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Identification of epidermal growth factor receptor-derived peptides recognised by both cellular and humoral immune responses in HLA-A24$^+$ non-small cell lung cancer patients ☆

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Abstract

The epidermal growth factor receptor (EGFR) is one of the most appropriate target molecules for cancer therapy because of its high expression in epithelial cancers. A novel EGFR-tyrosine-kinase inhibitor, ZD1839, has been approved as a drug for non-small cell lung cancer (NSCLC), and many other agents are now being tested in clinical trials. Cytotoxic T lymphocyte (CTL)-directed epitope peptides could be another class of useful compounds in EGFR-targeted therapies. However, at present, there are no data on CTL-directed peptides of EGFR. Therefore, this study aimed to identify immunogenic EGFR-derived peptides in HLA-A24$^+$ NSCLC patients. We report in this study three such EGFR-derived peptides at positions 54–62, 124–132 and 800–809. These peptides were recognised by both cellular and humoral immune responses in most of the peripheral blood mononuclear cells (PBMCs) and sera from NSCLC patients that we tested. These results may provide a scientific basis for the development of EGFR-based immunotherapy.

Keywords: EGFR; Peptides; CTL; Ab; Lung cancer; Cancer vaccine

1. Introduction

The epidermal growth factor receptor (EGFR) plays an important role in epithelial biology and in many human malignancies [1–3]. Evidence that EGFR plays a role in the pathogenesis of various cancers has led to the rational design and development of agents that selectively inhibit this receptor. Classes of compounds used in these EGFR-targeted therapies are mainly antibodies (Abs) and tyrosine-kinase inhibitors. Among them, ZD1839 (Iressa) is therapeutically effective for patients with advanced non-small cell lung cancer (NSCLC) [4,5]. In addition, cytotoxic T lymphocyte (CTL)-directed epitopes may also be useful in EGFR-targeted therapies as peptide vaccines for cancer patients whose tumours overexpress EGFR. However, there are no data indicating the CTL-directed epitopes of EGFR, although such peptides raised against HER2/neu, a member of the EGFR family, have been reported to be capable of inducing HLA-class I-restricted CTLs [6–9]. We previously reported that some CTL-directed peptides from non-mutated proliferation-related proteins had the ability to elicit both cellular and humoral im-

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mune responses *in vivo* in clinical studies [10–12]. Further more, levels of anti-peptide Abs in post-vaccination sera correlated with the overall survival rates of advanced cancer patients who had received peptide vaccinations [13]. In addition, there is evidence suggesting peptides have a high immunogenicity and are capable of inducing both cellular and humoral immune responses [14,15]. Therefore, with the view of developing peptide-based cancer therapies, we attempted in this study to identify peptides that might be vaccine candidates for HLA-A24$^+$ NSCLC patients.

2. Materials and methods

2.1. Samples and cell lines

Following written informed consent, sera and peripheral blood mononuclear cells (PBMCs) were collected from NSCLC patients at the Kurume University Hospital. PBMCs and sera were also obtained from healthy donors (HDs). All subjects were free from infection with the human immunodeficiency virus (HIV). Expression of HLA-class I antigens on these PBMCs was serologically defined by conventional methods as previously reported in [10]. Expression of EGFR in the tumour cell lines was examined by flow cytometric assay with an anti-EGFR monoclonal antibody (mAb) (Santa Cruz Biotechnology, Santa Cruz, CA) [16], and representative histograms are shown in Fig. 1. Based on these results, the following tumour cell lines were used as target cells in a 6-h ^{51}Cr-release assay: 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, lung small cell carcinoma, EG- FR$^+$), SKOV3 (HLA-A3/28, ovarian cancer, EGFR$^+$) and SKOV3-A24 (HLA-A24-transfected SKOV3). Phytohaemagglutinin (PHA)-blastoid T cells from PBMCs were also used as a negative control for the target cells in the ^{51}Cr-release assay. For peptide loading, the C1R-A2402 (HLA-A2402 transfectant cell line) cell line was also used in this study.

2.2. Peptides and quantification of peptide-specific IgG

The following peptides were purchased from Bio-Synthesis (Lewisville, USA): 18 EGFR-derived peptides with HLA-A24 binding motifs at 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, respectively. An HIV peptide with a HLA-A24 binding motif (RYLRDQQLL) was also used as a negative control. Anti-peptide-specific IgG levels in sera were measured by an enzyme-linked immunosorbent assay (ELISA) as previously reported in [12,13]. The reactivities of sera from 10 HDs to an HIV peptide were measured by the assays and the sum of this mean value (0.02) and the standard deviation (SD) value (0.02) was set as the cut-off point (0.04). To test the specificity of the anti-peptide IgG in the sera, 100 μl/well of sera (100× dilution with 0.05% phosphate-buffered solution (PBS)) were absorbed with immobilised peptides (20 μg/well)

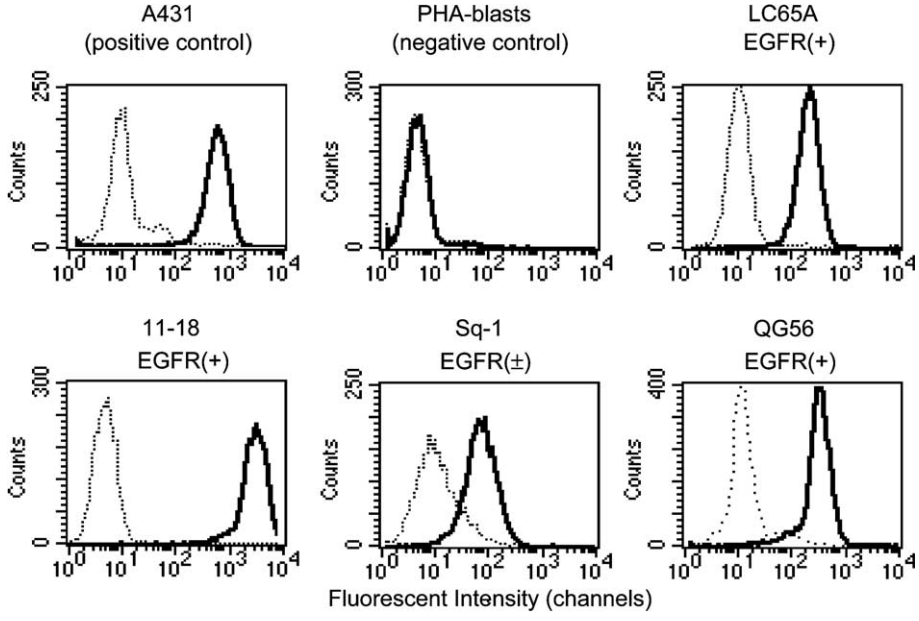

Fig. 1. Expression of epidermal growth factor receptor (EGFR) in tumour cell lines. A standard flow cytometric assay was used to detect EGFR expression on the tumour cells. The dotted line indicates the secondary antibody (Ab) alone. The black line indicates that anti-EGFR monoclonal Ab plus secondary Ab.

for 2-h at 37 °C, three times. Then, these absorbed sera were tested the anti-peptide IgG using an ELISA. To test the anti-peptide IgG response to the whole EGFR molecule, patients' sera containing anti-peptide activity were also absorbed with either immobilised EGFR protein (UPSTATE, Charlottesville, USA) or immobilised human albumin as a negative control, followed by measurement of the anti-peptide activity by ELISA.

2.3. Induction of cytotoxic T lymphocyte

PBMCs from HLA-A24$^+$ NSCLC patients and HDs served as subjects for the CTL induction assay. PBMCs (15×10^4 cells/well) were incubated with 10 μM of each peptide in a 96-well microculture plate (Nunc, Roskilde, Denmark) at a volume of 200 μl in culture medium containing 100 unit/ml of interleukin-2 (IL-2). Half of the culture medium was removed and replaced with the new medium containing a corresponding peptide (20 μM), every 3 days.

2.4. IFN-γ measurement using an ELISA and cytotoxicity analysis using the ^{51}Cr-release assay

These PBMCs as here were measured for peptide-specific interferon-2 (IFN-γ) production by the method previously described in [12]. In brief, cells in each well were separated into the two portions and co-cultured with a corresponding peptide or an HIV peptide for 14 days. After an 18-h incubation with C1R-A2402 cells pulsed with those peptides, the supernatant was collected and the level of IFN-γ was measured using duplicate ELISA assays. For each corresponding peptide, the assay was performed in the 4 independent wells. Then, cells that were producing IFN-γ in response to a corresponding peptide were collected and further cultured with IL-2 alone for 10–14 days to obtain a large number of cells for a standard 6-h ^{51}Cr-release assay against the various tumour cells described above. The method of ^{51}Cr-release assay was reported elsewhere in [12]. For the inhibition test, we used 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) mAbs as a negative control. For a competition assay to study the peptide specificity of the cytotoxicity, unlabelled C1R-A2402 cells pulsed with the corresponding peptide or an HIV peptide as a negative control were added to the ^{51}Cr-release assay at a cold to hot target cell ratio of 10–1.

2.5. ELISPOT analysis and CTL precursor analysis

PBMCs (5×10^4/well) from four patients, which had been cultured for 14 days by the methods described above, were further incubated for 24-h with the peptide-pulsed C1R-A2402 cells (1×10^5/well) in the sterile BD^{TM}ELISPOT plates coated with anti-human IFN-γ mAbs (BD Biosciences, San Diego, USA). The wells were then washed, coated with biotinylated anti-IFN-γ Ab (BD Biosciences), developed with avidin-alkaline phosphatase, stained and analysed using KS ELISPOT (Carl Zeiss, Hallbergmoos, Germany) [17]. These PBMCs from two patients were also provided for CTL precursor frequency analysis by the methods previously described in [18]. CTL precursor frequency was calculated using Taswell's method.

2.6. Statistics

The statistical significance of the data was determined by a two-tailed Student's t test and a P value of <0.05 was considered statistically significant throughout the study.

3. Results

We first investigated whether IgG reactive to each of the 18 EGFR-derived peptides could be detected in the sera of 13 NSCLC patients and 11 HDs. A summary of the results for 11 peptides, to which some of the sera showed a positive response, is given in Table 1 and representative results patients ((Pts) 2, 3 and 10, HD 2) are shown in Fig. 2. Significant levels of IgG (>0.04 OD values at a serum dilution of 100 times) reactive to the $EGFR_{800–809}$, $EGFR_{124–132}$ and $EGFR_{54–62}$ peptides were detected in the sera of 8, 7 and 6 of 13 patients, respectively. Sera from 9, 5 and 3 of 11 HDs tested also showed significant levels of IgG reactive to peptides $EGFR_{800–809}$, $EGFR_{124–132,}$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 in a few HDs. These humoral responses to EGFR peptides were observed in both HLA-A24-positive and -negative subjects, although most subjects in this study were HLA-A24-positive. In contrast, significant levels of IgG reactive to the remaining seven peptides were not detected in any of the sera tested (data not shown).

The peptide specificity of the anti-peptide IgG response to each of the $EGFR_{800–809}$, $EGFR_{124–132}$, and $EGFR_{54–62}$ peptides was confirmed using an absorption test (Fig. 3(a)). As a result, the activity of these sera reactive to each of the three peptides was absorbed with a corresponding peptide, but not with an HIV peptide used as a negative control. We also investigated using an absorption test whether the anti-peptide IgG reacts to the whole EGFR protein. However, the level of the anti-peptide IgG reactive to any of these three peptides was

Table 1
Humoral responses to EGFR peptides

Subjects	HLA	Responses to the EGFR peptides (OD values)[a]										
		$EGFR_{899–908}$	$EGFR_{1015–1024}$	$EGFR_{800–809}$	$EGFR_{269–277}$	$EGFR_{899–907}$	$EGFR_{124–132}$	$EGFR_{812–821}$	$EGFR_{625–633}$	$EGFR_{73–82}$	$EGFR_{54–62}$	$EGFR_{1015–1023}$
Pt. 1	A24/2	–[b]	–	–	–	–	–	–	–	–	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	–	–	–	0.15	–
Pt. 13	A24	–	–	0.13	–	–	0.12	–	–	–	0.14	–
HD 1	A24/33	0.08	0.09	0.08	–	–	–	–	–	–	–	–
HD 2	A24/26	–	0.20	0.16	0.10	–	0.10	–	–	–	0.06	–
HD 3	A2/26	0.22	1.50	0.20	0.18	0.34	0.09	0.15	0.05	–	0.13	0.66
HD 4	A24/26	–	–	0.09	–	–	0.05	–	–	–	–	–
HD 5	A2/24	–	–	0.08	–	–	–	–	–	–	–	–
HD 6	A11/33	–	–	–	–	–	–	–	–	–	–	–
HD 7	A2/24	–	–	0.05	0.05	–	–	–	–	–	–	–
HD 8	A31/33	–	–	–	–	–	–	–	–	–	–	–
HD 9	A2/24	–	–	0.19	–	–	–	–	–	–	–	–
HD 10	A2/11	–	–	0.11	–	–	0.05	–	–	–	–	–
HD 11	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	1

ELISA, enzyme-linked immunosorbent assay; OD, optical density; EGFR, epidermal growth factor receptor; Pt., patient; HLA, human leucocyte antigen; HD, healthy donors.
[a] Anti-peptide IgG was assayed by ELISA as described in Section 2. Values represent the OD value at a serum dilution of 100 times.
[b] The OD values lower than the cut-off point (0.04) were shown as (–).

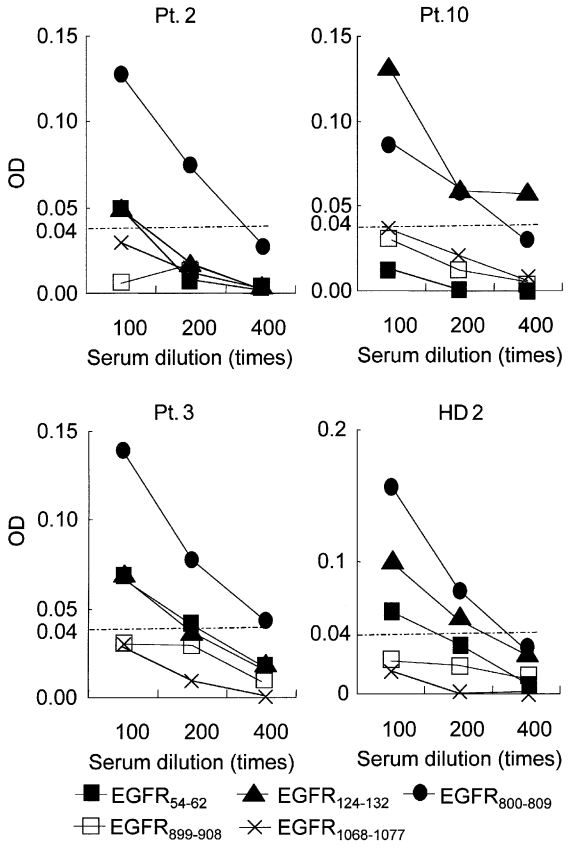

Fig. 2. Detection of anti-peptide IgG. The optical density (OD) value against a negative control human immuno defiencency virus (HIV peptide) was subtracted from the data. Representative results of patients (Pts) 2, 3, 10 and healthy donor (HD) 2 are shown.

not decreased at all by the absorption test (Fig. 3(b)), suggesting no cross-reactivity of the peptide IgG to the whole EGFR protein. Based on these findings, these three peptides were tested for their ability to induce CTL activity in PBMCs of HLA-A24$^+$ NSCLC patients and HDs in the following experiments. The two peptides ($EGFR_{43–51}$ and $EGFR_{943–952}$) to which no IgG response was detectable were also used as controls. Representative results of the four cases (Pts. 1, 11, 13 and HD 11) are shown in Fig. 4, and the summary of all subjects is given in Table 2. The $EGFR_{800–809}$, $EGFR_{54–62}$, and $EGFR_{124–132}$ peptides stimulated PBMCs from at least one of the four wells tested to produce statistically significant amounts of IFN-γ (P value of <0.05 and >100 pg/ml of IFN-γ) in response to C1R-A2402 cells pulsed with a corresponding peptide in 5, 5 and 4 of 8 cancer patients tested, respectively. These peptides also stimulated to produce significant levels of IFN-γ in 3, 4 and 4 of 5 HDs tested, respectively. The $EGFR_{43–51}$ and $EGFR_{943–952}$ also stimulated PBMCs to produce the significant levels of IFN-γ in 2 and 2 of 8 cancer patients tested, respectively (Table 2). The $EGFR_{43–51}$ and $EGFR_{943–952}$ stimulated PBMCs in 1 and 0 of 5 HDs tested, respectively.

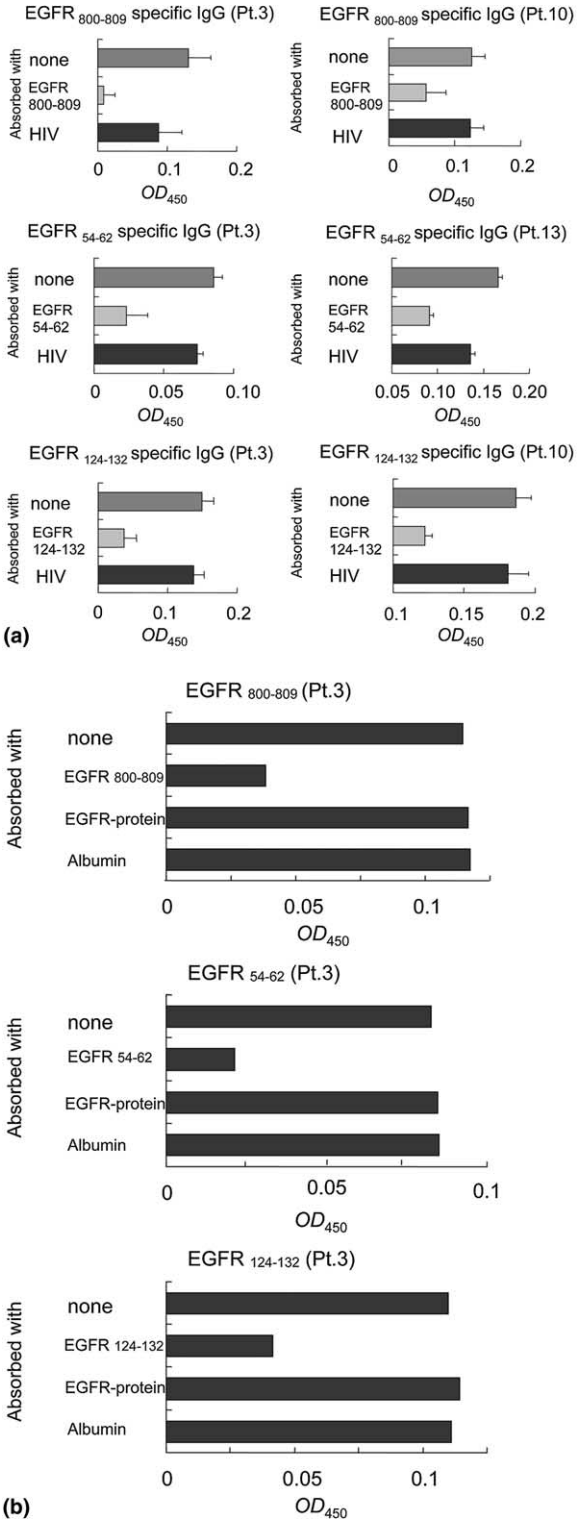

Fig. 3. Specificity of anti-peptide IgG: (a) absorption with either a corresponding peptide or an HIV peptide. The representative results from sera of Pts. 3, 10 and 13 are shown; (b) absorption with an EGFR protein or human albumin. The representative results from sera of Pt. 3 are shown.

To confirm the peptide-specific cellular responses measured by ELISA, we employed two different assays for the measurement of cellular responses; ELISPOT

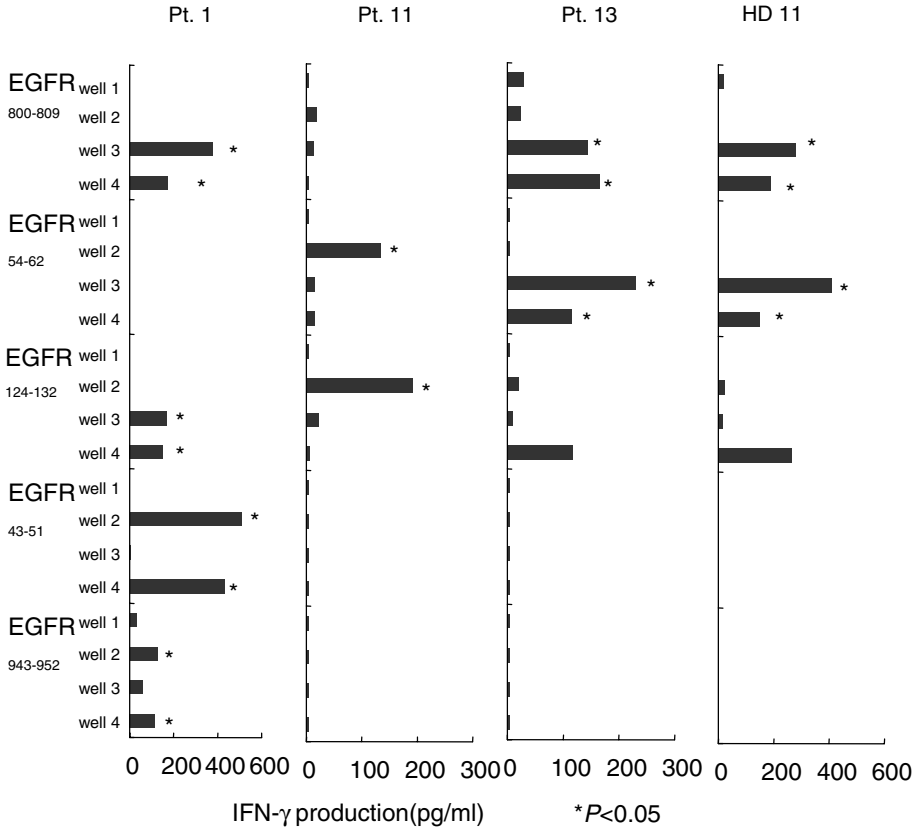

Fig. 4. Cellular response to peptide. The peptide-stimulated peripheral blood mono nuclear cells (PBMCs) were tested for their peptide-specific interferon (IFN)-γ production in quadruplicate assays. Background IFN-γ production in response to the HIV peptide (<50 pg/ml) was subtracted. * $P < 0.05$. The representative results of Pts. 1, 11, 13 and of HD 11 are shown.

Table 2
Cellular responses to EGFR peptides

Subjects	HLA	Responses to the EGFR peptides (IFN-γ production)[a]				
		$EGFR_{800–809}$	$EGFR_{54–62}$	$EGFR_{124–132}$	$EGFR_{43–51}$	$EGFR_{943–952}$
Pt. 1	A24/2	376/172	–[b]	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 S	A2/24	–	–	–	–	–
HD 11	A2/24	280/190	410/150	267	–	–

PBMCs, pheripheral blood mononuclear cells; CTLs, cytotoxic T lymphocytes; IFN, interferon.

[a] PBMCs from HLA-A24+ lung cancer patients and HDs were stimulated with each of the indicated EGFR peptides. On day 14, the cultured PBMCs of the quadruplicate wells were tested for their reactivity to C1R-A2402 cells, which were pre-pulsed with a corresponding peptide in the duplicate assays. Values represent IFN-γ productions (pg/ml). Background IFN-γ production (<50 pg/ml) in response to an HIV peptide (taken as a negative control) was subtracted from the data shown in the Table. Successful induction of peptide-specific CTLs was judged to be positive when the supernatant of the well showed more than 100 pg/ml IFN-γ production with *P*-value of < at least 0.05. The mean values of amounts of IFN-γ of the positive wells among the 4 wells tested are shown in the table.

[b] (–) indicates when none of the 4 wells showed a positive response.

and CTL precursor frequency analyses. PBMCs from 4 NSCLC patients (Pts. 1, 3, 7 and 12) were at first provided for IFN-γ ELISPOT assays to further estimate the activity of the $EGFR_{800–809}$ and $EGFR_{54–62}$ peptides. An HIV peptide served as a negative control. As a result, $EGFR_{800–809}$ and $EGFR_{54–62}$ peptides had the ability to induce CTL activity in 3 and 3 of the 4 patients, respectively (Fig. 5(a)). As non-specific reactions, some positive spots in culture with an HIV peptide were observed in Pts. 1 and 7, although the numbers of spots were definitely lower than those induced by the $EGFR_{800–809}$ in Pt. 1 and the $EGFR_{54–62}$ peptide in Pt. 7. For the 6 positive cases, the ELISA also provided positive results, as shown in Table 2. There was no response in either assay for the $EGFR_{54–62}$ peptide in Pt. 1, whereas the $EGFR_{800–809}$-induced CTL activity in Pt. 7 was detectable by ELISA only. All these results suggest that peptide-specific CTL activity was detectable by either ELISA or ELISPOT assay in most cases. Fig. 5(a) also shows the humoral responses to the peptides. We investigated the peptide-specific CTL precursor frequencies (in Pt. 3 and 12) by stimulation with the $EGFR_{800–809}$ and $EGFR_{54–62}$ peptides. The CTL precursor frequencies reactive to $EGFR_{800–809}$, $EGFR_{54–62}$ and HIV peptides in Pt. 3 were 1/1329, 1315 and <1/10 000, while those in Pt. 12 were 1/976, 1/1662, and <1/10 000, respectively (Fig. 5(b)).

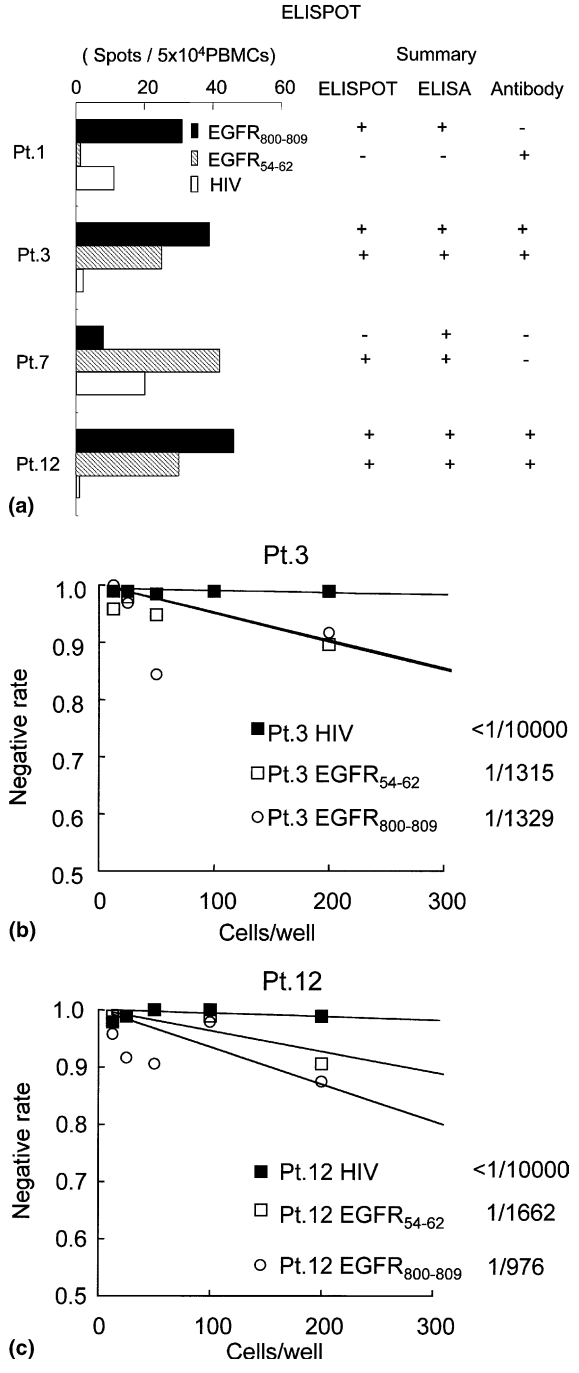

Fig. 5. IFN-γ ELISPOT and CTL precursor frequency: (a) peptide-stimulated PBMCs were tested for their peptide-specific IFN-γ production. The results of patients (Pts.) 1, 3, 7 and 12 are shown; (b) values represent the frequency of peptide-specific CTL precursor in Pts. 3 and 12.

The cytotoxicity of these peptide-stimulated PBMCs was confirmed by a 6-h ^{51}Cr-release assay, and representative results for 4 patients (Pts. 1, 2, 3 and 13) are shown in Fig. 6. These PBMCs showed significant levels of cytotoxicity against all the 11–18 NSCLC cells (HLA-$A24^+$, $EGFR^+$), LC65A small cell lung carcinoma cells (HLA-$A24^+$, $EGFR^+$) and SKOV3-A24 (HLA-$A24^+$, $EGFR^+$) tumour cells tested, but failed to kill any of the QG56 NSCLC cells (HLA-$A24^-$, $EGFR^+$), Sq-1 (HLA-$A24^+$, $EGFR^{\pm}$) NSCLC cells, or SKOV3 (HLA-$A24^-$, $EGFR^+$) tumour cells tested. These PBMCs also failed to kill PHA-blastoid T cells (HLA-$A24^+$, $EGFR^-$). PBMCs stimulated with an HIV peptide as a negative control did not show such HLA-A24-restricted cytotoxicity (Fig. 6, bottom left corner). These results suggest that these PBMCs possess HLA-A24-restricted cytotoxicity reactive to $EGFR^+$ tumour cells.

Further more, the restriction and peptide-specificity of the cytotoxicity were confirmed by inhibition and competition assays (Fig. 7). Namely, levels of cytotoxicity of these peptide-stimulated PBMCs were significantly inhibited by anti-class I (W6/32) or anti-CD8 mAb, but not by the other mAbs tested in the assay. Cytotoxicity was also inhibited by the addition of the corresponding peptide-pulsed C1R-A2402 cells, but not by that of the HIV peptide-pulsed cells. These results suggest that the CTL activity was largely mediated by the peptide-reactive $CD8^+$ T cells in an HLA-class I-restricted manner.

4. Discussion

We reported in this study that the $EGFR_{800–809}$, $EGFR_{124–132}$ and $EGFR_{54–62}$ peptides were recognised

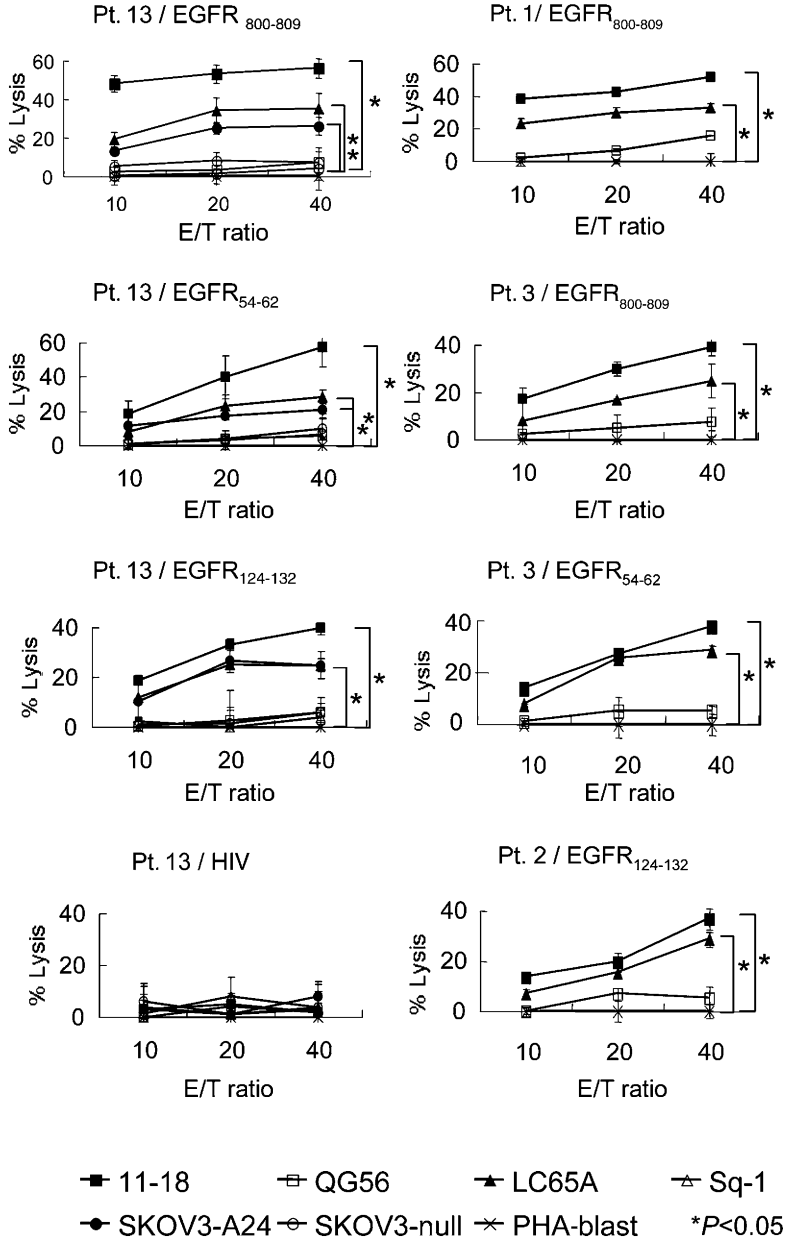

Fig. 6. Cytotoxicity. Peptide-stimulated PBMCs were tested for their cytotoxicity against various cancer cell lines using a standard 6-h ^{51}Cr-release assay. Representative results for Pts. 1, 2, 3 and 13 are shown. Values represent the mean ± standard deviation (SD) of % specific lysis. * $P < 0.05$.

by both cellular and humoral immune responses in >50% of PBMCs and sera, not only from NSCLC patients, but also from HDs. The immune responses in both cancer patients and HDs to EGFR peptides may not be surprising given that EGFR is expressed not only in epithelial cancer cells, but also in certain normal epithelial cells [1–3]. CTL precursors for peptides of HER2/neu, an EGFR family member, are also detectable in PBMCs from both cancer patients and HDs [6–9]. In addition to these three peptides, the $EGFR_{43–51}$ and $EGFR_{943–952}$ peptides also had the ability to induce peptide-reactive IFN-γ production in several NSCLC patients. However, their respective cytotoxicities were not investigated in this study, primarily due to the limited number of PBMCs available for the analysis. Cellular responses to the remaining 13 peptides with HLA-A24 binding motifs were also not investigated because of the limited number of PBMCs. Therefore, further studies will be needed to define the EGFR-derived peptides capable of inducing an HLA-A24-restricted cellular response alone.

It is generally accepted that ELISPOT and ELISA are well-established methods to measure cytokines, including IFN-γ, in the culture supernatants. Either assay is usually sufficient for measuring the CTL response if the ^{51}Cr-release assay, inhibition assay and peptide

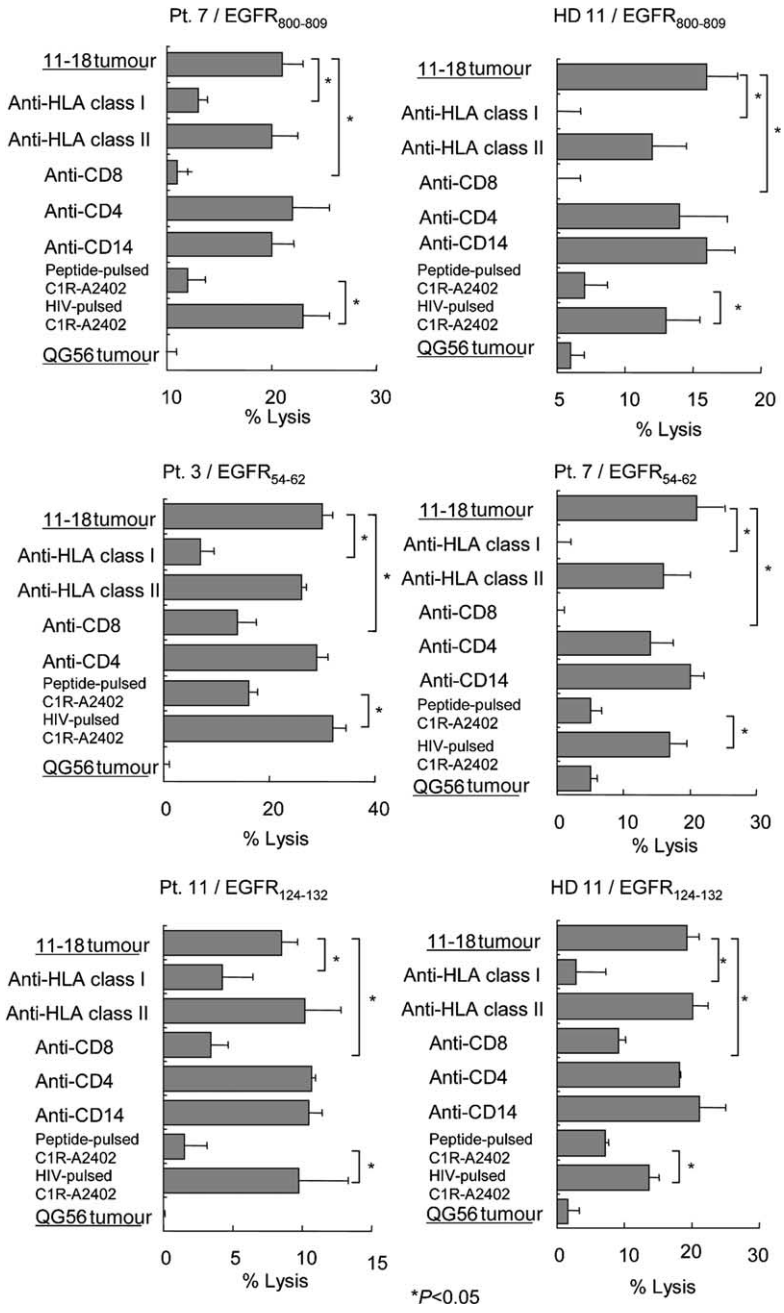

Fig. 7. Inhibition and competition assays. Peptide-stimulated PBMCs were tested for their restriction and peptide-specificity of cytotoxicity in the presence or absence of monoclonal antibody (mAb). For the competition assay, C1R-A2402 cells pulsed with the corresponding peptide or an HIV peptide were added at a cold to hot target cell ratio of 10–1. The 6-h ^{51}Cr-release assay was performed at an E/T ratio of 10 to 1. Values represent the mean ± SD of % specific lysis. *, $P < 0.05$.

specificity experiments are also conducted. To confirm whether the results obtained using an ELISA correlate well with those from other assays, we performed out both an IFN-γ ELISPOT and a classical CTL precursor frequency analysis in certain samples. As expected, the results obtained by these two assays correlated well with those from the ELISA.

We previously reported that IgG reactive against CTL epitope peptides was often detected in pre-vaccination sera of cancer patients and also in sera of HD, and there was no obvious HLA-class IA restriction involved [10–12,19,20]. Furthermore, some CTL-directed peptides have shown the ability to elicit both cellular and humoral immune responses *in vivo* in phase I clinical studies, and levels of anti-peptide IgG in post-vaccination sera have correlated well with the overall survival rates of advanced cancer patients who received peptide vaccinations [13]. In contrast, IgG reactive to these CTL

peptides is either lacking or unbalanced in the sera of patients with atopic disease [20]. These results suggest that the IgGs to these peptides play a role in the host-defense response against these diseases, although the underlying mechanism of anti-tumour immune responses in cancer patients is presently unclear. The underlying mechanisms of IgG production against CTL epitope peptides in HDs, as well as the disturbance of IgG production in patients with atopic disease, are also presently unclear.

However, we showed that the peptide-specific IgGs did not react with the mother proteins at least for those we tested. In addition, sera containing anti-peptide IgGs failed to show direct growth inhibition effects and antibody-dependent cell-mediated cytotoxicity [21]. The anti-peptide IgGs may not act directly on tumour cells, but may facilitate the infiltration of immunocompetent cells into tumour sites through the induction of inflammatory reactions. This assumption is based on the fact that inflammatory reactions have been observed near tumours during surgery (radical prostatectomy) in prostate cancer patients who had entered into the peptide vaccination programme prior to undergoing prostatectomy (data not shown from our institute). Increased levels of IgG reactive to the vaccinated peptides were observed in the post-vaccination, but pre-surgery, sera of these prostate cancer patients.

As expected, positive cellular responses to peptides were not always associated with humoral responses. Namely, $EGFR_{800–809}$ and $EGFR_{124–132}$ peptides were recognised by both cellular and humoral responses in 6 and 5 subjects, whereas each peptide was recognised by either cellular or humoral responses alone in a few subjects. Similar results were observed for the $EGFR_{54–62}$ peptide, with a trend for a higher rate of cellular responses. One of the reasons for this discrepancy between cellular and humoral responses could be due to the differing sensitivities of the assays. Namely, the frequencies of circulating CTL precursors reactive to peptides are very low (less than 1/5000) [18] (data in this study), whereas Ig molecules (if they exist) are consistently detected in sera with a higher sensitivity. (if they exist). In addition, there are many biases affecting the detection of CTL precursors, including the condition of the frozen PBMCs and the culture conditions. Consequently, repeated experiments of CTL assays with different assay systems, if relatively large numbers of PBMCs are available, may increase the positive rate of peptide-specific cellular responses. It is also likely that a T cell response to a peptide will not always induce a B cell response in some subjects.

Although further studies are needed to clarify the biological role, as well as the mechanism of action of the peptide Abs, the three peptides recognised by both cellular and humoral immune responses can be presumed to be more immunogenic than those recognised by the cellular response alone. This assumption is, in part, supported by the fact that PBMCs of both cancer patients and HDs recognised each of the three peptides more frequently than they did either of the two peptides not recognised by serum IgG. The HLA-A24 allele is found in 60% of Japanese (with 95% of these cases being genotypically A2402), 20% of Caucasians and 12% of Africans [22]. Thus, these findings may provide new insight in to the development of EGFR-based immunotherapy that could be beneficial for substantial numbers of NSCLC patients through out the world.

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