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(54) Titre : PEPTIDE PROVENANT DU RECEPTEUR DU FACTEUR DE CROISSANCE EPIDERMIQUE (EGFR)
(54) Title: PEPTIDE ORIGINATING IN EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR)

(57) Abrégé/Abstract:
It is intended to provide an EGFR-origin peptide usable in EGFR-based immunotherapy for cancer. Namely, an EGFR-origin peptide capable of inducing both cellular and humoral immune responses or a mutant peptide thereof; a polypeptide containing the above peptide; a nucleic acid molecule encoding the same; and a medicinal composition containing the same.

ABSTRACT

The object of the invention is to provide an EGFR-derived peptide useful for EGFR-based immunotherapy.

The invention provides an EGFR-derived peptide capable of inducing both cellular and humoral immune responses and mutant peptide thereof and a polypeptide comprising said peptide, a nucleic acid molecule encoding the same, and a pharmaceutical composition comprising the same.

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SPECIFICATION

EPIDERMAL GROWTH FACTOR RECEPTOR-DERIVED PEPTIDES

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

[0001]

The invention relates to an EGFR-derived peptide useful for EGFR-based immunotherapy for cancer. In addition, the invention relates to a polypeptide comprising the EGFR-derived peptide capable of inducing both cellular and humoral immune responses and also a cancer vaccine containing said peptide.

BACKGROUND OF THE INVENTION

[0002]

Epithelial growth factor receptor (EGFR) plays an important role in epithelial biology and in many human malignancies (References 1-3). EGFR is a member of the receptor family comprising four, highly homologous proteins, HER2, HER3, and HER4 as well as EGFR. Those proteins in this family consist of an extracellular domain, a transmenbrane domain, and an intracellular tyrosine kinase domain (Reference 20). Binding of the ligand such as epithelial growth factor (EGF) activates the intracellular tyrosine kinase domain to induce autophosphorylation of the receptor, which initiates the signaling cascade involved in cell proliferation and survival (Reference 20). The activation of EGFR highly involved in the processes of tumor proliferation and progression, including cell proliferation, inhibition of apoptosis, angiogenesis and metastasis (Reference 19). EGFR shows relatively high expression in approximately one-third of all types of epithelial cancers and the expression correlates with tumor progression, and therefore it is one of the most suitable targets in cancer therapy (References 21, 22).

[0003]

As EGFR-targeted therapies, monoclonal antibodies which bind to the

extracellular ligand binding site of the receptor and inhibitors for the intracellular tyrosine-kinase domain were intensively studied. Among them, a novel EGFR-tyrosine-kinase inhibitor ZD1839 is known to be effective for advanced non-small cell lung cancer (NSCLC) (References 4, 5).

[0004]

It has been known that a living body has an immune system to eliminate tumor cells developed and that cytotoxic T lymphocyte (CTL) plays the central role in the system. CTL specifically recognizes an antigen presented on a tumor cell via a major histocompatibility complex (HLA in human) to kill the tumor cell. Taking advantage of the immune system for tumor cells, vaccine therapies, which include immunization of a body with epitope peptides of tumor antigens, have been attempted to potentiate the cytotoxicity against tumor cells.

[0005]

Epitope peptides of HER2/neu, a member of the receptor family of EGFR, capable of inducing HLA-class I-restricted CTL were reported in the past decade (References 6-9). The inventers of the present invention previously reported that some CTL-directed peptides derived from non-mutated proliferation-related proteins had the ability to elicit both cellular and humoral immune responses in vivo in clinical studies (References 10-12). Further, levels of anti-peptide Abs in post-vaccination sera were well correlated with overall survival of advanced lung cancer patients who received peptide vaccination (Reference 12). In addition, there is a line of evidence for higher immunogenicity of a peptide capable of inducing both cellular and humoral immune responses (References 13-15), which can be expected to have more potent therapeutic activity.

[0006]

The CTL epitope peptide of EGFR may be useful in cancer therapies in a different way from existing compounds, because it can be used as a peptide vaccine in EGFR-targeted therapies for cancer patients with tumors overexpressing EGFR. So far, however, there is no information about CTL epitopes of EGFR.

[0007]

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DISCLOSURE OF THE INVENTION

PROBLEM TO BE SOLVED BY THE INVENTION

[0008]

An object of the invention is to provide a peptide which is useful as a cancer vaccine in view of the development of EGFR-based cancer therapy.

MEANS OF SOLVING THE PROBLEM

[0009]

To identify EGFR-derived peptides capable of inducing humoral immune response, the inventors first investigated whether specific antibodies against EGFR-derived peptides are present in sera of NSCLC patients and healthy donors (HDs). Those peptides could induce CTLs which specifically kill EGFR-expressing tumor cells, and thus the present invention was accomplished.

[0010]

Accordingly, the invention provides:

(1) an EGFR-derived peptide or mutant peptide thereof which is capable of inducing a CTL and an antibody specific for said peptide, preferably the peptide which is an HLA-A24- or HLA-A2-restricted peptide;

(2) the peptide of (1), wherein the EGFR-derived peptide consists of at least 8 consecutive amino acid residues derived from the amino acid sequence of $EGFR_{800-809}$, $EGFR_{124-132}$, $EGFR_{54-62}$, $EGFR_{479-488}$ or $EGFR_{1138-1147}$;

(3) a polypeptide consisting of 8 to 50 amino acid residues, which comprises the peptide of (1) or (2) and is capable of inducing a CTL and an antibody specific for said peptide;

(4) a nucleic acid molecule encoding the peptide of (1) or (2) or a polypeptide comprising said peptide;

(5) a vector comprising the nucleic acid molecule of (4);

[0011]

(6) a pharmaceutical composition comprising the peptide of (1) or (2) , the polypeptide of (3), or the nucleic acid molecule of (4) for inducing a CTL and an antibody specific for said peptide;

(7) the pharmaceutical composition of (6), which is used as a cancer vaccine;

(8) an EGFR-reactive CTL which recognizes a complex between the peptide of (1) or (2) or the polypeptide of (3) and an HLA molecule;

(9) a method of inducing an EGFR-reactive CTL using the peptide of (1) or (2) or the polypeptide of (3);

(10) an antibody which specifically recognizes the peptide of (1) or (2) or the polypeptide of (3).

BRIEF DESCRIPTION OF THE DRAWINGS

[0012]

Fig. 1 shows the representative histograms demonstrating EGFR expression on tumor cells analyzed by a flow cytometry assay. The dot line shows the result with the second antibody (FITC-bound control antibody) only, and the black line shows that with an anti-EGFR monoclonal antibody plus the second antibody.

Fig. 2 shows the detection of the anti-peptide IgGs in the serum samples.

Fig. 3A shows the peptide specificity of the anti-peptide IgGs in the serum samples.

Fig. 3B shows the result of the assay for cross-reactivity of the anti-peptide IgGs to the whole EGFR protein.

Fig. 4 shows the CTL induction by the EGFR-derived peptides. * $P<0.05$ (Student's t-test).

Fig. 5 shows the cytotoxic activity of peptide-stimulated PBMCs against tumor cell lines. * $P<0.05$ (Two-tailed student's t-test).

Fig. 6 shows HLA-restricted and peptide specific cytotoxicity demonstrated by the inhibition assay and the competition assay.

Fig. 7 shows the detection of anti-peptide IgGs in the serum samples.

Fig. 8 shows the peptide specificity of the anti-peptide IgGs in the serum samples.

Fig. 9 shows the CTL induction by the EGFR-derived peptides. PBMCs derived from HLA-$A2^+$ cancer patients were stimulated with any of the peptides and their IFN-γ production against T2 cells (HLA-A2, T-B hybridoma) pulsed with the corresponding peptide were determined. * $P<0.05$ (Student's t test).

Fig. 10 shows the cytotoxic activity of peptide-stimulated PBMCs against tumor cell lines. SKOV3-A2(HLA-$A2^+$, $EGFR^+$) and SKOV3 (HLA-$A2^-$, $EGFR^+$) were used as the tumor cell lines. * $P<0.05$ (Two-tailed student's t-test).

Fig. 11 shows HLA-restricted and peptide specific cytotoxicity demonstrated by the inhibition assay and the competition assay. Peptide-pulsed T2 cells were used for the competition assay.

BEST MODE FOR CARRYING OUT THE INVENTION

[0013]

Peptide and Polypeptide

The EGFR-derived peptide of the invention is capable of inducing both cellular and humoral immune responses and has a high immunogenicity. The inventers have reported that IgGs reactive against CTL epitope peptides were often detected in pre-vaccination sera of cancer patients and HDs (References 10 -12, 16, 17). Further, some CTL-directed peptides have the ability to elicit both cellular and humoral immune responses in vivo in the phase I clinical studies, and the levels of anti-peptide Ab in post-vaccination sera well correlated with over-all survival of advanced cancer patients who received peptide vaccination (References 11,12). In addition, the adverse events found in a number of clinical studies of EGFR-targeted cancer therapies, such as acne-like eruption and diarrhea (References 23, 24, 25), have not been observed in the phase I clinical studies of vaccine therapy performed by the inventers with EGFR-derived peptides. Those results indicate that the peptides of the invention are useful as cancer vaccines in EGFR-targeted cancer therapy.

Preferably, the peptide of the invention is a HLA-A24- or HLA-A2-restricted peptide. The peptide capable of inducing both cellular and humoral immune responses is, for example, $EGFR_{800-809}$ (SEQ ID NO. 1), $EGFR_{124-132}$ (SEQ ID NO. 2), $EGFR_{54-62}$

(SEQ ID NO. 3), $EGFR_{479-488}$ (SEQ ID NO. 4) or $EGFR_{1138-1147}$ (SEQ ID NO. 5). The whole amino acid sequence of EGFR is deposited in GeneBank with the deposition number of CAA25240 (SEQ ID NO. 6).

Other EGFR-derived peptides capable of inducing their specific CTLs and antibodies can be easily identified and selected according to the working examples hereinafter. Considering the activity of immune response induction, $EGFR_{43-51}$ and $EGFR_{943-952}$ are also potential peptides of the invention.

[0014]

The invention also includes the mutant peptide of any one of the above peptides of SEQ ID NOS. 1 to 5, wherein the mutant peptide has the CTL- and antibody-inducing activities equivalent to those of its original peptide. The alteration may be deletion, substitution, addition, or insertion of one or more of amino acids in the EGFR-derived peptide of the invention, and the methods for such alteration are well known in the art. The mutant peptide can be selected according to the recognition by CTL. The number of amino acid residues of the mutant peptide may be that sufficient to be presented on an antigen presenting cell and to work as a CTL-recognizing epitope, and at least 8, preferably at least 9 and more preferably 9 or 10.

[0015]

The invention further provides the polypeptide comprising the EGFR-derived peptide of the invention or mutant peptide thereof, wherein the polypeptide is capable of inducing the specific CTL and antibody. The polypeptide generally has the length of amino acid residues of 8-50, preferably 8-30, more preferably 9-10 or 8-10. The polypeptide preferably comprises the EGFR-derived peptide selected from $EGFR_{800-809}$ (SEQ ID NO. 1), $EGFR_{124-132}$ (SEQ ID NO. 2), $EGFR_{54-62}$ (SEQ ID NO. 3), $EGFR_{479-488}$ (SEQ ID NO. 4) and $EGFR_{1138-1147}$ (SEQ ID NO. 5).

[0016]

The peptide and polypeptide of the invention may be modified on their constituent amino acids or carboxyl groups to the extent that their functions are not significantly damaged.

The peptide and polypeptide of the invention may be synthesized by any of

usual methods known in peptide chemistry.

[0017]

Nucleic acid molecule

The nucleic acid molecule of the invention includes a single-stranded polynucleotide (including complementary strand thereof) and a double-stranded polynucleotide encoding the amino acid sequence of the EGFR-derived peptide of the invention, the mutant peptide thereof or the polypeptide comprising the same. The nucleic acid molecule of the invention may be DNA or RNA. The peptide having the amino acid sequence encoded by the nucleic acid molecule can be recognized by CTL to activate the CTL and function as a tumor antigen.

In addition, the nucleic acid molecule of the invention may be the polynucleotide or complementary strand thereof consisting of at least 24 bases corresponding to the coding region for the peptide of the invention. The polynucleotide can be selected, for example, by checking the peptide expressed from the polynucleotide by any of known protein expression systems.

[0018]

Antibody

The antibody of the invention specifically recognizes a peptide consisting of at least 5 consecutive amino acid residues which is derived from any of the amino acid sequences of the EGFR-derived peptides or the polypeptides of the invention. The antibody can be prepared using its epitope peptide which consists of at least 5, preferably at least 8-10 amino acids. The present invention encompasses said peptide consisting of at least 5 amino acids and also the nucleic acid molecule encoding said peptide. The amino acid sequence of the epitope is not necessarily identical to the amino acid sequence of any of SEQ ID NOS. 1 to 5, but the peptide consisting of the amino acid sequence has to be recognized by CTL.

The antibody of the invention can be prepared by immunizing a suitable animal, such as mouse, rat, rabbit, goat and the like, with the epitope peptide of EGFR or that of an EGFR-derived peptide or polypeptide, alone or in conjunction with any suitable carrier, in the absence or presence of adjuvant, to induce the antibody production. The

polyclonal antibodies obtained can be collected from the serum of the animal by any of known methods.

Further, a monoclonal antibody can be prepared by fusing the antibody-producing cells collected from the immunized animal as above to tumor cells which replicate endlessly. This method is well known in the art.

Those polyclonal and monoclonal antibodies are useful for purification or as a reagent, a labeling marker, and the like. As far as we examined, anti-EGFR peptide IgGs fail to directly inhibit tumor growth in vitro and to elicit antibody-dependent cell-mediated cytotoxicity against tumor cells (data is not shown herein). The anti-EGFR peptide IgG, therefore, may not act on tumor cells. The anti-peptide IgG, however, may facilitate infiltration of immunocompetent cells into tumor sites through induction of inflammatory reactions around tumor sites; inflammatory reactions around tumors were observed at the time of surgery (radical prostatectomy) for prostate cancer patients who had received the peptide vaccination prior to the prostatectomy; in the same patients, increased levels of IgG reactive to the vaccinated peptides were observed in the sera of post-vaccination but pre-surgery (Noguchi et al., unpublished results). The antibody of the invention is thereby assumed to have a potential to help the anti-tumor activity. Moreover, IgGs reactive to CTL epitope peptides were either lacking or unbalanced in sera of patients with atopic disease (Reference 17). These results suggest that IgGs to CTL peptides are involved in host-defense against various diseases, although underlying mechanism of anti-tumor immune responses in cancer patients is presently unclear.

[0019]

<u>Pharmaceutical composition</u>

The pharmaceutical composition of the invention can be prepared with the EGFR-derived peptide or polypeptide of the invention, the nucleic acid molecule encoding the same, the vector prepared based on the sequence of said nucleic acid molecule, or the antibody of the invention, or combination thereof.

Particularly, the EGFR-derived peptide of the invention or mutant peptide thereof and the polypeptide comprising said peptide can be used as cancer vaccines.

The pharmaceutical composition of the invention is useful as a cancer vaccine in EGFR-based immunotherapy, and it can be used for treating epithelial cancer, such as non-small-cell lung cancer, ovarian cancer, prostate cancer, breast cancer, gastric cancer, GIST tumor (gastrointestinal stromal tumor), pancreas cancer and the like. "EGFR-targeted therapy" is herein used in a broad sense and includes not only therapies using antibodies but also those using antagonists of the ligand (in this case EGFR) or inhibitors of signal transducers (including receptors and any component of receptor-meditated signal transductions, as EGFR is a receptor of cell growth factor EGF). In contrast, "EGFR-based immunotherapy" is used in a narrower sense, wherein EGFR is the target molecule of the antibody or T cells.

For the pharmaceutical composition of the invention, combinations of more than one peptide are preferably used, although a single peptide is still useful as a cancer vaccine. This is because CTLs of a cancer patient consist of groups of cells each recognizing different tumor antigens and therefore such combination are expected to be more effective than a single peptide as cancer vaccines. The peptides of the invention may be combined each other.

[0020]

The peptide or polypeptide of the invention as a cancer vaccine may be used in the presence or absence of any suitable adjuvant, alone or as a mixture or conjugate with any pharmaceutically acceptable carrier. The carrier is not limited as long as it has no adverse effect on a human body, and the examples are cellulose, amino acid polymers, and albumin. The dosage form may be selected from those well known for peptide drugs. The dose is 0.01-100mg/day/adult human, preferably 0.1-10mg/day/adult human, although it may vary depending on the recognition by CTL, and it may be administered once in several days or several months.

[0021]

The pharmaceutical composition of the invention may comprise an appropriate vector which includes the nucleic acid sequence encoding the peptide of the invention. The composition can be used in vivo or ex vivo. The vector may be retrovirus, adenovirus or vaccinia virus, and preferably retrovirus. The dose is 0.1μg-

100mg/day/adult human, preferably 1μg -50mg/day/adult human, although it may vary depending on the recognition by CTL. It may be administered once in several days or several months.

[0022]

Method for induction of CTL

The EGFR-reactive CTL is induced, for example, with the peptide of the invention from peripheral blood cells (PBMCs) of a NSCLC patient.

In brief, PBMCs isolated from a NSCLC patient are incubated with antigen presenting cells (APCs) pulsed with the peptide of the invention to induce CTLs, and the induction is evaluated by IFN-γ production of the cells. The activity of the CTLs induced can be confirmed by ^{51}Cr release assay which indicates the tumor cytotoxicity of the cells

The above method may be useful for adoptive immunotherapy in which antigen-specific CTLs induced in vitro are returned to the patient to kill his tumor cells.

[0023]

The present invention is further described by the following Examples, but not limited by them in any sense.

EXAMPLES

[0024]

Example 1

Immunogenic EGFR-derived peptides

A. Identification of the activity of inducing humoral immune response

This study was made to determine whether Immunogloblin G (IgG)s reactive to EGFR-derived peptides could be detected in sera of 13 NSCL cancer patients and 11 HDs.

The following 18 EGFR-derived peptides with HLA-A24 binding motif were purchased from BioSynthesis (Lewisville, TX). Those peptides correspond to positions 43-51, 54-62, 68-76, 73-82, 111-119, 124-132, 269-277, 625-633, 722-730, 800-809, 812-821, 899-907, 899-908, 943-952, 960-969, 1015-1023, 1015-1024, and

1068-1077 of EGFR, respectively. An HIV peptide with HLA-A24 binding motif (RYLRDQQLLGI) was also provided as a negative control.

[0025]

After written informed consent was obtained, sera and peripheral blood mononuclear cells (PBMCs) were collected from NSCLC patients and HDs at Kurume University Hospital, and the sera and PBMCs were cryopreserved at − 80℃ and - 196 ℃ until use, respectively. All subjects were free from HIV infection. Expression of HLA-class I antigens on these PBMCs was serologically defined by the conventional methods as reported previously (Reference 10).

Peptide-specific IgG levels in sera were measured by an enzyme-linked immunosorbent assay (ELISA) as reported previously (Reference 11). Briefly, serum samples were serially diluted with 0.05% Tween 20-Block Ace (Yukijirushi nyugyo, Hokkaido, Japan), and 100 μl /well of the diluted serum were added to the peptide (20 μg/well)-immobilized Nunc Covalink plates (Fisher Scientific, Pittsburgh, PA). Anti-peptide Abs were detected with rabbit anti-human IgG (γ-chain-specific) (DAKO, Glostrp, Denmark). For determining the limit of sensitivity of ELISA, sera from 10 healthy donors (HIV-negative) were measured for their reactivity to an HIV peptide by the assays. The mean ± SD of absorbance (A) indicated at 0.020 ± 0.02, and the mean + SD value (0.04) was then determined as the cut-off value.

[0026]

Fig. 2 shows the representative results of three NSCLC patients (Pts. 2, 3, and 10) and three HDs (HDs 2, 4, and 10). The A value against the HIV peptide used as a negative control was subtracted from the data.

Summary of the results on 11 peptides, to which sera of some subjects showed positive responses, is given in Table 1.

[0027]

[Table 1]

Table 1 Humoral responses to the EGFR peptides

		Responses to the EGFR peptides (OD values)										
Subjects	HLA	EGFR899-908	EGFR1015-1024	EGFR800-809	EGFR269-277	EGFR899-907	EGFR124-132	EGFR812-821	EGFR625-633	EGFR73-82	EGFR54-62	EGFR1015-1023
Pt.1	A24/2	-	-	-	-	-	-	-	-	-	0.05	-
Pt.2	A24/33	-	-	0.13	-	-	0.05	-	-	-	0.05	-
Pt.3	A24/2	-	-	0.14	0.06	-	0.07	-	-	-	0.07	-
Pt.4	A2/11	0.05	0.16	0.06	0.05	0.05	0.05	0.05	0.05	-	0.05	0.06
Pt.5	A24/31	-	-	-	-	-	-	-	-	-	-	-
Pt.6	A2	-	-	-	-	-	-	-	-	-	-	-
Pt.7	A1/24	0.13	-	-	-	-	-	-	-	0.06	-	-
Pt.8	A24	-	-	0.05	-	-	0.49	-	-	-	-	-
Pt.9	A24/2	-	-	0.05	-	-	-	-	-	-	-	-
Pt.10	A24/11	-	-	0.09	-	-	0.13	-	-	-	-	-
Pt.11	A24/2	-	-	-	-	-	-	-	-	-	-	-
Pt.12	A24/2	-	-	0.13	-	-	0.08	-	-	-	0.09	-
Pt.13	A24	-	-	0.13	-	-	0.12	-	-	-	0.14	-
HD1	A24/33	0.08	0.09	0.08	-	-	-	-	-	-	-	-
HD2	A24/26	-	0.20	0.16	0.10	-	0.10	-	-	-	0.06	0.05
HD3	A2/26	0.22	1.50	0.20	0.18	0.34	0.09	0.15	0.05	-	0.13	0.66
HD4	A24/26	-	-	0.09	-	-	0.05	-	-	-	-	-
HD5	A2/24	-	-	0.08	-	-	-	-	-	-	-	-
HD6	A11/33	-	-	-	-	-	-	-	-	-	-	-
HD7	A2/24	-	-	0.05	0.05	-	-	-	-	-	-	-
HD8	A31/33	-	-	-	-	-	-	-	-	-	-	-
HD9	A2/24	-	-	0.19	-	-	-	-	-	-	-	-
HD10	A2/11	-	-	0.11	-	-	0.05	-	-	-	-	-
HD11	A2/24	-	-	0.18	-	-	0.07	-	-	-	0.14	-
Anti-peptides Abs	Pt.(n=13)	2	1	8	2	1	7	1	1	1	6	1
	HD (n=11)	2	3	9	3	1	5	1	1	0	3	2

The OD values lower than the cut-off (0.04) were shown as -.

[0028]

Significant levels of IgG reactive to the $EGFR_{800-809}$, $EGFR_{124-132}$, and $EGFR_{54-62}$ peptides (A value>0.04 at serum dilution of 1:100) were detected in sera of 8, 7, and 6 patients, respectively. Sera from 9, 5, and 3 out of 11 HDs tested also showed the significant levels of IgG reactive to $EGFR_{800-809}$, $EGFR_{124-132}$, and $EGFR_{54-62}$, respectively. In addition, significant levels of IgG reactive to the $EGFR_{899-908}$, $EGFR_{1015-1023}$, $EGFR_{269-277}$, $EGFR_{899-907}$, $EGFR_{812-821}$, $EGFR_{625-633}$, $EGFR_{73-82}$, and $EGFR_{1015-1023}$ peptides were detected in sera from one or two cancer patients as well as a few HDs. The immune response to EGFR peptides observed in both cancer patients and HDs may not be surprising since EGFR is expressed not only in epithelial cancer cells but also in certain normal epithelial cells (References 1-3). Humoral responses to these EGFR peptides were observed in both HLA-A24 positive and -A24 negative subjects, although the majority of subjects were HLA-A24 positive. In contrast, significant levels of IgG reactive to the remaining 7 peptides were not detectable in any serum tested (data not shown).

These results indicate that, among the 18 synthetic peptides, $EGFR_{800-809}$, $EGFR_{124-132}$, and $EGFR_{54-62}$ are more preferable for induction of the immune response aimed by the present invention.

[0029]

B. Evaluation of the peptide specificity of anti-peptide antibodies

The peptide specificity of anti-peptide IgG in the serum sample to each of the $EGFR_{800-809}$, $EGFR_{124-132}$, and $EGF\text{-}R_{54-62}$ peptides was confirmed by an absorption test.

100 μl/well of serum samples (x100 dilution with 0.05% PBS) were absorbed with immobilized peptides (20 μg/well) in wells of plate for 2h at 37°C. The absorption was repeated three times followed by testing of the anti-peptide IgG with ELISA.

Representative results from sera of Pts.2, 3, 8 and 10 are shown in Fig 3A. The activities of these sera reactive to each of the three peptides were absorbed with the corresponding peptide, but not with the HIV peptide taken as a negative control (Fig 3A).

To test whether the anti-peptide IgGs are reactive to the whole molecule of EGFR, patients' sera possessing anti-peptide activity were also absorbed with either immobilized EGFR isolated from human A431 cells with the purity of 85% (Upstate Charlottesville, USA) or immobilized human albumin as a negative control followed by measuring their anti-peptide activities by ELISA. Representative results from sera of Pts.3 and 12 are shown in Fig 3B. The level of the anti- peptide IgG reactive to any of the three peptides was not decreased at all by the absorption test (Fig. 3B), suggesting no cross-reactivity of the peptide IgG to the whole EGFR protein.

The results indicate that $EGFR_{800-809}$, $EGFR_{124-132}$, and $EGFR_{54-62}$ peptides of the invention can induce peptide specific humoral immune response.

[0030]

Example 2

<u>CTL induction with EGFR-derived peptides</u>

A. IFN-γ production

$EGFR_{800-809}$, $EGFR_{124-132}$, and $EGFR_{54-62}$ peptides were tested for their ability to induce CTL in PBMCs of HLA-$A24^+$ NSCLC patients and HDs, utilizing IFN-γ production as an indicator of the induction.

For induction of peptide-specific CTLs, PBMCs ($15x10^4$ cells/well) were incubated with 10 μM of each peptide in the four different wells of a 96-well microculture plate (Nunc, Roskilde, Denmark) in 200 μl culture medium containing IL-2, as reported previously. For peptide loading, C1R-A2402 cell line (HLA-2402 transfectant) was used (Reference 12). On the 14th day, the cells from each well were independently harvested and washed. These cells were divided into the four parts and each two of them were incubated for 18h with C1R-A2402 cells pulsed in duplicate with a corresponding peptide or the negative control (HIV) peptide, and then the supernatants were collected for measurement of IFN-γ by ELISA. Background IFN-γ production in response to the HIV peptide (< 50pg/ml) was subtracted from the data. As a control for the ability to induce CTL activity, the two peptides ($EGFR_{43-51}$ and $EGFR_{943-952}$), to which IgG response was not detectable at all, were also tested.

[0031]

Representative results of the four cases (Pts.1, 11, 13 and HD11) are shown in Fig. 4, in which the results from each of the four wells were provided.

The summary of all subjects is given in Table 2. The well of successful induction of peptide-specific CTLs was judged to be positive when the supernatant of well showed more than 100pg/ml IFN-γ production with *p*-value of < at least 0.05. The mean values of the amount of IFN-γ of the positive wells among the 4 wells tested are shown in Table 2.

[0032]

[Table 2]

Table 2 Cellular responses to the EGFR peptides

		Responses to the EGFR peptides (INF-γ production (pg/ml))				
Subjects	HLA	EGFR800-809	EGFR54-62	EGFR124-132	EGFR43-51	EGFR943-952
Pt-1	A24/2	376/172	-	168/150	509/432	126/115
Pt-2	A24/33	-	-	138	-	-
Pt-3	A24/2	430	154/170	-	160	-
Pt-7	A1/24	572	116	-	-	122/376
Pt-10	A24/11	-	-	-	-	-
Pt-11	A24/2	-	134	192	-	-
Pt-12	A24/2	122	202	-	-	-
Pt-13	A24	166/144	231/115	118	-	-
HD-1	A24/33	-	110/116	166	130	-
HD-2	A24/26	161/863/184	316/314	1375/724	-	-
HD-4	A24/26	164	132/116	176/206	-	-
HD-5	A2/24	-	-	-	-	-
HD-11	A2/24	280/190	410/150	267	-	-

(-) indicates when none of the 4 wells showed the positive response.

[0033]

The $EGFR_{800-809}$, $EGFR_{54-62}$, and $EGFR_{124-132}$ peptides stimulated PBMCs in at least one of four wells to produce significant amounts of IFN-γ in response to C1R - A2402 cells pulsed with the corresponding peptide in 5, 5, and 4 of cancer patients tested, respectively. These peptides also stimulated to produce IFN-γ in 3, 4, and 4 of 5 HDs tested, respectively. The $EGFR_{43-51}$ and $EGFR_{943-952}$, to which IgG responses were not observed, also stimulated PBMCs to produce significant amounts of IFN-γ in response to C1R - A2402 cells pulsed with the corresponding peptide in 2 of 8 cancer patients tested, respectively (Table 2). The $EGFR_{43-51}$ or $EGFR_{943-952}$, stimulated PBMCs in 1 or 0 of 5 HDs tested.

These results indicate that $EGFR_{800-809}$, $EGFR_{124-132}$, and $EGFR_{54-62}$, and are capable of inducing CTLs.

[0034]

B. Anti-tumor cytotoxic activity

The cytotoxicity of the peptide-stimulated PBMCs was evaluated by a 6 h ^{51}Cr-release assay to confirm the CTL induction.

Expression of EGFR on tumor cell lines was tested by flowcytometoric assay with immunofluorescence-labeled anti-EGFR monoclonal antibody (mAb) (Santa Cruz Biotechnology, Santa Cruz, CA) (Reference 13). A431 tumor cells and Phytohemagglutinin (PHA)-blastoid T cells were used as a positive and negative control, respectively. The representative results of histograms were shown in Fig. 1. Based on these results, the following tumor cell lines were used as target cells in the 6 hr-^{51}Cr-release assay in this study; 11-18 (HLA-A24/2, human lung adenocarcinoma, $EGFR^{+}$), QG56 (HLA -A26, lung squamous cell carcinoma (SCC), $EGFR^{+}$), Sq-1 (HLA -A24/11, lung SCC, $EGFR^{\pm}$) , LC65A (HLA -A24/11, non-small cell lung carcinoma, $EGFR^{+}$), SKOV3 (HLA -A3/28, ovarian cancer, $EGFR^{+}$) and SKOV3-A24 (HLA-A24-transfected SKOV3). PHA-blastoid T cells from PBMCs were used as a negative control of target cells for the 51Cr-release assay.

[0035]

The cells producing IFN-γ in response to the corresponding peptide in the

assay described above (A) were collected from the wells and further cultured with IL-2 alone for 10-14 days to obtain a large number of cells for the 6 h ^{51}Cr-release assay. The standard 6-h ^{51}Cr-release assay was performed at three E/T (effecter cells/target cells) ratios. This method was reported previously (Reference 12). Two-tailed Student's-t test was employed for the statistical analysis.

The representative results of the 4 patients (Pts. 1, 2, 3, and 13) are shown in Fig. 5. The values represent the mean ± SD of specific lysis (%). These peptide-stimulated PBMCs showed significant levels of cytotoxicity against all of the 11-18 NSCLC cells (HLA-A24$^+$, EGFR$^+$), LC65A non-small cell lung carcinoma cells (HLA-A24$^+$, EGFR$^+$), and SKOV3-A24 tumor cells (HLA-A24$^+$, EGFR$^+$), but failed to kill any of the QG56 NSCLC cells (HLA-A24$^-$, EGFR$^+$), Sq-1 NSCLC cells (HLA-A24$^+$, EGFR$^+$) and SKV3 tumor cells (HLA-A24$^-$, EGFR$^+$) tested. These PBMCs also failed to kill PHA-blastoid T cells (HLA-A24$^+$, EGFR$^-$). PBMCs stimulated with the HIV peptide, taken as a negative control, did not show the HLA-A24-restricted cytotoxicity (Fig. 5, the lowest left column). Those results suggest that the PBMCs possess HLA-A24-restricted cytotoxicity reactive to EGFR$^+$ tumor cells.

[0036]

Furthermore, the HLA-restricted and peptide-specific cytotoxicity were confirmed by the inhibition and competition assays, respectively.

For the inhibition assay, 20 μg/ml of anti-HLA-class I (W6/32, IgG2a), anti-HLA-class II (H-DR-1, IgG2a), anti-CD8 (Nu-Ts/c, IgG2a), anti-CD4 (Nu-Th/i, IgG1), and anti-CD14 (JML-H14, IgG2a) (as a negative control) mAbs were used. For the competition assay to study the peptide specific cytotoxicity, unlabeled C1R - 2402 cells pulsed with the corresponding peptide or the HIV peptide as a negative control were added to the ^{51}Cr-release assay at a cold to hot target cell ratio of 10 to 1. The values represent the mean ± SD of specific lysis (%), and two-tailed Student's-t test was employed for the statistical analysis.

The cytotoxicities of these peptide- stimulated PBMCs were significantly inhibited by anti-class I (W6/32) or anti-CD8 mAb, but not by the other mAb in the assay. The cytotoxicities were also inhibited by addition of the corresponding peptide-

pulsed C1R-A2402 cells, but not by the HIV peptide-pulsed cells (Fig. 6).

These results suggest that the CTL activities induced by $EGFR_{800-809}$, $EGFR_{124-132}$, and $EGFR_{54-62}$ peptides were largely mediated by the peptide-reactive $CD8^+$ T cells with an HLA-class I-restricted manner.

[0037]

Example 3

In a similar way to Examples 1 and 2, $EGFR_{479-488}$ and $EGFR_{1138-1147}$ were identified to be capable of inducing humoral immune response and HLA-A2-restricted cytotoxic immune response. The results were shown in Table 3 and Fig. 7 to 11.

[Table 3]

Table 3 Humoral responses to the peptides

			Responses to the EGFR peptides (OD values)										
Subjects	HLA	Subtype	EGFR10-18	EGFR61-70	EGFR110-118	EGFR479-488	EGFR599-607	EGFR653-662	EGFR654-662	EGFR729-738	EGFR765-776	EGFR852-861	EGFR1138-1147
Pt.1	A2/24	A0207	-[2]	-	-	-	-	-	-	-	-	-	-
Pt.2	A2/24	A0206	-	-	-	-	-	-	-	-	-	-	-
Pt.3	A2/24	A0206	-	-	0.09	0.15	-	-	-	0.17	0.08	0.07	0.15
Pt.4	A2/11	A0206	0.26	-	0.08	0.49	-	-	-	0.10	-	-	0.10
Pt.5	A2	A0201	-	-	0.09	0.33	-	-	-	-	-	-	0.08
Pt.6	A2/24	A0206	-	-	-	0.22	-	-	-	0.24	0.10	-	0.14
Pt.7	A2/3	A0201	-	-	-	-	-	-	-	-	-	-	-
Pt.8	A2/24	A0206	-	-	-	0.07	-	-	-	-	0.16	-	0.07
Pt.9	A2/24	A0201	-	-	-	0.15	-	-	-	0.13	-	-	0.09
Pt.10	A2	A0207	-	0.07	0.14	0.12	0.13	0.07	-	0.07	-	-	0.13
Pt.11	A24/33		-	0.07	-	0.10	0.17	-	0.09	0.17	-	-	0.16
Pt.12	A24		-	-	-	0.21	0.08	-	-	-	-	-	0.07
Pt.13	A24		-	-	-	-	0.07	-	-	-	-	-	-
Pt.14	A24		-	-	-	-	-	-	-	-	0.18	-	-
Pt.15	A24		-	-	-	0.55	-	-	-	0.59	-	-	-
Pt.16	A24		-	-	-	0.17	-	-	-	-	-	-	-
Pt.17	A24/31		-	-	-	0.24	-	-	-	0.10	-	0.07	0.10
Pt.18	A24		-	-	-	0.07	-	-	-	-	-	-	-
Pt.19	A24/33		-	-	-	-	-	1.22	-	0.31	-	-	-
Pt.20	A24/11		-	-	-	-	-	-	-	0.30	-	-	-
HD1	A2/24	A0206	-	-	-	-	0.20	-	0.07	-	-	-	0.13
HD2	A2/24	A0206	-	-	-	-	0.09	-	-	-	-	-	-
HD3	A2/11	A0206	-	-	-	0.26	-	-	-	-	-	-	-
HD4	A2/26	A0201	-	-	-	0.13	-	-	-	-	-	-	0.08
HD5	A2	A0206	-	-	-	-	-	-	-	-	-	-	-
HD6	A2/24	A0201	-	-	-	-	-	-	-	-	-	-	-
HD7	A24/33		-	-	-	-	-	-	-	-	-	-	-
HD8	A24/26		-	-	-	-	-	-	-	-	-	-	-
HD9	A24/26		0.07	0.07	-	-	0.20	-	0.12	-	-	-	0.26
HD10	A24		-	-	-	-	-	-	-	-	-	-	0.17
HD11	A11/33		-	0.09	0.09	-	0.13	-	0.07	-	-	-	0.15
Anti-peptide Abs		Pt (n=20)	1	2	4	13	4	2	1	10	4	2	10
		HD(n=11)	1	2	1	2	4	0	3	0	0	0	5

The OD values lower than the cut-off (0.06) were shown as -.

CA 02554195 2006-07-21

The above results indicate that EGFR-derived peptides of the present invention are useful as cancer vaccines in EGFR-based immunotherapy.

INDUSTRIAL APPLICABILITY

[0038]

$EGFR_{800-809}$、$EGFR_{124-132}$、$EGFR_{54-62}$、$EGFR_{479-488}$, and $EGFR_{1138-1147}$ of the invention have the ability to elicit both cellular and humoral immune responses, suggesting the higher immunogenecity of them as compared to previous HER2/neu-derived CTL epitope peptides (References 6-9).

[0039]

Although only a part of NSCLC patients respond to a tyrosine kinase inhibitor ZD1839, there is no suitable laboratory marker to predict the clinical response to it. It has been observed that the level of immune response to the EGFR peptides of the invention correlates to the clinical response to ZD1839, and therefore the EGFR peptides of the invention may be useful in the prediction of the clinical response to ZD1839 (References 4, 5).

[0040]

HLA-A24 allele was found in 60% of Japanese (with 95% of these cases being genotypically HLA-A2402), in 20% of Caucasians, and 12% in Africans (Reference 18). These findings may provide a new insight for development of the EGFR- based immunotherapy for substantial numbers of NSCLC patients in the world.

DEMANDES OU BREVETS VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS COMPREND PLUS D'UN TOME.

CECI EST LE TOME __1___ DE __2___

NOTE: Pour les tomes additionels, veillez contacter le Bureau Canadien des Brevets.

JUMBO APPLICATIONS / PATENTS

THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE THAN ONE VOLUME.

THIS IS VOLUME __1___ OF __2___

NOTE: For additional volumes please contact the Canadian Patent Office.

WHAT IS CLAIMED IS:

1. An epidermal growth factor receptor (EGFR)-derived peptide or mutant peptide thereof which is capable of inducing a cytotoxic T lymphocyte and an antibody specific for said peptide.

2. The peptide of Claim 1, wherein the EGFR-derived peptide consists of at least 8 consecutive amino acid residues derived from the amino acid sequence of $EGFR_{800-809}$, $EGFR_{124-132}$, $EGFR_{54-62}$, $EGFR_{479-488}$ or $EGFR_{1138-1147}$.

3. A polypeptide consisting of 8 to 50 amino acid residues, which comprises the peptide of Claim 1 or 2 and is capable of inducing a cytotoxic T lymphocyte and an antibody specific for said peptide.

4. A nucleic acid molecule encoding the peptide of Claim 1 or 2 or the polypeptide of Claim 3.

5. A vector comprising the nucleic acid molecule of Claim 4.

6. A pharmaceutical composition comprising the peptide of Claim 1 or 2, the polypeptide of Claim 3, or the nucleic acid molecule of Claim 4, for inducing a cytotoxic T lymphocyte and an antibody specific for said peptide.

7. The pharmaceutical composition of Claim 6, which is used as a cancer vaccine.

8. An EGFR-reactive cytotoxic T lymphocyte which recognizes a complex between the peptide of Claim 1 or 2 or the polypeptide of Claim 3 and an HLA molecule.

9. A method for inducing an EGFR-reactive cytotoxic T lymphocyte using the peptide of Claim 1 or 2 or the polypeptide of Claim 3.

10. An antibody which specifically recognizes the peptide of Claim 1 or 2 or the polypeptide of Claim 3.

Fig. 1

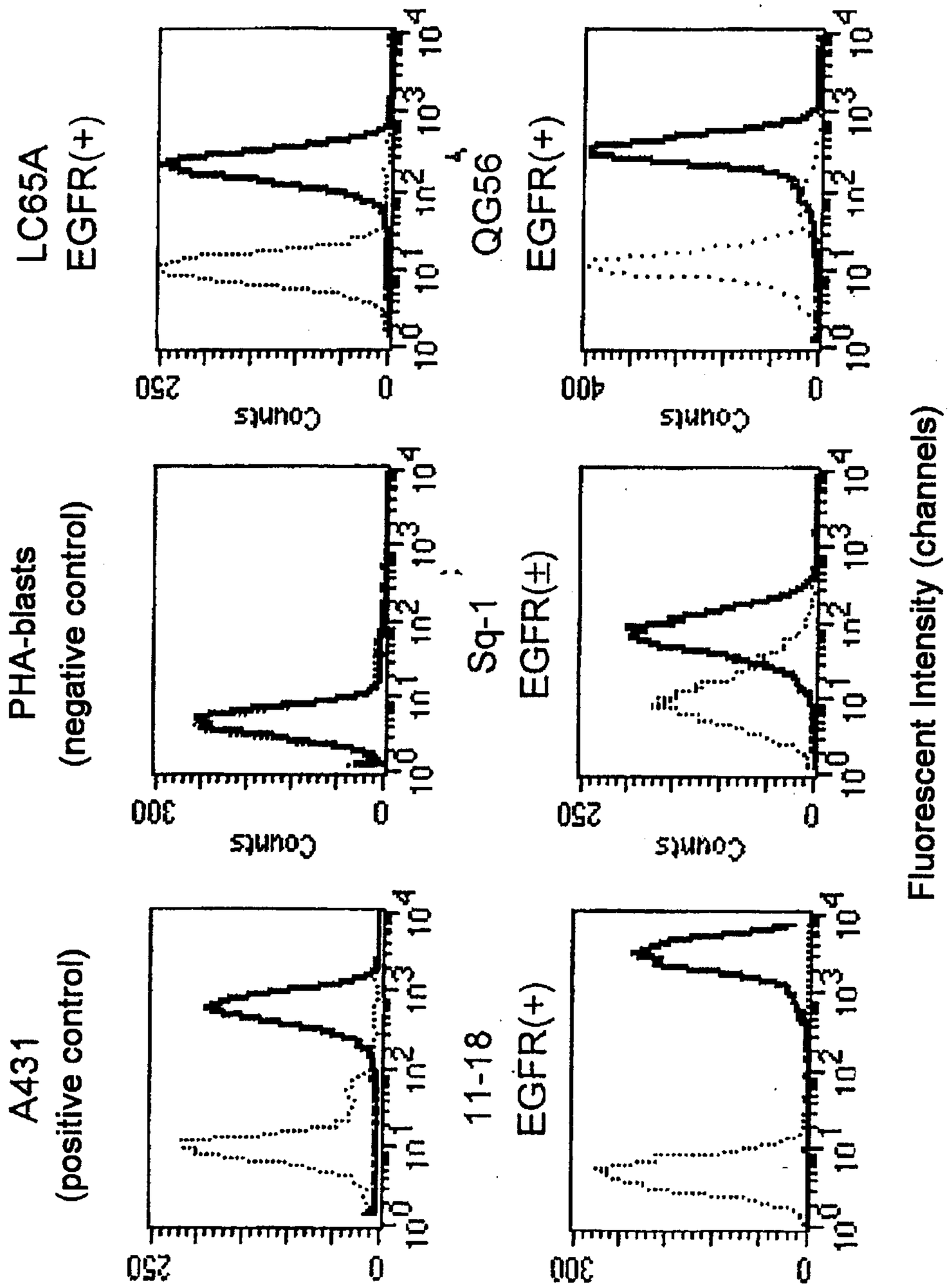

LC65A
EGFR(+)
QG56
EGFR(+)
PHA-blasts
(negative control)
Sq-1
EGFR(±)
A431
(positive control)
11-18
EGFR(+)
Counts
Fluorescent Intensity (channels)

Fig. 2

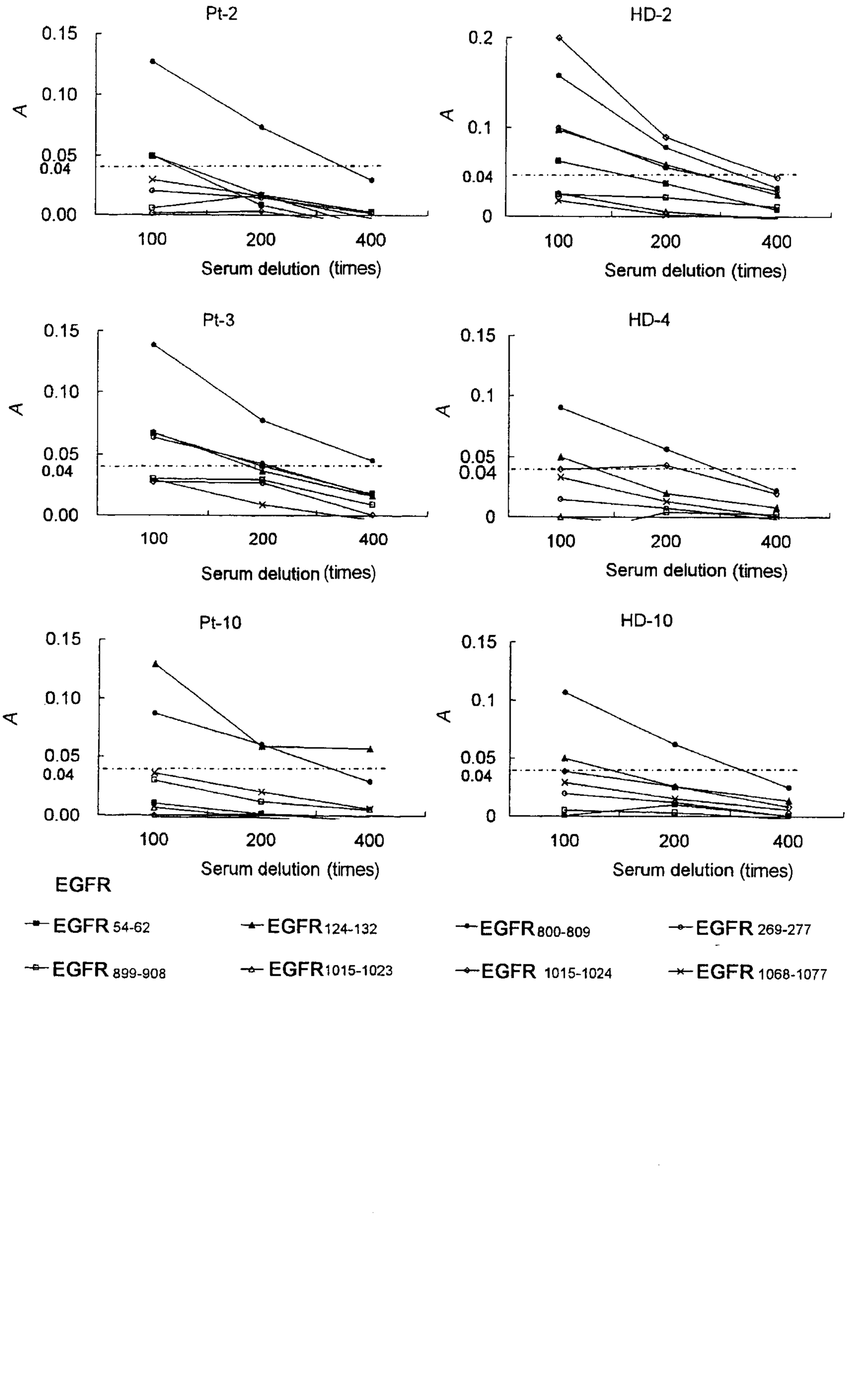

Pt-2
HD-2
Pt-3
HD-4
Pt-10
HD-10
A
0.15
0.10
0.05
0.04
0.00
0.2
0.1
0
100
200
400
Serum delution (times)
EGFR
EGFR 54-62
EGFR 124-132
EGFR 800-809
EGFR 269-277
EGFR 899-908
EGFR 1015-1023
EGFR 1015-1024
EGFR 1068-1077

Fig. 3A

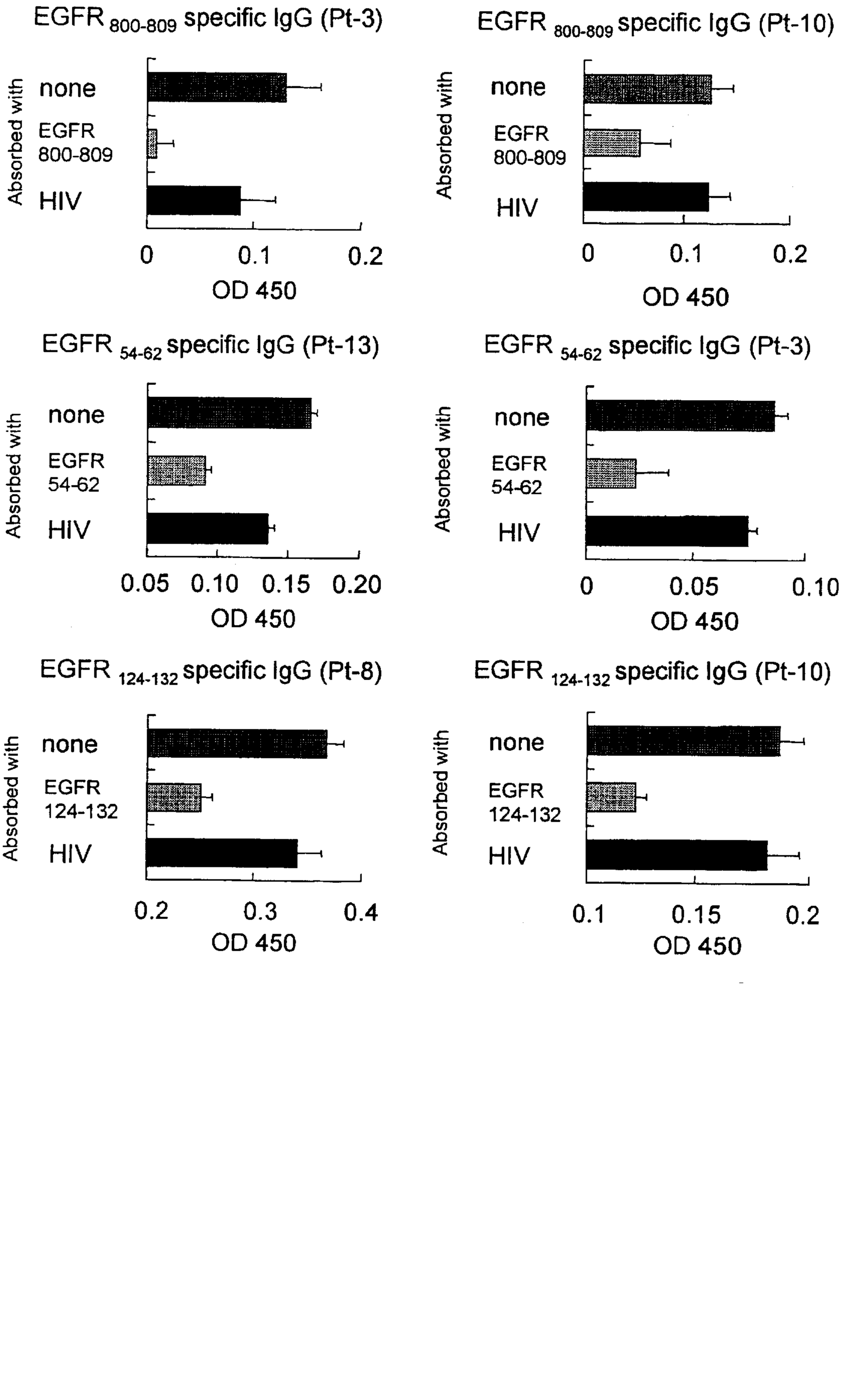
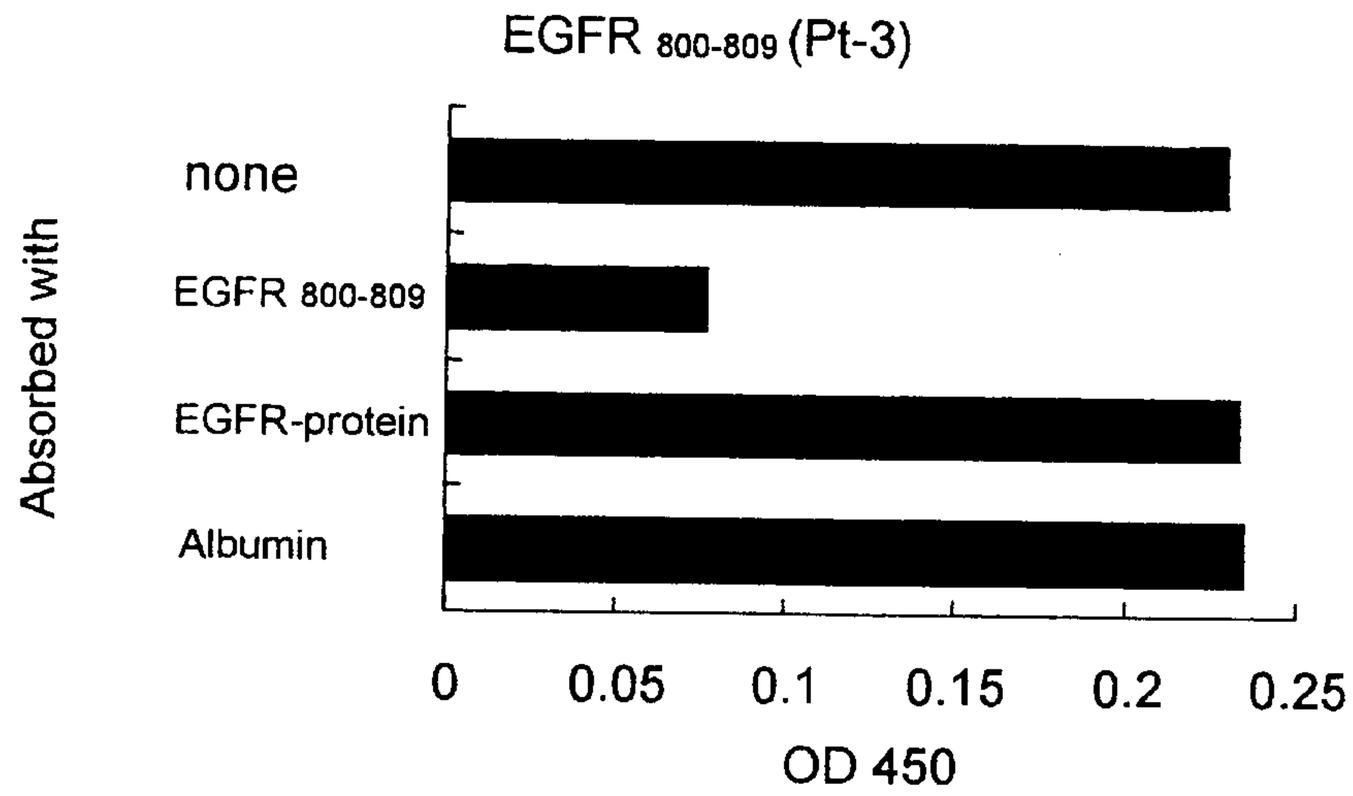
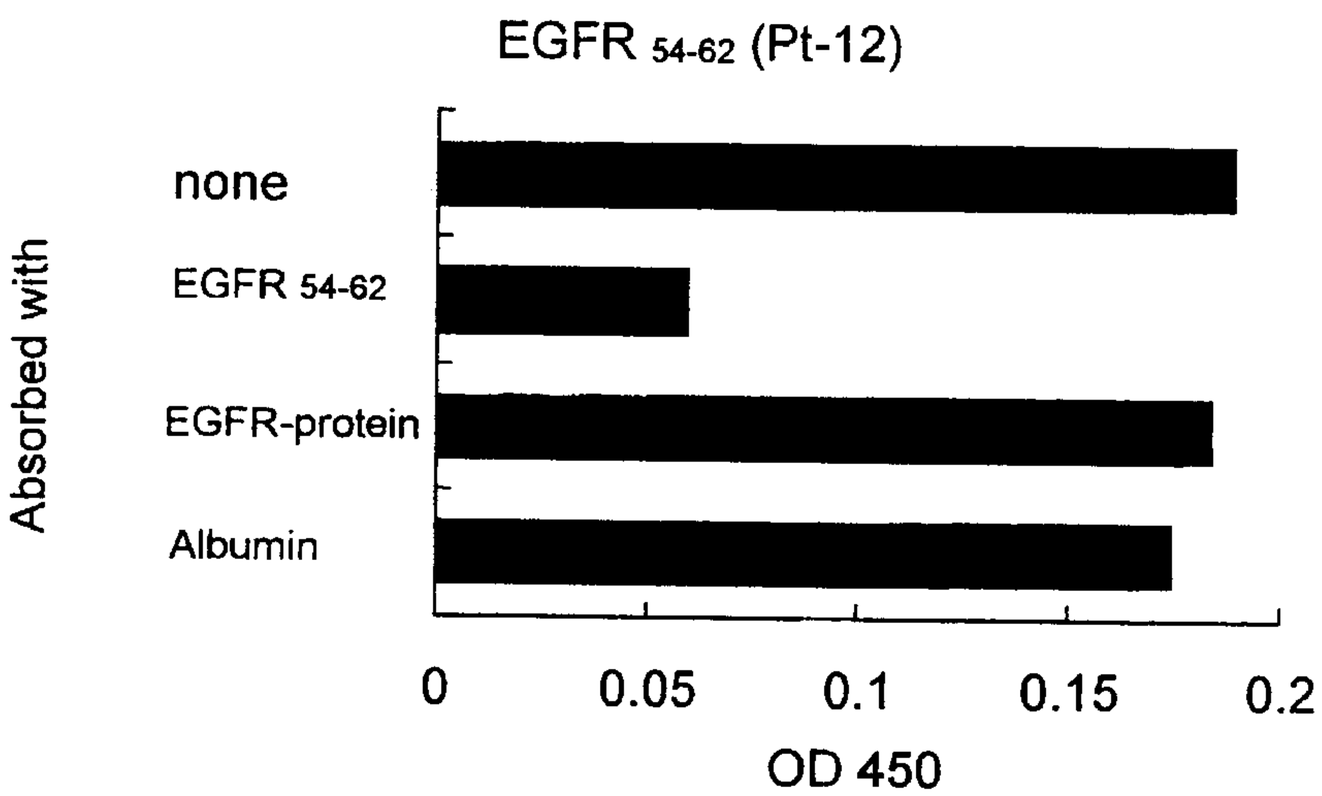

EGFR 800-809 specific IgG (Pt-3)
Absorbed with
none
EGFR 800-809
HIV
0
0.1
0.2
OD 450
EGFR 800-809 specific IgG (Pt-10)
Absorbed with
none
EGFR 800-809
HIV
0
0.1
0.2
OD 450
EGFR 54-62 specific IgG (Pt-13)
Absorbed with
none
EGFR 54-62
HIV
0.05
0.10
0.15
0.20
OD 450
EGFR 54-62 specific IgG (Pt-3)
Absorbed with
none
EGFR 54-62
HIV
0
0.05
0.10
OD 450
EGFR 124-132 specific IgG (Pt-8)
Absorbed with
none
EGFR 124-132
HIV
0.2
0.3
0.4
OD 450
EGFR 124-132 specific IgG (Pt-10)
Absorbed with
none
EGFR 124-132
HIV
0.1
0.15
0.2
OD 450

Fig. 3B


EGFR 800-809 (Pt-3)
Absorbed with
none
EGFR 800-809
EGFR-protein
Albumin
0
0.05
0.1
0.15
0.2
0.25
OD 450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EGFR 54-62
EGFR-protein
Albumin
0
0.05
0.1
0.15
0.2
OD 450

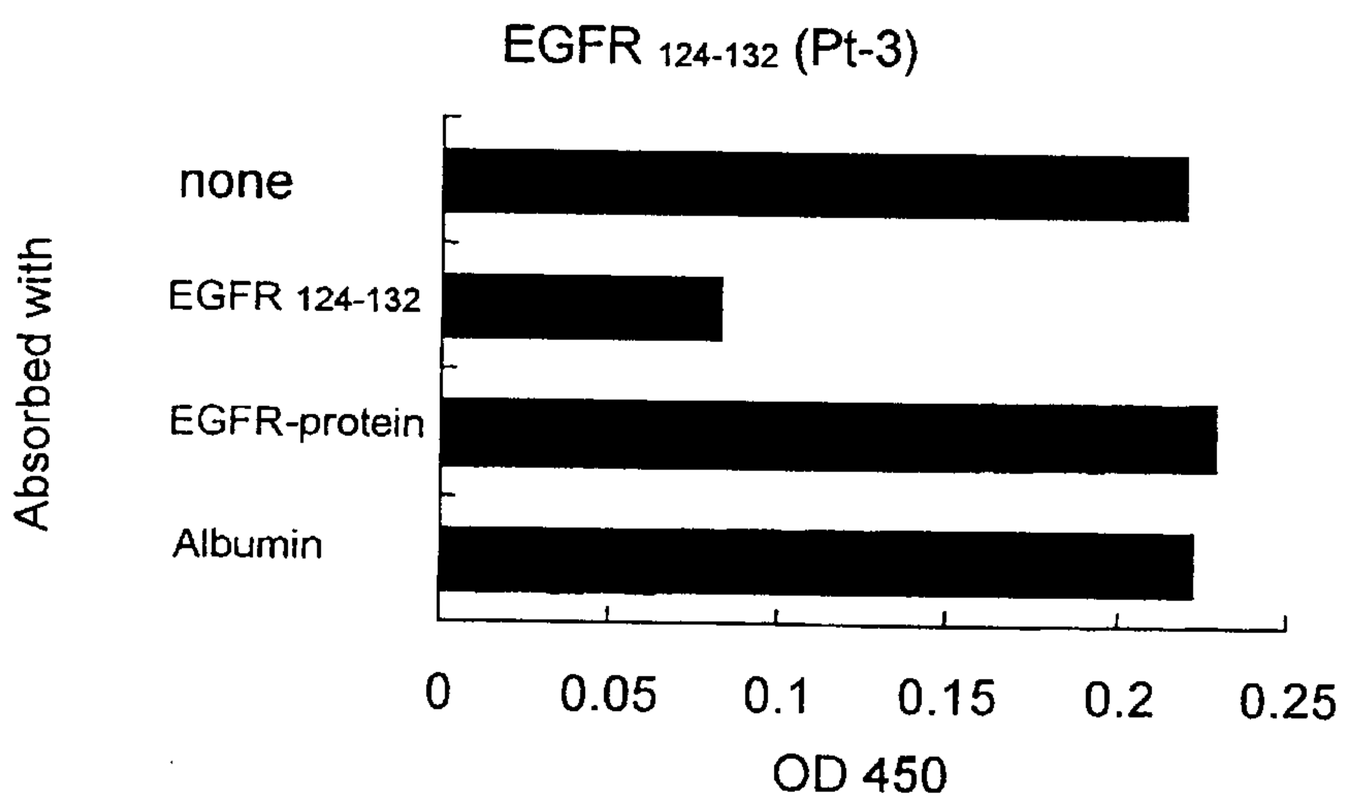

EGFR 124-132 (Pt-3)
Absorbed with
none
EGFR 124-132
EGFR-protein
Albumin
0
0.05
0.1
0.15
0.2
0.25
OD 450

Fig. 4

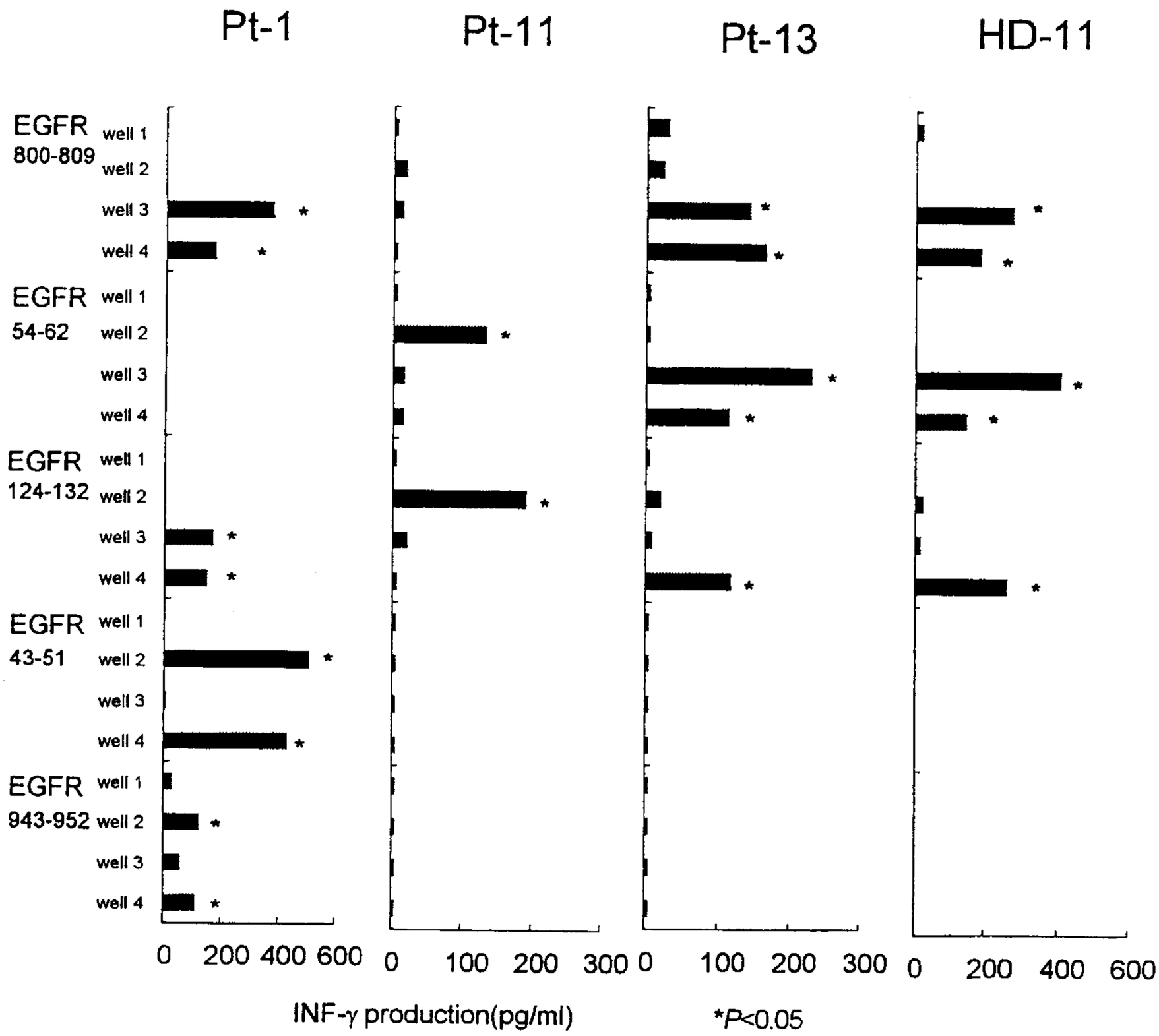

Pt-1
Pt-11
Pt-13
HD-11
EGFR 800-809
EGFR 54-62
EGFR 124-132
EGFR 43-51
EGFR 943-952
well 1
well 2
well 3
well 4
0 200 400 600
0 100 200 300
0 100 200 300
0 200 400 600
INF-γ production(pg/ml)
*P<0.05

Fig. 5

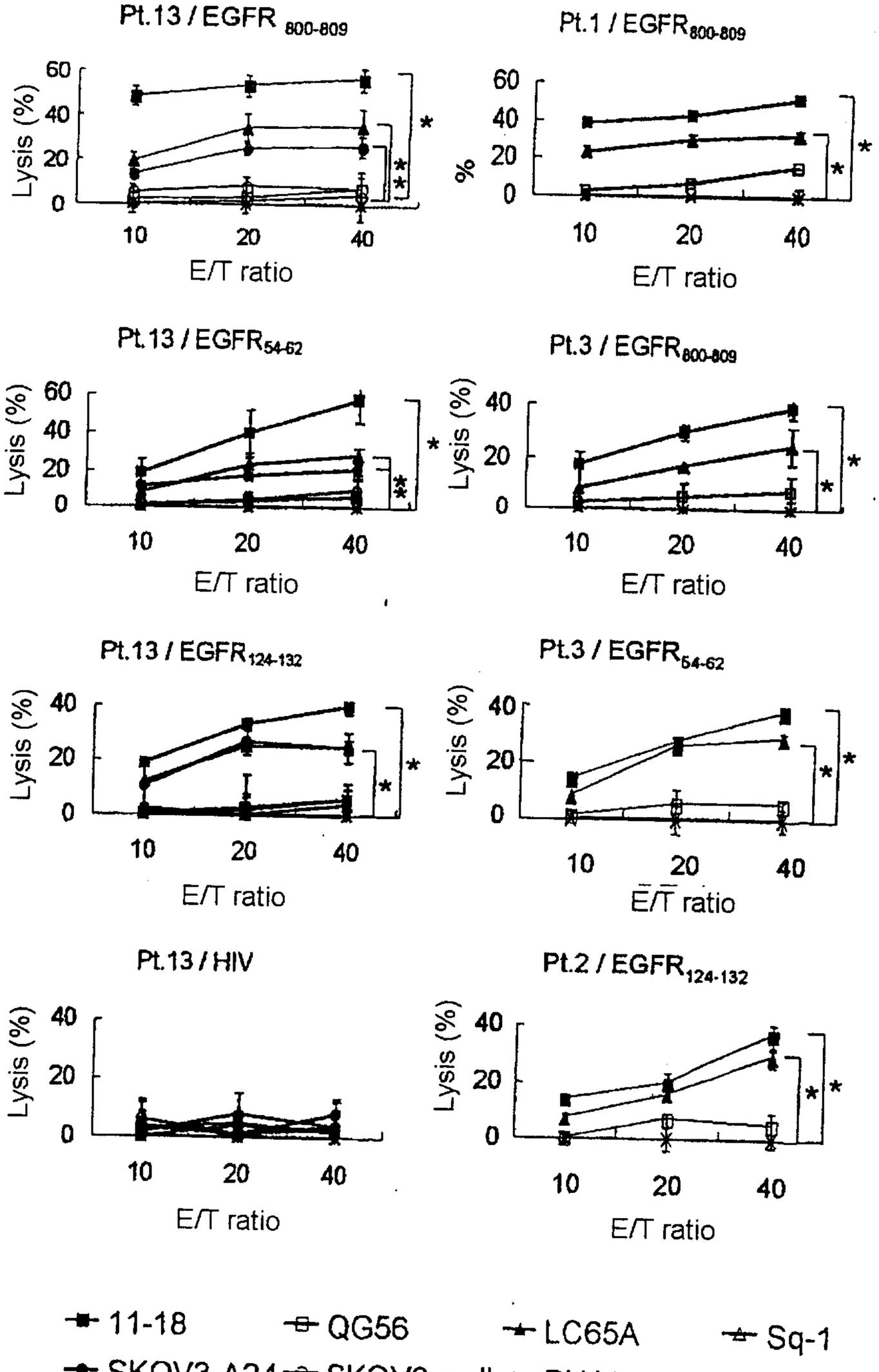

Pt.13 / EGFR 800-809
Pt.1 / EGFR800-809
Pt.13 / EGFR54-62
Pt.3 / EGFR800-809
Pt.13 / EGFR124-132
Pt.3 / EGFR54-62
Pt.13 / HIV
Pt.2 / EGFR124-132
Lysis (%)
E/T ratio
10
20
40
0
60
40
20
*
11-18
QG56
LC65A
Sq-1
SKOV3-A24
SKOV3-null
PHA blasts
*P<0. 05

Fig. 6

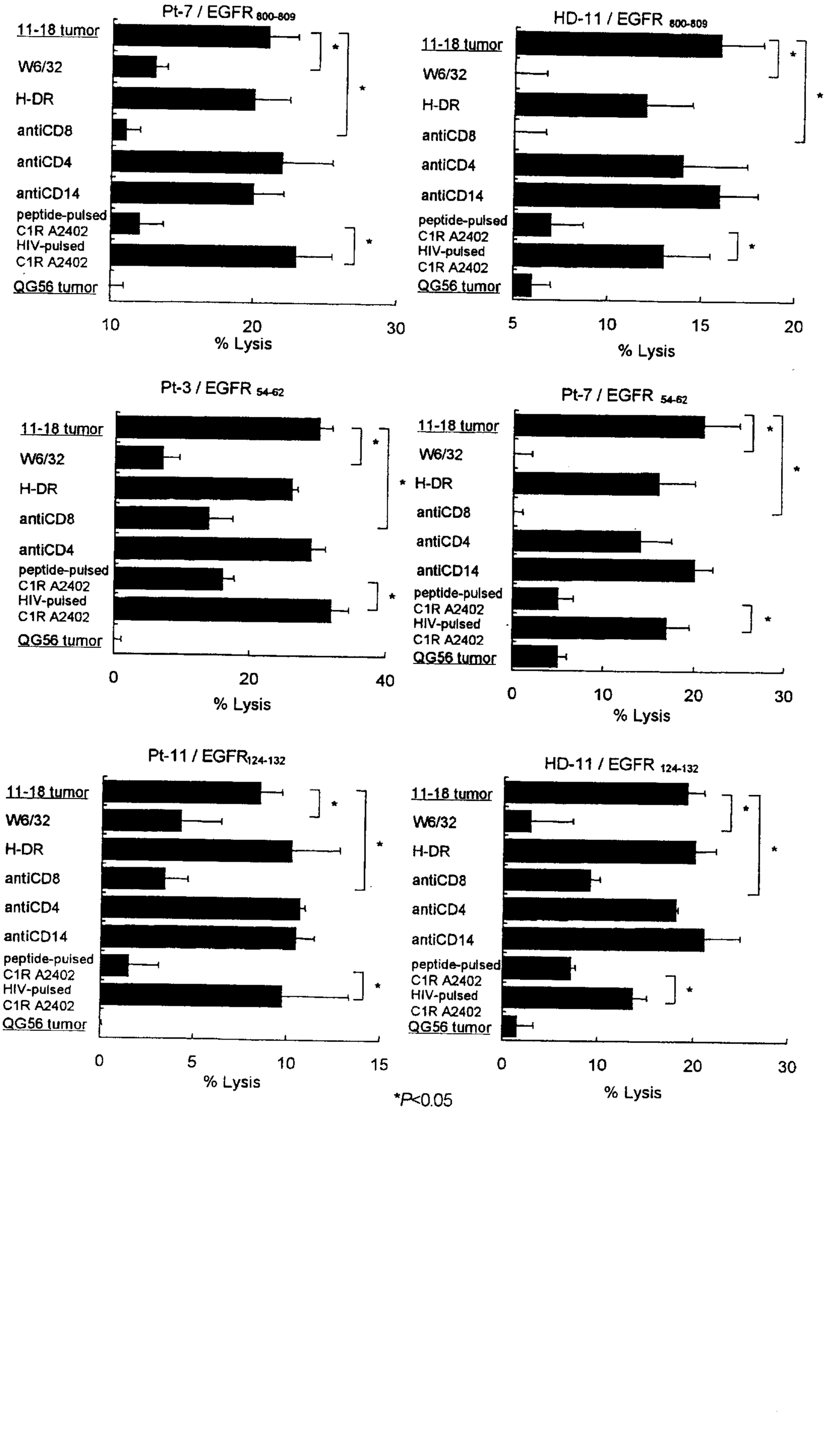

Pt-7 / EGFR 800-809
11-18 tumor
W6/32
H-DR
antiCD8
antiCD4
antiCD14
peptide-pulsed C1R A2402
HIV-pulsed C1R A2402
QG56 tumor
10
20
30
% Lysis
HD-11 / EGFR 800-809
5
10
15
20
Pt-3 / EGFR 54-62
0
20
40
Pt-7 / EGFR 54-62
Pt-11 / EGFR124-132
HD-11 / EGFR 124-132
*P<0.05

Fig. 7

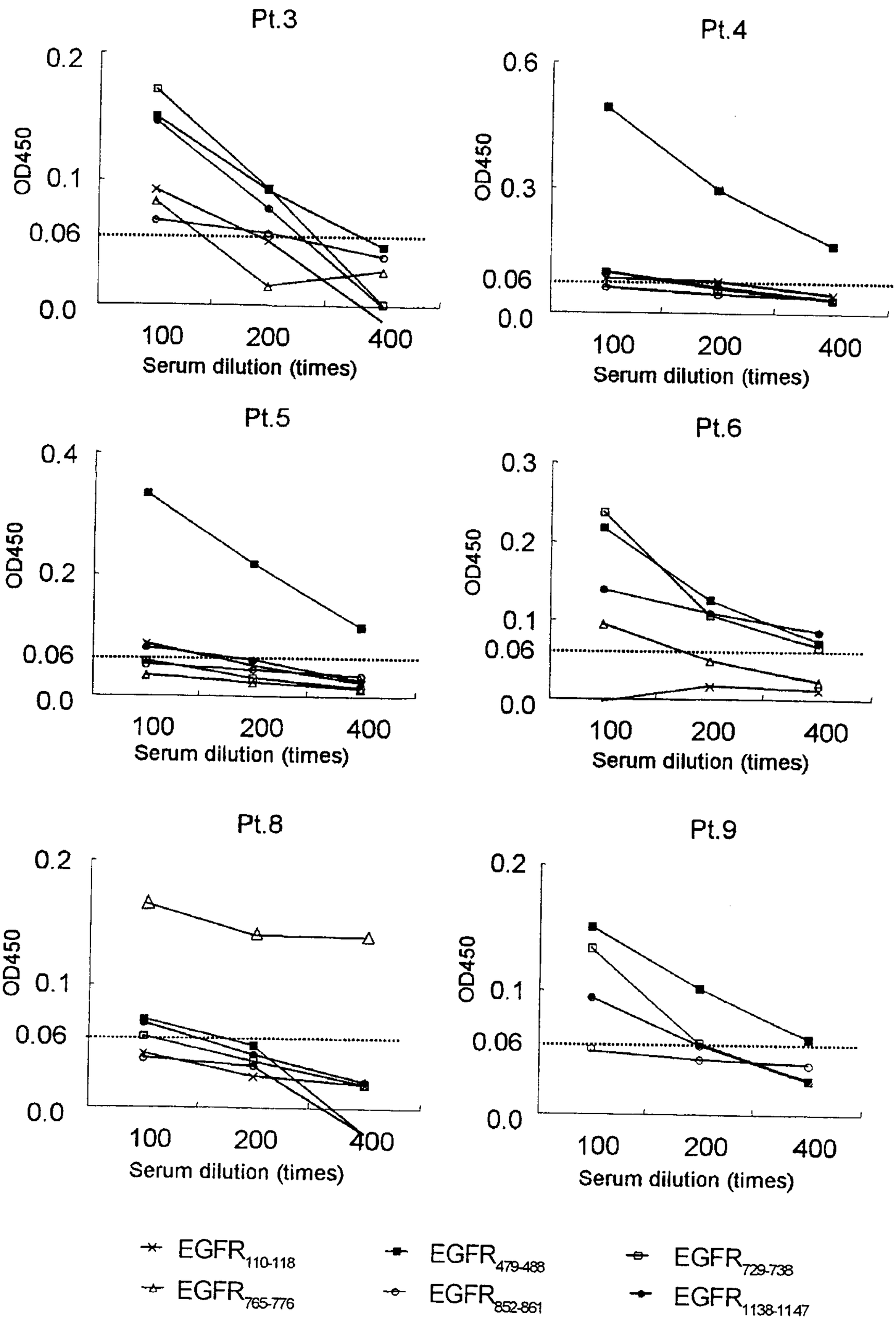

Pt.3
Pt.4
Pt.5
Pt.6
Pt.8
Pt.9
OD450
Serum dilution (times)
100
200
400
0.0
0.06
0.1
0.2
0.3
0.4
0.6
EGFR110-118
EGFR479-488
EGFR729-738
EGFR765-776
EGFR852-861
EGFR1138-1147

Fig. 8

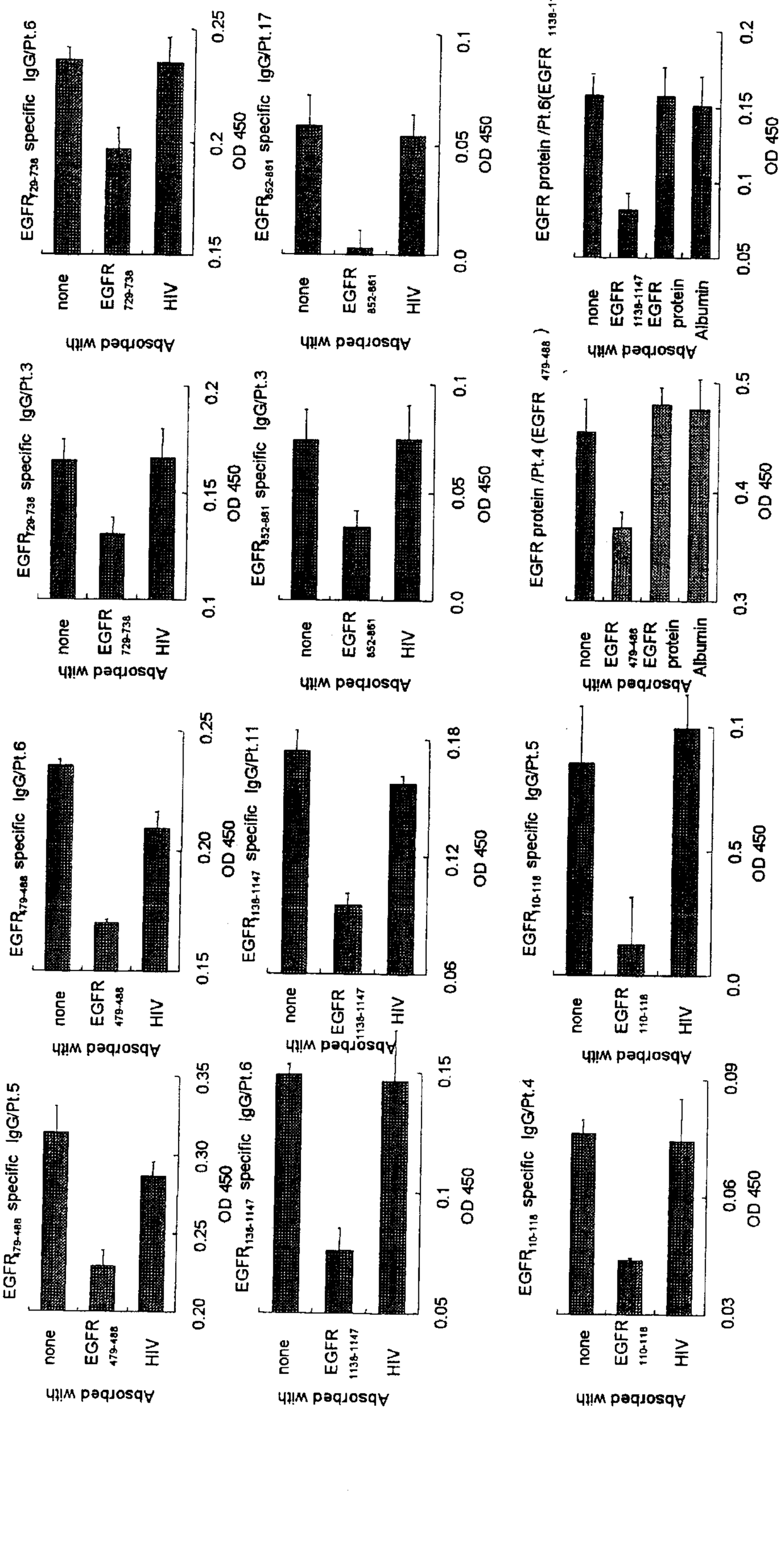

EGFR729-738 specific IgG/Pt.6
EGFR852-861 specific IgG/Pt.6
EGFR protein /Pt.6(EGFR 1138-1147)
EGFR729-738 specific IgG/Pt.3
EGFR852-861 specific IgG/Pt.3
EGFR protein /Pt.4 (EGFR 479-488)
EGFR479-488 specific IgG/Pt.5
EGFR1138-1147 specific IgG/Pt.11
EGFR10-118 specific IgG/Pt.5
EGFR479-488 specific IgG/Pt.5
EGFR1138-1147 specific IgG/Pt.6
EGFR10-118 specific IgG/Pt.4
Absorbed with
none
EGFR 729-738
EGFR 852-861
EGFR 1138-1147
EGFR protein
Albumin
HIV
EGFR 479-488
EGFR 110-118
OD 450

Fig. 9

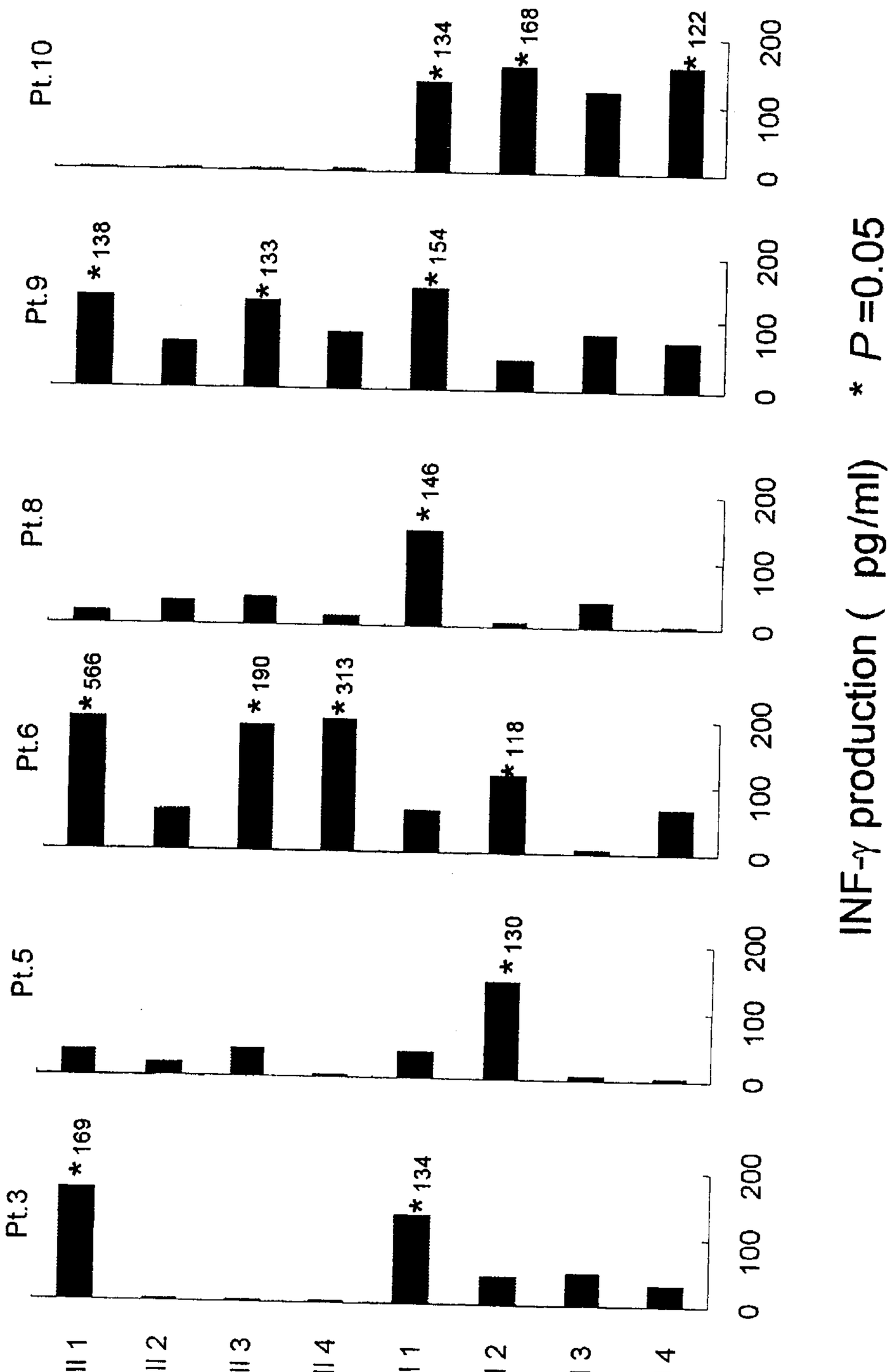
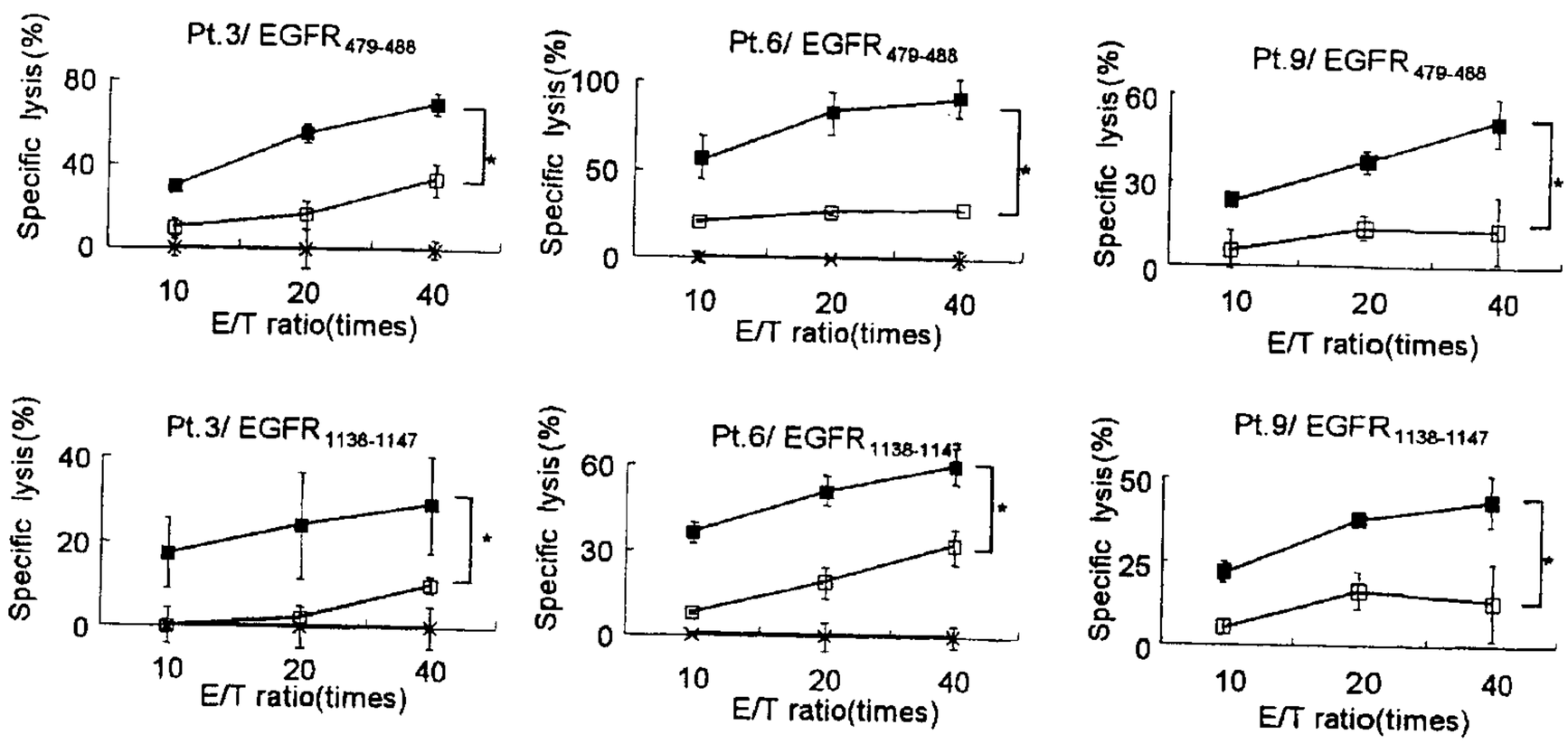

EGFR 479-488
well 1
well 2
well 3
well 4
EGFR 1138-1147
well 1
well 2
well 3
well 4
Pt.3
Pt.5
Pt.6
Pt.8
Pt.9
Pt.10
* 169
* 134
* 130
* 566
* 190
* 313
* 118
* 146
* 138
* 133
* 154
* 134
* 168
* 122
0 100 200
INF-γ production (pg/ml)
* P=0.05

Fig. 10


Pt.3/ EGFR479-488
Pt.6/ EGFR479-488
Pt.9/ EGFR479-488
Pt.3/ EGFR1138-1147
Pt.6/ EGFR1138-1147
Pt.9/ EGFR1138-1147
Specific lysis (%)
E/T ratio(times)
11-18
QG56
PHA blast
*P <0.05

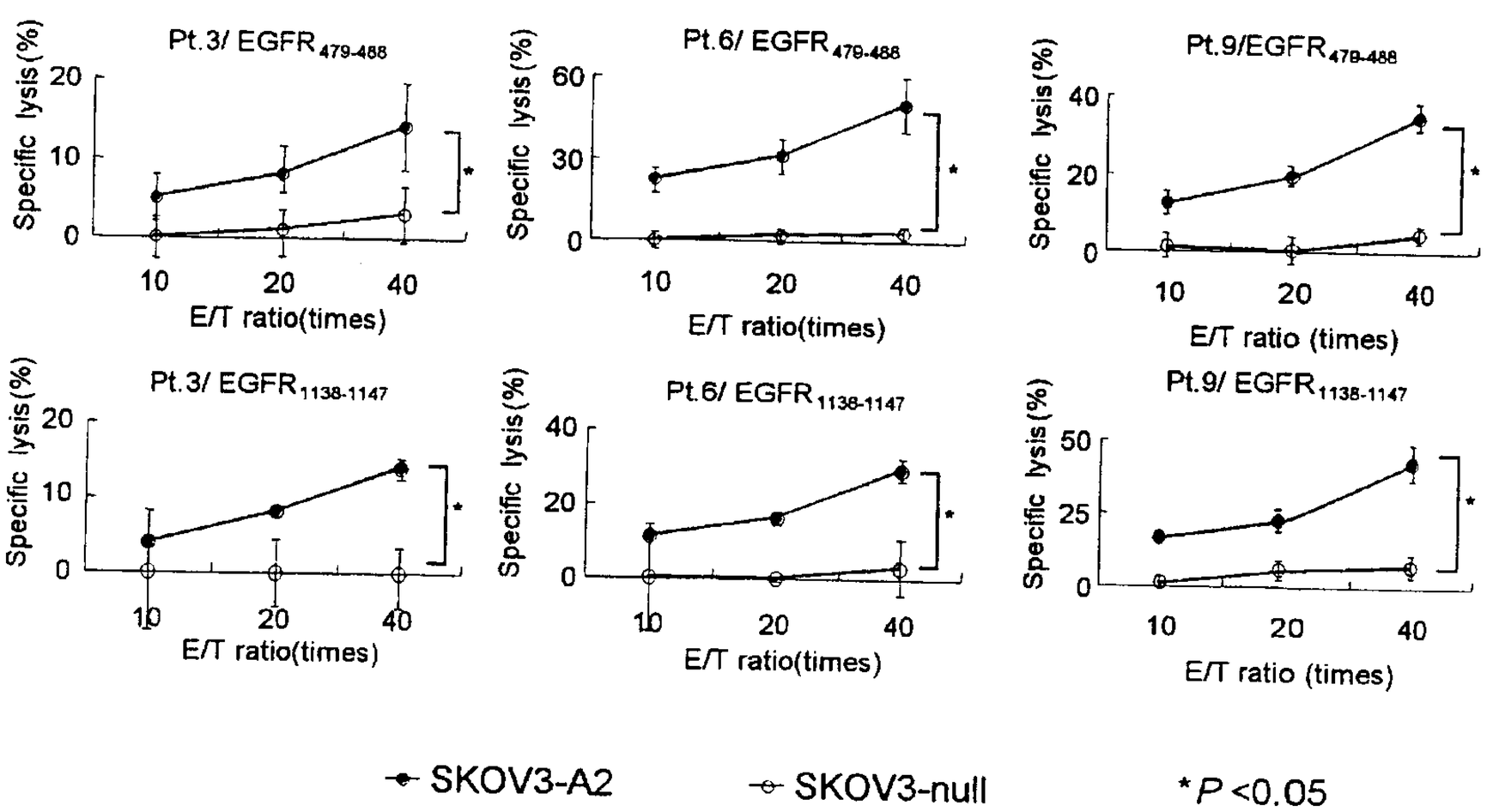
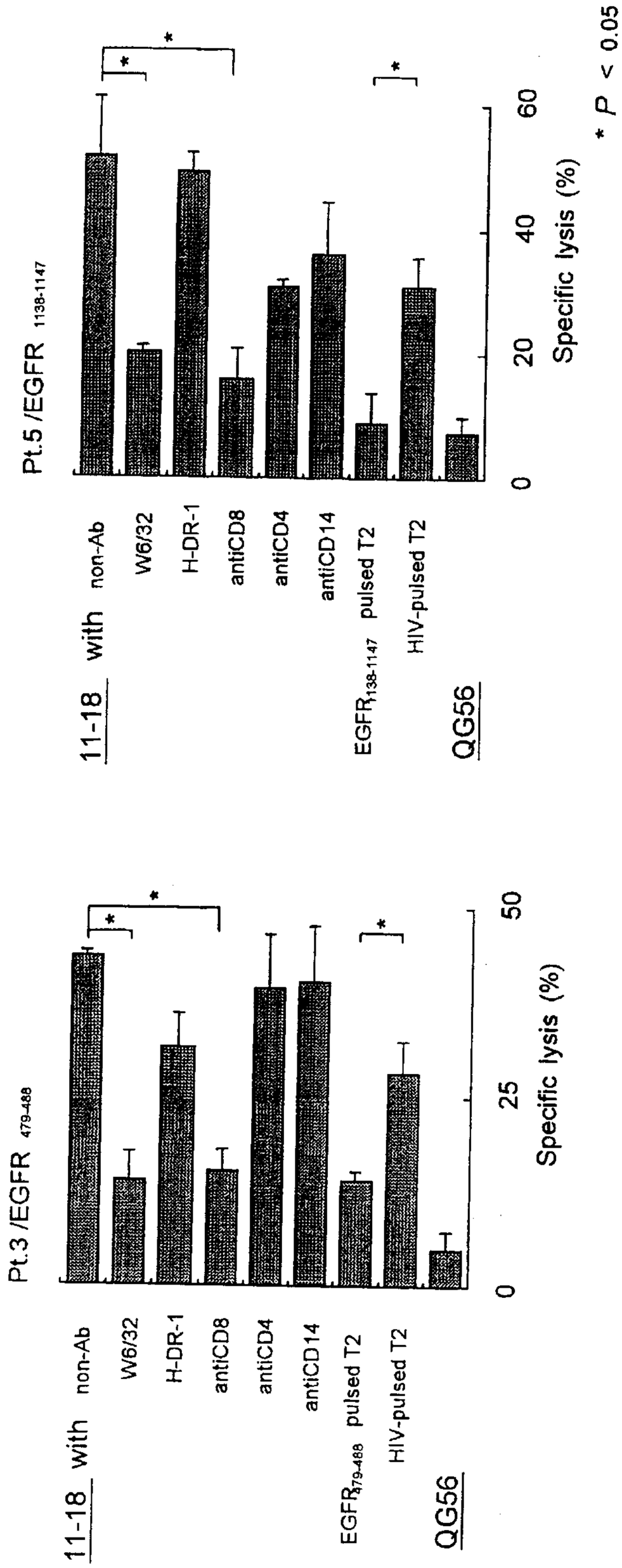

Pt.3/ EGFR479-488
Pt.6/ EGFR479-488
Pt.9/EGFR479-488
Pt.3/ EGFR1138-1147
Pt.6/ EGFR1138-1147
Pt.9/ EGFR1138-1147
Specific lysis (%)
E/T ratio(times)
SKOV3-A2
SKOV3-null
*P <0.05

Fig. 11


11-18 with
non-Ab
W6/32
H-DR-1
antiCD8
antiCD4
antiCD14
EGFR1138-1147 pulsed T2
HIV-pulsed T2
Pt.5 /EGFR 1138-1147
0
20
40
60
Specific lysis (%)
* P < 0.05
11-18
QG56
EGFR479-488 pulsed T2
Pt.3 /EGFR 479-488
0
25
50
Specific lysis (%)