid inducing of cancer cell death compared with bacterial toxin-based immunotoxin, and might be able to induce bystander effect or innate immunity at the treatment site *in vivo*.

Although it is suggested that peptides are relatively easily inactivated by serum components in human body, it has been shown that diastereomeric peptides are relatively free from inactivation in serum,[21] and a lytic diastereomeric peptide administered *i.v.* reduces the tumour growth of animal model of human prostate and breast cancer without rapid degradation of the peptide in blood at the dose of 5–9 mg/kg.[27] There are many anticancer peptides targeting intracellular molecules or organelle.[18,19] In this study, we first designed a novel peptide drug targeting extracellular target of cancer cells which accordingly attacks the cell with lytic moiety, and showed enough antitumour activity *in vivo*.

Immunotoxin is composed of a targeting moiety such as a ligand or an antibody for the cancer cell selectivity linked to a killing moiety such as a protein toxin. Immunotoxins can be divided into two categories which are the chemical conjugates as first-generation and the recombinant immunotoxins as second-generation.[28] These conventional immunotoxins usually show hurdles in the clinical use, e.g. immunogenicity, undesirable toxicity, difficulty in manufacture, limited half-life and production of neutralising antibody.[4,29,30] However, because peptides can be synthesised chemically, production of peptides can be performed with affordable cost compared to protein drugs. Furthermore, because the peptides are easy to produce, a variety of combinations of candidate peptides for targeting and toxic moieties can be tested generally easily in preclinical settings. For example, the toxic moiety of mitochondriotoxic[18] or antibiotics peptides[31] with tumouricidal activity can be utilised as the toxic moieties. In addition to EGFR, interleukin-11[32] and prostate-specific membrane antigen (PSMA)[33] can also be targeted, but the candidate peptides with the toxic moiety need more powerful cytotoxic activity like plant or bacterial toxin, and must be widely exploited together with candidate peptides for the targeting on cancer cells.

In conclusion, hybrid peptide that targets unique protein on the cancer cells would be a new possible tool for the cancer

target therapy. With our proposed concept for this hybrid peptide, we will proceed to reintroduce the next generation of immunotoxin. The research and development of hybrid targeted-peptide would enable personalised treatment of cancer according to the individual profile in future, targeting tumour-unique target of the surgically resected tumour from patients with specific hybrid peptide. Ultimately, this strategy may be useful for the treatment of not only cancers but also other diseases.

Conflict of interest statement

Koji Kawakami is a founder and stock holder of Upstream Infinity, Inc. The other authors disclose no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.10.021.

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