Novel EGFR-targeted strategy with hybrid peptide against oesophageal squamous cell carcinoma

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Supplementary Information

Supplementary figure legends

Supplementary Fig. S1 Real-time cell viability of single cells after exposure to EGFR(2R)-lytic hybrid peptide *in vitro*.

TE-11R cells were transiently transfected with pGL4.50 vector and time-course analysis of ATP bioluminescence was performed with (right panel) or without (left panel) exposure to EGFR(2R)-lytic hybrid peptide (10 μM) for the indicated durations. All data represent the relative luminescent intensity of the ROIs. Squares in the luminescence images indicate the ROIs. Note that luminescence had disappeared within 30 minutes following exposure to EGFR(2R)-lytic hybrid peptide. All scale bars are 200 μm.

Supplementary Fig. S2 Body weight change of BALB/c mice after exposure to EGFR(2R)-lytic hybrid peptide.

EGFR(2R)-lytic hybrid peptide (■: 4 mg/kg), lytic peptide (◇: 4 mg/kg), or PBS (○) was intravenously injected twice a week for a total of six doses, as indicated by the arrows. The body weights were monitored twice a week. Each

point represents the mean ± SEM (bars). n = 5.

Supplementary Fig. S3 Effects of inhibition of EGFR signalling on cytotoxicity of EGFR(2R)-lytic hybrid peptide

(A) Phospho-EGFR and total-EGFR expression levels in whole-cell lysates of TE-11R cells in the presence or absence of preincubation with EGFR-tyrosine kinase inhibitor (Erlotinib). The phospho-EGFR and total-EGFR protein expression levels were determined by Western blotting. The relative density of phospho-EGFR was calculated by densitometry. β-Actin served as a loading control. Phospho-EGFR protein levels in TE-11R were reduced by Erlotinib (1 μM, 60 min) preincubation.

(B) Affinity of EGFR-binding peptide for TE-8 and TE-11R cells with or without preincubation with EGFR-tyrosine kinase inhibitor. TE-8 and TE-11R cells were exposed to Fluorescein (FLC)-labelled EGFR-binding peptide in the presence or absence of Erlotinib preincubation (1 μM, 60 min). The fluorescence intensity was assessed by flow cytometry. Note that the affinity of EGFR-binding peptide to the cell membrane of those cells was not influenced by treatment with Erlotinib. The assays were repeated three times, and data are shown as the

mean ± SD.

(C) TE-8 and TE-11R cells were cultured with various concentrations (0–50 μM) of EGFR(2R)-lytic hybrid peptide for 30 minutes with or without preincubation with Erlotinib (1 μM, 60 min), and cell viability was determined by the WST-1 assay. A viability of 100% was defined as the amount of absorption at 450 nm found in control cells. Each point represents the mean ± S.D. (bars) from experiments conducted in sextuplicate, and the assays were repeated three times. Note that the inhibition of EGFR signalling does not affect the cytotoxicity of EGFR(2R)-lytic hybrid peptide against OSCC cells.

Supplementary Fig. S4 The amino acid sequence alignment of human and mouse EGFRs.

The alignment of the EGFR extracellular domain (25 - 633 amino acids) against human (NP_005219.2) and mouse (NP_997538.1) EGFRs. Amino acid sequence alignment was performed using clustalw (http://www.genome.jp/tools/clustalw/), and the identity of these sequences was confirmed as 89% consistent by NCBI Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi), respectively. " * " indicates positions that

have a single, fully conserved residue. " : " indicates that one of the following 'strong' groups is fully conserved: STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW (single amino acid code). " . " indicates that one of the following 'weak' groups is fully conserved: CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY (single amino acid code).

Supplementary Fig. S5 Affinity of EGFR-binding peptide for mouse-derived cells *in vitro*.

HEK293 or Colon26 cells (mouse-derived cells) were exposed to fluorescein (FLC)-conjugated EGFR-binding peptide (10 μM). The affinity of EGFR-binding peptide for cell membranes was assessed by flow cytometry. Note that EGFR-binding peptide showed an affinity for Colon26 cells.

Supplementary table legend

Supplementary Table S1. Inhibitory concentration (IC_{50}) of EGFR(2R)-lytic hybrid peptide for OSCC and HEK293 cells.

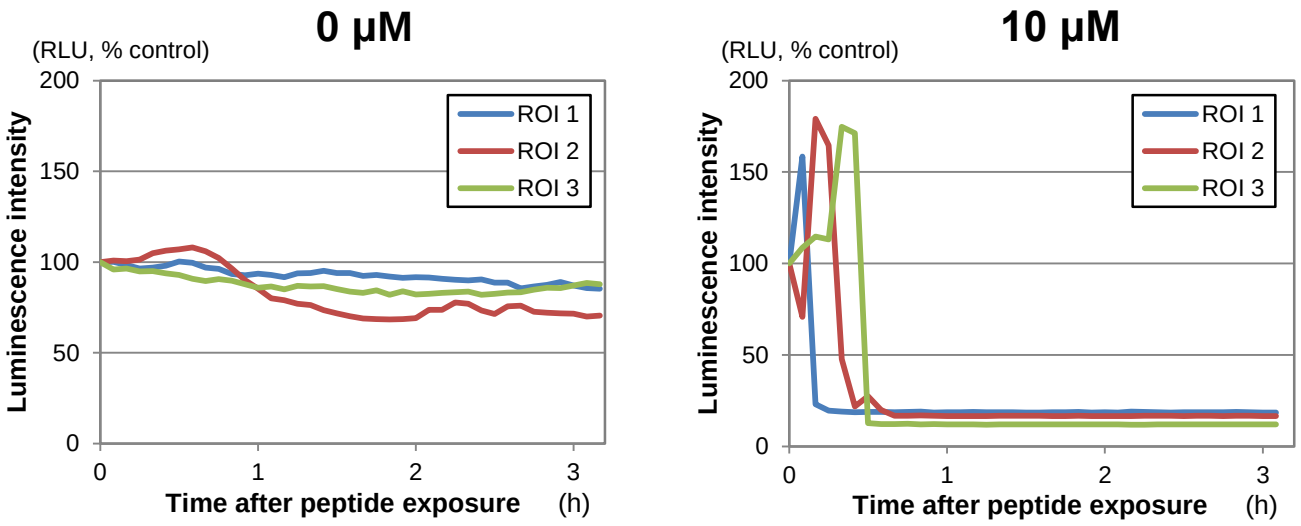
The inhibitory concentration (IC_{50}) of EGFR(2R)-lytic hybrid peptide for OSCC cells was calculated from the results of the WST-1 assay (Fig.3). Data are

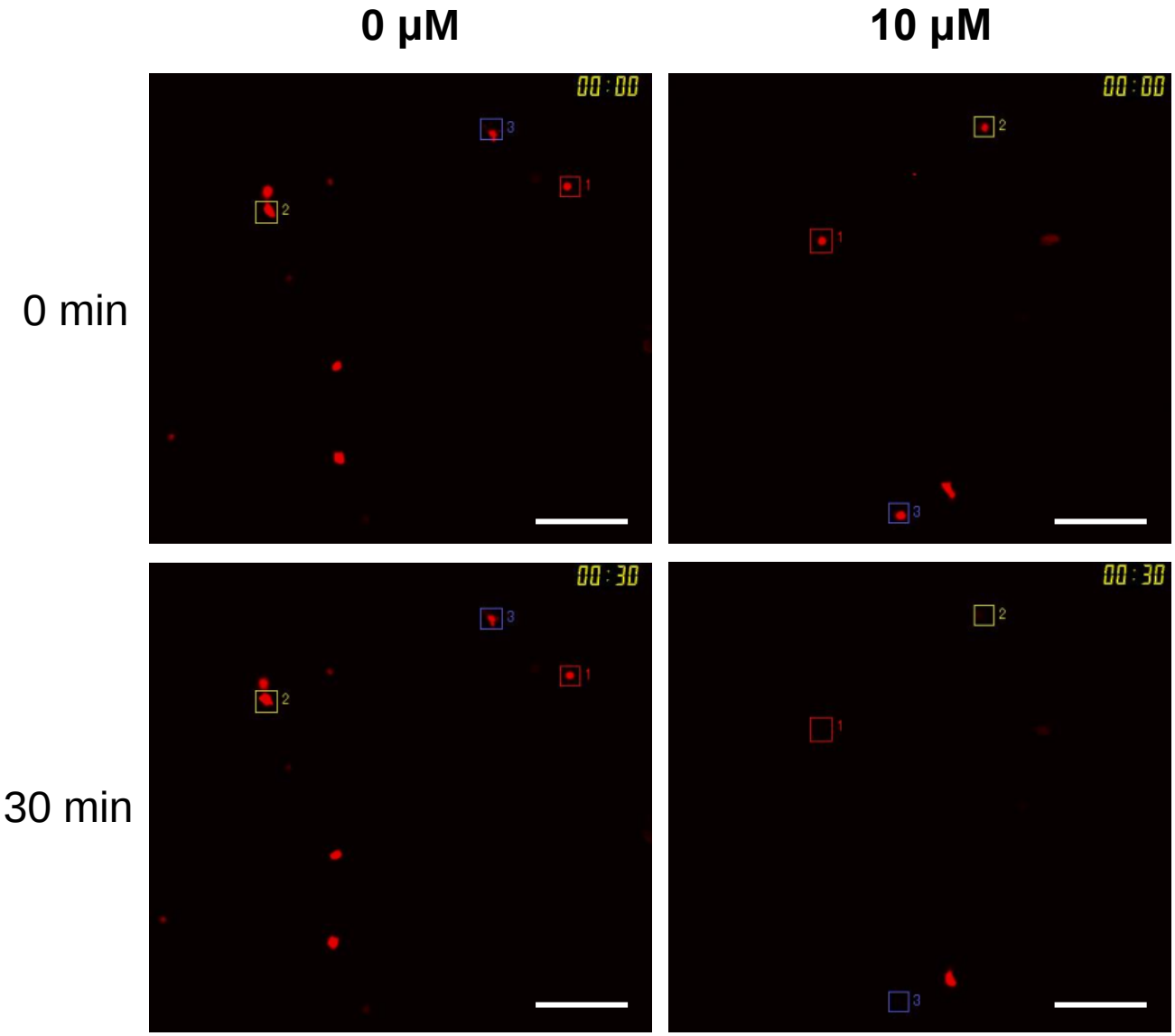
shown as the mean ± S.D. of three independent experiments, each performed in sextuplicate. Note that EGFR(2R)-lytic hybrid peptide exhibits potent cytotoxicity against all OSCC cells at very low concentrations of less than 10 μM.

Supplementary methods

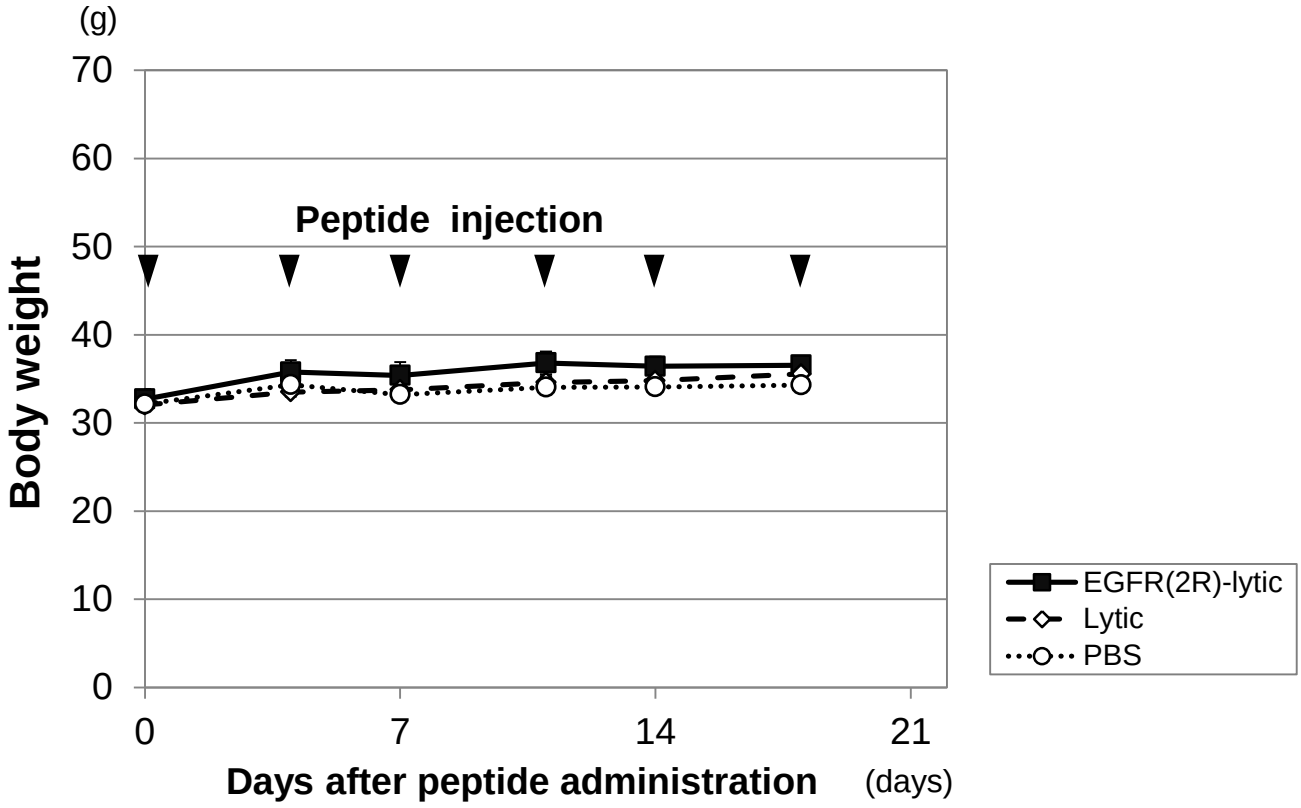
Primary antibodies and the titres used in the studies of Supplementary Fig. S3 were as follows: rabbit monoclonal phospho-EGFR (Tyr1068) antibody (D7A5, #3777, Cell Signaling; 1:1,000), rabbit monoclonal EGFR antibody (D38B1, #4267, Cell Signaling; 1:1,000), rabbit monoclonal anti-β-actin antibody (13E5, #5125, Cell Signaling; 1:5,000). β-Actin served as a loading control for whole-cell lysates.

Supplement Figure S1.



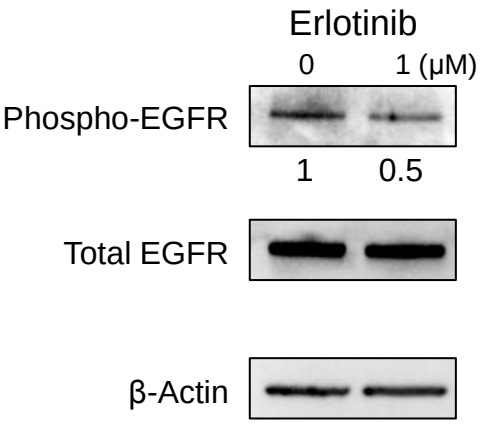


Supplement Figure S2.



Supplement Figure S3.

(A)



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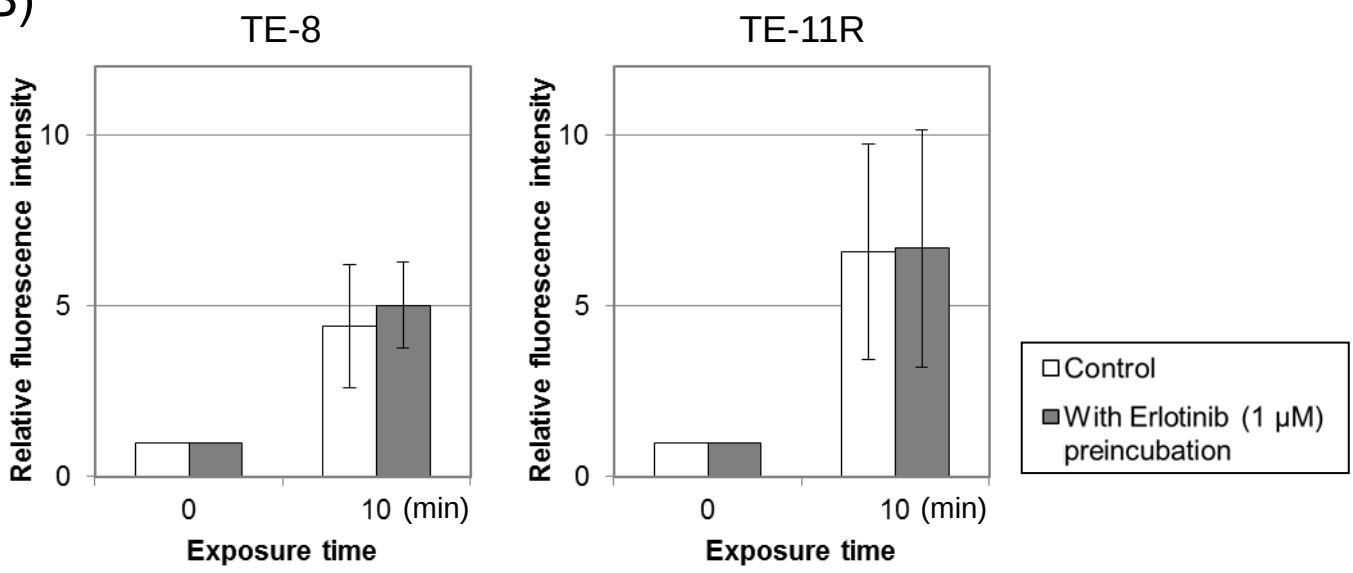

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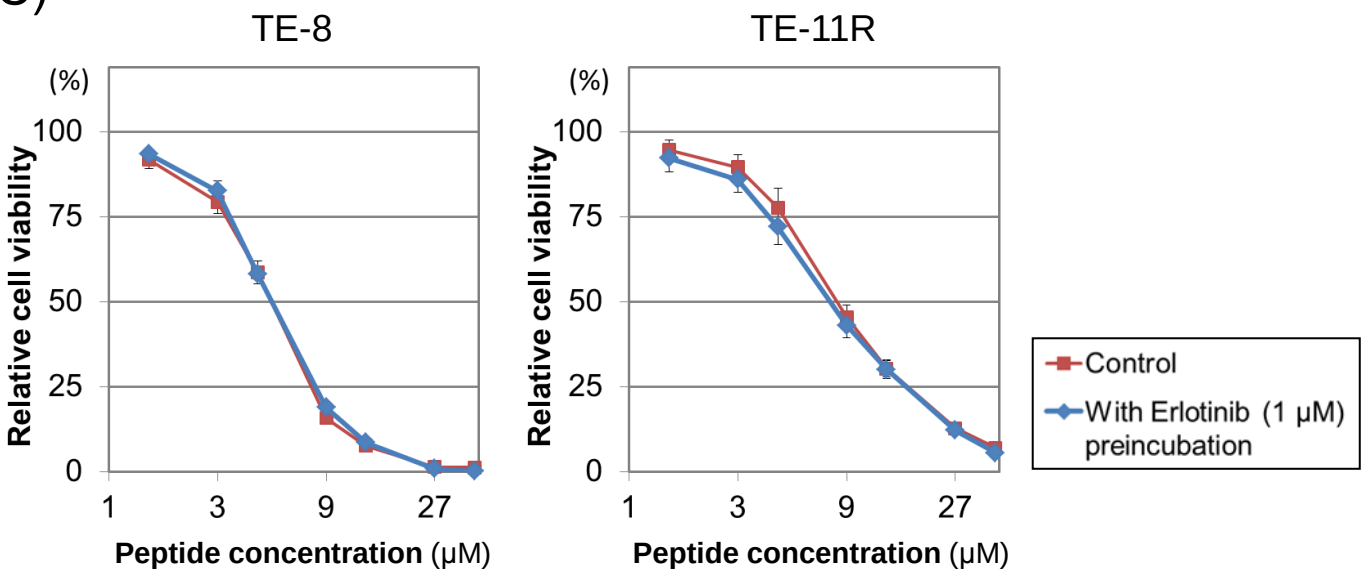
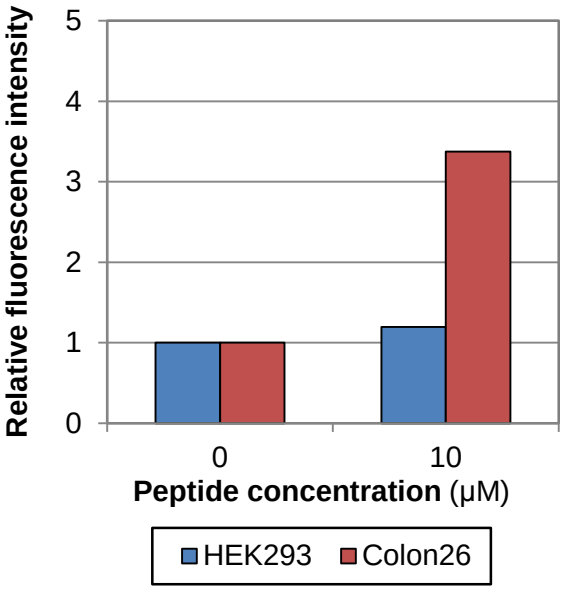


Supplement Figure S4.

Supplement Figure S5.



Supplement Table S1.

Inhibitory concentration (IC_{50}) of EGFR(2R)-lytic hybrid peptide

Cell	**IC_{50} (μM)**
HEK293	29.8 ± 8.6
TE-5	4.0 ± 0.1
TE-8	4.5 ± 0.2
TE-10	6.3 ± 0.6
TE-11	6.9 ± 0.3
TE-5R	3.6 ± 0.1
TE-11R	4.2 ± 0.4