厚生労働科学研究費補助金（がん臨床研究事業）
分担研究報告書

広汎性子宮全摘術における自律神経温存手技の確立と術後排尿機能について

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研究要旨
広汎性子宮全摘術における効果的自律神経温存手技を確立し、術後の排尿機能障害を防止することを試みた。われわれの自律神経温存手技は治療後生存率に悪影響を及ぼすことなく術後排尿機能障害の発生を低下させ、頸癌患者の治療後 QOL 向上に有用であることが示された。

A. 研究目的

広汎性子宮全摘術および傍大動脈リンパ節郭清を施行した Ib - IIb 期頸癌の予後因子を明らかにする。

B. 研究方法

2000 年から 2002 年の期間に自律神経温存手技を併用し広汎性子宮全摘術を施行した子宮頸癌患者 27 名を対象とした。臨床進行期の内訳は Ib1 期 10 例、Ib2 期 6 例、IIa 期 3 例、IIb 期 8 例である。年齢は 31-64 歳である。観察期間中央値は 29 か月（12-48 か月）である。再発リスクが高いと考えられる症例に対しては術後全身化学療法あるいは全骨盤照射を追加した。自律神経温存は下腹神経、骨盤内臓神経、骨盤神経叢、骨盤神経叢膀胱枝を対象として行った。術前ならびに術後排尿機能は Urolab Spectrum を用いて尿流動態検査を行って評価した。

C. 研究結果

27 名中 22 名に少なくとも一側の自律神経を系統的に全て温存することができた。5 名では自律神経を温存できなかった。術後 1 年の時点で温存群では非温存群と比較して有意に排尿機能が改善した。非温存群では尿失禁あるいは尿意鈍麻が 5 名中 3 名に認められた（その内の 2 名は両方の症状を有していた）のに対して、温存群では尿失禁は認められず（$p=0.0034$）、2 名で膀胱知覚の亢進が認められたのみ（$p=0.030$）であった。両群の生存曲線には差を認めなかった。

D. 考察

症例数や観察期間は未だ十分とは言えないがわれわれが開発した自律神経温存手技は術後排尿機能を維持するのに有用であると考えられる。

E. 結論

広汎性子宮全摘術施行患者の術後 QOL 改善のために自律神経温存は試みられるべき手技である。

F. 健康危険情報

特記すべきことなし

G. 研究発表

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H. 知的財産権の出願・登録状況（予定含）

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分担研究報告書

子宮頸がんの予後向上を目指した集学的治療における標準的化学療法の確立に関する研究

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研究要旨
塩酸イリノテカン（CPT-11）＋ネダプラチン（CDGP）併用療法の in vitro における抗腫瘍効果を、3 種のヒト子宮頸癌細胞株（ME-180, CaSki, SiHa：全て扁平上皮癌）を用いて MTT assay、isobologram により評価したところ、両剤の併用抗腫瘍効果が認められた。CDGP は同様なプラチナ製剤である CDDP よりも腎障害などの副作用が軽減されており、臨床応用が期待される。

A. 研究目的

子宮頸癌に対する新規の抗癌剤併用化学療法を開発することを目的とし、CPT-11 と CDGP を併用した時の in vitro 細胞増殖抑制活性を評価した。

B. 研究方法

3 種のヒト子宮頸癌細胞株（ME-180, CaSki, SiHa：全て扁平上皮癌）を用いて、SN－38（CPT－11 の活性代謝物）と CDGP を併用（同時投与）した時の in vitro 細胞増殖抑制活性を MTT assay 法により評価し、併用効果（synergistic、additive、sub-aditive、antagonistic）の判定には isobologram を用いた。細胞を抗癌剤と 37 度 C、5%二酸化炭素下で 72 時間培養し、生細胞を MTT assay 法を用いて判定した。dose-response curve から 50%阻止濃度（IC50）を算出した。実験は 2～7 回施行し、IC50 の標準偏差を算出した。isobologram は、SN-38 および CDGP 単剤における IC50 値から modeI、modeIIa および modeIIb 曲線を作成し、単剤における IC50 値に対する、両剤併用時の IC50 値の割合をグラフ上にプロットすることにより併用効果を判定した。

C. 研究結果

ME-180, CaSki, SiHa における IC50 値（ng/ml）は、SN-38 では、1.34±0.180、18.29±9.70、1.76±0.503、CDGP では、409±25.1、6410±1770、2790±1060 であった。SN-38 に対して、ME-180 と CaSki は同様な感受性を示したが、SiHa の感受性は低かった。isobologram による SN-38 と CDGP 併用効果の判定は、細胞株により変化した。ME-180 に対する併用効果は synergistic、SiHa に対しては additive であった。一方 CaSki に対しては synergistic から antagonistic までを示し一定しなかった。

D. 考察

抗腫瘍効果を高めるためには、異なった作用機序を有する抗癌剤を組み合わせることが重要である。CPT-11 は、DNA topoisomerase-I と結合体を形成することにより DNA 合成を阻害する。効果は S 期に特異的で時間依存性である。CDGP は

DNA 塩基に結合することにより DNA 複製を阻害する。isobologram による SN-38 と CDGP 併用効果の判定は、CaSki に対しては synergistic から antagonistic までを示し一定しなかったものの、ME-180 に対する併用効果は synergistic、SiHa に対しては additive であった。CPT-11 (SN-38) と CDGP の併用は高い効果を有することが示唆された。CDGP は CDDP と同様のプラチナ製剤であるが、CDDP に比較して腎障害が軽減されているため大量の補液が不要で外来治療も可能である。また、消化器障害、末梢神経障害、聴覚障害も軽減されており、患者の QOL の向上・維持も期待されその有用性が示唆される。

E. 結論

塩酸イリノテカン (CPT-11) ＋ネダプラチン (CDGP) 併用療法の in vitro における高い抗腫瘍効果が示唆された。CDGP は同様なプラチナ製剤である CDDP よりも腎障害などの副作用が軽減されており、臨床応用が期待される。

F. 健康危険情報

特記すべきことなし

G. 研究発表

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H. 知的財産権の出願・登録状況（予定含）

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子宮頸がんの予後向上を目指した集学的治療における標準的化学療法の確立に関する研究

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研究要旨

局所進行子宮頸癌に対する術前化学療法の有用性を検証するための研究において、cisplatin/irinotecan 併用療法での phase II study、さらに、cisplatin を用いた放射線化学療法のパイロット研究を検討した。術前化学療法は、奏効率は８０％を超え、down-staging 効果を有することが示された。放射線化学療法も短期間の観察であるが安全に行えることが示唆されたが、長期に渡り晩期毒性を監視していく必要がある。

A. 研究目的

局所進行子宮頸癌の臨床進行期別の予後の改善はみられていない。多くの RCT にて主治療を放射線療法とした neoadjuvant chemotherapy の効果は否定されたが、術前化学療法の効果に関しては概ね有効と考えられているが、エビデンスに乏しい。多施設での無作為化比較試験が計画されているが、本研究では臨床第２相試験として術前化学療法の効果と安全性を検討することを主目的とした。また、高齢者、合併症を有する患者や手術を希望しない患者に対しては cisplatin を用いた放射線化学療法のパイロット研究を行い、その安全性についても同時に検討している。

B. 研究方法

Ib2～IVa 期の患者を対象として、cisplatin 単剤で検討してきたが、2002 年より Ib2～bulky IIb 期と卵巣機能温存が必要な４０歳以下の III 期を対象として cisplatin70mg/m^2(day 1) +irinotecan 70mg/m^2(day 1,8)静注、２コースでの phase II study を開始した。放射線化学療法（パイロット研究）では cisplatin 30mg/m2 毎週併用で行っている。

（倫理面への配慮）

全ての患者から十分なインフォームドコンセントが得られており、カルテに記載した上で治療を施行した。

C. 研究結果

研究期間中にインフォームドコンセントを取得した 12 例中、適格例 9 例で評価でき、7 例の奏効(奏効率 78%)が確認された。Grade3 以上の好中球減少が 5 例に見られたが、G-CSF 投与により速やかに回復した。Grade3 以上の下痢や grade 2 以上の血小板減少は認めなかった。奏効した症例はすべて広汎性子宮全摘術が施行されたが、効果を認めなかった（NC）２例は放射線療法を行った。

放射線化学療法は６例に行われ、grade 3 の好中球減少を１例に認めたが、放射線療法は予定どうり完遂された。

D. 考察

現在のところ、３週ごとの cisplatin/irinotecan 併用療法は、安全に行え、術前化学療法として短期間での

治療であり、cisplatin dose intensity (DI) も 30mg/week で、その有効性が示唆される。さらに症例を増やしてこの結果を確認する必要があると思われる。また、術後、病理学的な検討を加えねばならない。
放射線化学療法も短期間の観察であるが安全に行えることが示唆されたが、長期に渡り晩期毒性を監視していく必要がある。

E. 結論

3週ごとの cisplatin/irinotecan 併用療法は、安全かつプラチナ DI も 30 以上あり、術前化学療法の有用性が示唆された。また、cisplatin 30mg/m2 毎週併用での放射線化学療法も安全に行えることが示唆される。

F. 健康危険情報

特記すべきことなし

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H. 知的財産権の出願・登録状況（予定含）

なし

研究成果の刊行に関する一覧表レイアウト

雑誌

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Co-expression of Y box-binding protein-1 and P-glycoprotein as a prognostic marker for survival in epithelial ovarian cancer

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Abstract

Objectives. This study aimed to observe the expressions of Y box-binding protein-1 (YB-1) and P-glycoprotein (P-gp) in primary ovarian tumor and to determine whether they act as biomarkers for survival in epithelial ovarian cancer.

Methods. The expressions of YB-1 and P-gp were examined immunohistochemically in 59 patients who were treated from 1997 to 2000 at Kurume University Hospital. Samples were paraffin-embedded primary ovarian cancer tissue taken from the surgical specimens.

Results. Of the 59 primary ovarian tumors examined, 32 (54.2%) and 18 (30.5%) were positive for YB-1 and P-gp, respectively. Co-expression of these two proteins was observed in 20.3% (12/59) cases. Patients showing this co-expression had a worse 3-year survival than those without co-expression (40.0% vs. 73.1%, $P = 0.0447$). This co-expression significantly correlated with poor prognosis according to multivariate analysis ($P = 0.0007$).

Conclusion. Co-expression of YB-1 and P-gp emerged as a promising relevant biomarker for unfavorable prognosis in ovarian cancer.

Keywords: Ovarian cancer; Y box-binding protein-1; P-glycoprotein; Multidrug resistance; Prognosis

In the Western world, ovarian cancer is the leading cause of death from gynecologic malignancies. According to the *Cancer Facts and Figures 2003* from American Cancer Society, it caused 26,800 female deaths in the United States. One of the reasons for this high mortality rate could be the appearance of resistance against anticancer drugs. Approximately 20% of ovarian cancers are considered to be intrinsically resistant to first-line chemotherapy [1]. Therefore, overcoming drug resistance could be the key for developing a more effective chemotherapy for ovarian cancer. The expression of various proteins could function to express an intrinsic resistance to chemotherapy [2]. The overexpression of P-glycoprotein (P-gp) has been found in many chemoresistant tumors. This protein encoded by the MDR1 gene, functions as efflux pump, and reduces the accumulation of anticancer drugs in tumor cell [3].

Recently, it was reported that the Y-box, an inverted CCAAT box, was located on the promoter area of MDR1 genes, and that Y box-binding protein-1 (YB-1), a member of the DNA binding protein family, was associated with expression of drug resistance in human tumors [4–7]. In a clinical study, patients with YB-1 nuclear-positive tumors had a poor prognosis when compared with those with YB-1 nuclear-negative tumors in ovarian serous adenocarcinoma [8]. These results suggest the presence of intrinsic drug resistance expressed by YB-1 protein. However, it remained unclear if YB-1 would play an important role in ovarian carcinomas, other than serous adenocarcinoma.

Therefore, the aim of this study was (1) to examine the expression of YB-1 and its target genes such as P-gp in all types of primary epithelial ovarian carcinoma, and (2) to investigate a relation between expression of these proteins and clinicopathologic factors as well as survival in epithelial ovarian cancer.

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Materials and methods

Patients

From 1997 to 2000, 59 patients with histologically proven ovarian cancer were treated at the Department of Obstetrics and Gynecology, Kurume University Hospital, Japan. All patients received primary surgery and adjuvant chemotherapy. The following data were collected from their medical records, including age, clinical stage, histological subtypes and grade, size of residual tumor at the primary surgery, and regimens of postsurgical chemotherapy. Histological grading was determined according to The International Federation of Gynecology and Obstetrics (FIGO, 1988) grading system. The patients' characteristics are presented in Table 1.

Primary antibody

Polyclonal anti-YBC antibody to YB-1 was generated as described previously [4]. Briefly, a synthetic peptide composed of 15 amino acids (residues 299–313) in the tail domain of the YB-1 protein was used to generate antibodies in a rabbit. The rabbit polyclonal antibody induced by this antigen was affinity-purified on columns prepared from the same peptide. YB-1 was diluted in PBS at a dilution of 1:5000 for immunohistochemical staining. JSB-1 antibody (1:20) monoclonal antibody directed against P-gp was obtained from Nichirei (Japan).

Table 1
Patient characteristics

Number of patients	59
Age: mean ± SD (years)	51.4 ± 14.6
Clinical stage (FIGO)	No. (%)
I	24 (40.6)
II	5 (8.5)
III	25 (42.4)
IV	5 (8.5)
Histology	
Serous	18 (30.5)
Mucinous	19 (32.2)
Endometrioid	9 (15.3)
Clear cell	8 (13.5)
Others	5 (8.5)
Grading	
Grade 1	17 (28.8)
Grade 2	20 (33.9)
Grade 3	22 (37.3)
Residual tumor after first surgery	
No	27 (45.8)
Yes	32 (54.2)
Chemotherapy	
CP/CAP[a]	19 (32.2)
CDDP/CBDCA + TAXOL	21 (35.6)
CDDP + CPT-11	19 (32.2)

[a] C: cyclophosphamide; P: cisplatin; A: adriamycin.

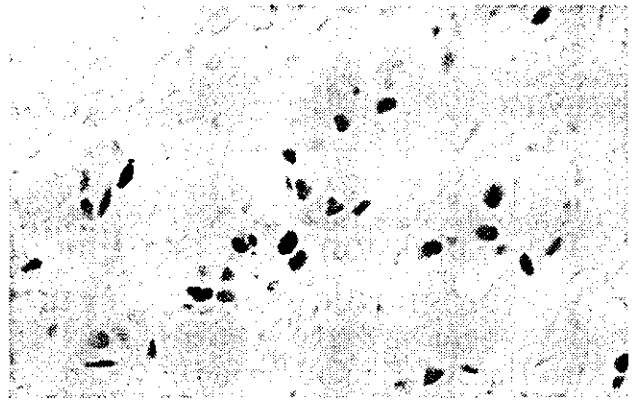

Fig. 1. Immunohistochemical YB-1 staining of ovarian cancer. The nuclei were strongly stained and cytoplasm was weakly stained (original magnification ×400).

Immunohistochemical staining

A formalin-fixed, paraffin-embedded, 4-μm section was obtained from each sample of primary epithelial ovarian cancer. Immunohistochemical (IHC) staining was performed with a streptavidin–biotin–peroxidase complex method. Samples were deparaffinized in xylene and dehydrated in a graded series of alcohol. To recover the antigenicity of YB-1, the sections were immersed in a citrate buffer and pretreated for 15 min at 120°C by an autoclave, and thereafter exposed to the primary antibody at room temperature for 60 min. A subsequent reaction was made using a streptavidin–biotin–peroxidase kit (Nichirei). For the detection of P-gp, the sections were exposed at room temperature for 15 min and a catalyzed signal amplification kit (CSA kit, DAKO) was used. The sections were stained with freshly prepared diaminobenzidine solution and then counterstained with hematoxylin.

The cells with strongly stained nuclei and weakly stained cytoplasm were interpreted as positive cells for YB-1. We defined those only with weakly stained cytoplasm as negative for YB-1. Either cytoplasm or nuclear staining was

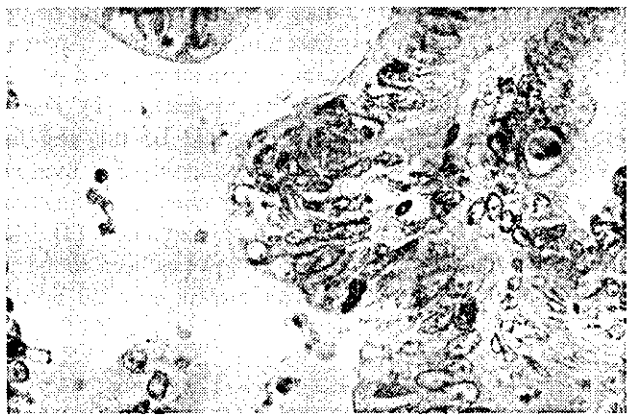

Fig. 2. Immunohistochemical P-gp staining of ovarian cancer. The cytoplasm was stained (original magnification ×400).

Table 2
Clinical-pathologic features and IHC data for 59 cases of ovarian cancer

Prognostic factors	YB-1 expression			P-gp expression		
	Negative	Positive	P^a	Negative	Positive	P^a
FIGO stage						
Stage I + II	15 (51.7.)	14 (48.3)	0.366	18 (62.1)	11 (37.9)	0.223
Stage III + IV	12 (40.0)	18 (60.0)		23 (76.7)	7 (23.3)	
Histology						
Serous	7 (38.9)	11 (61.1)		10 (55.6)	8 (44.4)	
Mucinous	11 (57.9)	8 (42.1)	0.513	15 (78.9)	4 (21.1)	0.184
Endometriod	4 (44.4)	5 (55.6)		8 (88.9)	1 (11.1)	
Clear cell	2 (25.0)	6 (75.0)		6 (75.0)	2 (25.0)	
Others	3 (60.0)	2 (40.0)		2 (40.0)	3 (60.0)	
Grade						
1	10 (58.8)	7 (41.2)	0.07	13 (76.5)	4 (23.5)	0.223
2	5 (25.0)	15 (75.0)		11 (55.0)	9 (45.0)	
3	12 (54.5)	10 (45.5)		17 (77.3)	6 (22.7)	
Residual						
No	13 (48.1)	14 (51.9)	0.735	19 (70.4)	8 (29.6)	0.893
Yes	14 (43.7)	18 (56.3)		22 (68.8)	10 (31.2)	
Total	27 (45.8)	32 (54.2)		41 (69.5)	18 (30.5)	

[a] Chi-square test.

regarded as positive for P-gp. Both the positive controls of YB-1 and P-gp were samples from the colorectal cancer.

Statistical analysis

The SPSS for Windows 10.0 computer program was used for statistical analysis. The statistical significance between clinical and pathological characteristics was evaluated using the chi-square test. The Spearman rank correlation test was used to determine the correlation between two variables. The survival distribution was calculated using the Kaplan–Meier method, and survival curves were compared using log-rank statistics. All variables that were significant at the 5% level in the univariate analysis were included in a Cox proportional hazard regression model. Model selection for identifying the variables having important effects on survival was based on a forward stepwise procedure. All tests were performed at a significance level of $\alpha = 0.05$.

Results

All 59 ovarian carcinoma showed positive immunoreaction for anti-YBC in the cytoplasm. In 32 of 59 cases (54.2%), YB-1 was expressed in the nucleus (Fig. 1). P-gp expression was found in 30.5% (18/59) ovarian cancers (Fig. 2). There was no significant correlation between the expression of YB-1 or P-gp and clinicopathologic factors, such as clinical stage, presence of residual tumor, type of histology, and tumor grade (Table 2).

Table 3
Type of expressions of YB-1 and P-gp ($n = 59$)

	YB-1(−)	YB-1(+)
P-gp(−)	21(35.6%)	20(33.9%)
P-gp(+)	6(10.2%)	12(20.3%)

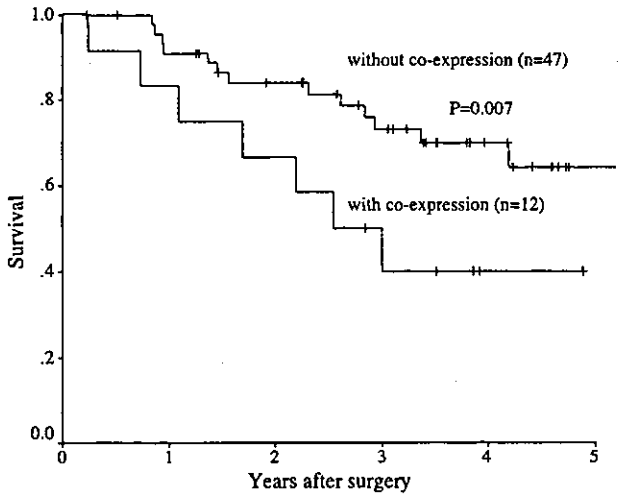

Fig. 3. Kaplan–Meier survival probability stratified by co-expression of YB-1 and P-gp.

Neither YB-1 nor P-gp expression was found in 21/59 (35.6%) of cases. In 26/59 (44.1%) patients, either YB-1 or P-gp expression was found. The co-expression of both factors was recognized in the same specimens from 12/59 (20.3%) patients (Table 3). There was no relationship between the expression of YB-1 and that of P-gp ($P = 0.204$).

No significant difference in overall survival was seen between the patients with YB-1 or P-gp expression and those without either expression. However, the patients with co-expression of YB-1 and P-gp showed lower 1-, 2-, and 3-

Table 4
Univariate survival analysis by Kaplan–Meier

	Factors	Total	Dead cases	P value
YB-1	(−)	27	5	0.0851
	(+)	32	16	
P-gp	(−)	41	14	0.5034
	(+)	18	7	
Co-expression	no	47	14	0.0447
	yes	12	7	
Stage	I + II	29	4	0.0028
	III + IV	30	17	
Histology	serous	18	5	0.5894
	mucinous	19	8	
	others	22	8	
Grade	1	17	3	0.2795
	2	20	9	
	3	22	9	
Residual	no	27	5	0.0034
	yes	32	16	
Distant metastasis	no	51	14	0.0029
	yes	8	7	
Recurrence	no	34	4	0.0000
	yes	25	17	

Table 5
Prognostic factors for overall survival by Cox regression analysis

Variable	B	SE	Wald	RR	95% CI for RR		P value
					Lower	Upper	
YB-1 + P-gp	1.379	0.514	7.200	3.972	1.450	10.878	0.007
Stage	1.265	0.601	4.429	3.542	1.091	11.505	0.035
Recurrence	1.670	0.580	8.279	5.312	1.703	16.571	0.004

YB-1 + P-gp: co-expression of YB-1 and P-gp.

year survival rates than did patients without co-expression (91.1%, 84.0%, 73.1% vs. 83.3%, 66.7%, 40.0%, respectively) (Fig. 3). Besides co-expression of YB-1 and P-gp, stage, residual presence at primary surgery, distant metastasis, and recurrence also were significantly different according to univariate analysis (Table 4).

Five variables that were significant at the 5% level in the univariate analysis were entered into a multivariate analysis model (Table 5). Co-expressions of YB-1 and P-gp, as well as stage and recurrence, were three significant independent prognostic factors.

Discussion

In the present study, 52% of ovarian cancer patients expressed YB-1 in the nucleus of the cancer cell. This was slightly higher than the results reported by other studies including 30% in serous carcinoma [8] and 45.7% in all other types of all ovarian cancer [9]. There was no significant relationship between YB-1 expression and any clinicopathologic prognostic factors, such as clinical stage, histological types or grade, and residuals after first surgery. The same results were also observed in ovarian serous carcinoma [8] and osteosarcoma [6]. In the present study, no relationship was seen between patient survival and YB-1 expression. These results disagreed with the report of stage III ovarian serous carcinoma [8]. We had only seven cases with stage III serous ovarian cancer in our series, including two patients with negative nuclear YB-1 that showed relatively longer survival (3.39 and 5.68 years, respectively). Both of them were also P-gp negative. We were unable to prove the significance between YB-1 and prognosis in stage III serous carcinoma in our series because of the limitation of cases. In breast cancer and colorectal carcinoma, no relationship between YB-1 expression and survival was observed [7,10].

The overexpression of P-gp appears to be closely associated with multidrug resistance in human cancers [11,12]. In ovarian tumors, the frequency of P-gp expression varied over a wide range (7–80%) [3,8,13–15]. A higher detectable ratio of P-gp expression was reported with amplification of MDR1 gene using the polymerase chain reaction (PCR) method than using the IHC method because of the higher sensitivity in PCR method [15]. We found P-gp expression in 30.5% of our untreated ovarian cancers. Review of the literatures suggests that our result was close to values reported for P-gp in the literatures, which were also using IHC in untreated ovarian cancer [16–18]. Nevertheless, no relationship had been found between survival and P-gp expression in our paper and the literature regarding primary ovarian tumors [3,13,19].

It was reported that overexpression of P-gp was linked to YB-1 expression because MDR-1 which encodes P-gp is activated by nuclear localization of YB-1 [4,20]. Correlation between overexpression of nuclear YB-1 and P-gp was reported in osteosarcoma and breast cancer [6,7]. But there was no correlation in ovarian cancer or lung cancer [8,21]. This is probably due to tissue-specific characteristics of lung and ovarian cancer. On the other hand, in the present study, the co-expression of YB-1 and P-gp significantly correlated with patient survival. Moreover, the co-expression of YB-1 and P-gp was shown to be an independent prognostic factor by both univariate and multivariate analysis as were clinical stage and history of recurrence. Many parameters are involved in the mechanism of drug resistance. Although this mechanism is complicated, the simultaneous appearance of multiple factors of drug resistance would be a further unfavorable combination. YB-1 is translocated from cytoplasm into the nucleus of tumor cells by exogenous stress such as ultraviolet irradiation or cytotoxic drugs [22]. Some tumors would acquire transient drug resistance after chemotherapy. In the present study, the analyzed drug resistance was only an intrinsic, not an acquired one, because all our samples were taken at primary surgery without having undergone chemotherapy. For the next step, tissue sampling from various points during treatment, such as at second-look operation, secondary cytoreductive surgery, or surgery for recurrent tumor, should be done. The change of drug resistance during chemotherapy may be detected from those samples.

In conclusion, co-expression of YB-1 and P-gp could be a biomarker predicting poor prognosis in ovarian cancer. Further studies are now in progress, which will help in choosing the most appropriate drugs for patients with persistent or recurrent tumor.

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Vaccination with Predesignated or Evidence-Based Peptides for Patients with Recurrent Gynecologic Cancers

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Abstract: Two different trials of peptide vaccination were conducted for patients with recurrent gynecologic cancers. In the first regimen, four HLA-A24$^+$ patients (two with cervical cancer and two with ovarian cancer) were vaccinated with peptides that were predesignated before vaccination. Three patients exhibited with a grade 1 adverse effect, and no clinical response was observed in any patients. In the second regimen, six HLA-A24$^+$ and four HLA-A2$^+$ patients (five with cervical cancer, one with endometrial cancer, one with uterine sarcoma, and three with ovarian cancer) were vaccinated with peptides (maximum four) to which preexisting cytotoxic T lymphocyte precursors in the periphery were confirmed before vaccination. With this regimen, grade 1 adverse effects were observed in eight patients, a grade 2 adverse effect in one patient, and a grade 3 adverse effect (ie, rectal bleeding) in one patient. However, this regimen was able to enhance peptide-specific cytotoxic T lymphocytes in seven of ten patients, and three of five cervical cancer patients showed objective tumor regression. Analysis of immunoglobulin G -reactive to administered peptides suggested that the induction of peptide-specific immunoglobulin G was correlated with clinical responses. Overall, these results suggest that peptide vaccination of patients showing evidence of preexisting peptide-specific cytotoxic T lymphocyte precursors could be superior to vaccination with predesignated peptides, and that the evidence-based regimen is applicable for clinical trials in treatment of patients with recurrent gynecologic cancers.

Key Words: peptide, vaccination, immunotherapy, gynecologic cancer

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Recent advances in molecular biology and tumor immunology have resulted in identification of many tumor antigens and epitopes recognized by tumor-reactive cytotoxic T lymphocytes (CTLs).[1,2] In the field of gynecology, vaccination has been conducted with human papilloma virus (HPV)16 E7-derived peptides for HLA-A2$^+$ patients with cervical cancer, although clinical responses have been unsatisfactory.[3–5] We previously identified a panel of antigenic peptides having the potential to induce peptide-specific and tumor-reactive CTLs in patients with epithelial cancers,[6–16] and several antigens have been shown to be expressed in gynecologic cancers and to have the potential to induce CTLs reactive to gynecologic cancers.[17]

In most protocols of peptide-based vaccination, no consideration has been paid to whether or not peptide-specific CTL precursors are preexistent in cancer patients. Since priming of naive CTLs generally takes longer than boosting of primed CTLs, vaccination with peptides after confirmation of preexisting peptide-specific CTL precursors might be therapeutically beneficial because it would promptly induce peptide-specific CTLs. To put this idea into practice, we developed a new culture protocol to screen a panel of antigenic peptides with a limited number of peripheral blood mononuclear cells (PBMCs),[18] and we confirmed that peptide-specific CTL precursors can be detected in most patients with pancreatic or gastric cancer.[19,20] In this study, gynecologic cancer patients were vaccinated with peptides according to two different regimens: vaccination with predesignated peptides or vaccination with peptides to which preexisting CTL precursors in the periphery were confirmed before vaccination. Our results suggest that the latter evidence-based regimen is effective for patients with recurrent gynecologic cancers, especially cervical cancer.

MATERIALS AND METHODS

Patients and Eligibility Criteria

Two different regimens were approved by the Institutional Ethical Review Boards of Kurume University. Com-

plete written informed consent was obtained from all patients at the time of enrollment. According to the protocol, patients were required to be positive for either HLA-A2 or HLA–A24. The expression of HLA-A24 or HLA-A2 molecules on PBMCs of cancer patients was first determined by flow cytometry, and HLA-A2 subtypes were determined by the sequence-specific oligonucleotide probe method. All patients were pathologically confirmed to have gynecologic cancer (cervical cancer, endometrial cancer, uterine carcinosarcoma, or ovarian cancer). Eligibility criteria included an age of 85 years or younger, serum creatinine of <1.4 mg/dL, bilirubin of <1.5 mg/dL, platelet count of ≥100,000/μL, hemoglobin of ≥8.0 g/dL, total WBC of ≥3000/μL, and negativity for hepatitis B and hepatitis C antigens. All patients had been untreated for at least 4 weeks before the study, and had an Eastern Cooperative Oncology Group performance status of 0 to 1. Patients with evidence of serious illness, an active secondary malignancy during five years before entry, immunosuppression, or autoimmune disease were excluded from the study.

Screening of Peptide-Specific CTL Precursors

Thirty milliliters of peripheral blood was obtained before and after the third, sixth, ninth, and twelfth vaccinations, and PBMCs were isolated by means of Ficoll-Conray density gradient centrifugation. Peptide-specific CTL precursors in PBMCs were detected using a previously reported culture method.[18] Briefly, PBMCs (1×10^5 cells/well) were incubated with 10 μM of a peptide in 200 μL of culture medium in U-bottom-type 96-well microculture plates (Nunc, Roskilde, Denmark). The culture medium consisted of 45% RPMI-1640 medium, 45% AIM-V medium (GIBCO BRL), 10% FCS, 100 U/mL of interleukin-2 (IL-2), and 0.1 μM MEM nonessential amino acid solution (GIBCO-BRL). Half of the medium was removed and replaced with new medium containing a corresponding peptide (20 μM) every three days. After incubation for twelve days, these cells were harvested and tested for their ability to produce IFN-γ in response to CIR-A2402 or T2 cells that were preloaded with either a corresponding peptide or HIV peptides (RYLRQQLLGI for HLA-A24 and LLFGYPVYV for HLA-A2) as a negative control. The level of IFN-γ was determined by enzyme-linked immunosorbent assay (ELISA) (limit of sensitivity: 10 pg/mL). All assays were performed in quadruplicate. A two-tailed Student's *t* test was used for the statistical analyses. Based on the results of this test, up to four positive peptides were selected for each patient, and then a skin test was performed. Peptides, which were negative for the skin test, were vaccinated into cancer patients. To evaluate the effects of immunization and newly determined peptides for vaccination, patients were re-screened for peptide-specific CTL precursors after the sixth and the twelfth vaccinations.

Peptides and Vaccination

The peptides used in the present study were prepared by Multiple Peptide Systems (San Diego, CA) under the conditions of Good Manufacturing Practice. The sequences of the peptides are shown in Table 1. All of these peptides have previously been shown to induce HLA-A24- or HLA-A2-restricted and tumor-reactive CTLs in PBMCs of cancer patients.[6–16] Although all peptides for HLA-$A2^+$ patients were selected based on the binding motif to HLA-A*0201 molecules, these peptides are immunogenic not only in HLA-A*0201 patients but also in those with other HLA-A2 subtypes such as HLA-A*0206 or HLA-A*0207.[13–16] Montanide ISA-51 adjuvant was manufactured by Seppic, Inc. (Franklin Lake, NJ). The peptides were supplied in vials containing three mg/mL sterile solution for injection. Three milligrams of peptide with sterile saline was added in a 1:1 volume to the Monotide ISA-51 and then mixed in a Vortex mixer (Fisher Inc., Alameda, CA). The resulting emulsion was injected subcutaneously in the lateral thigh using a glass syringe. Patients were vaccinated initially with three injections every two weeks to determine the toxicity levels. For the patients with no toxicity, the vaccinations were then given every two weeks after obtaining additional informed written consent.

Delayed-type Hypersensitivity (DTH) Skin Test

A skin test was performed using 50 μg of each peptide injected intradermally in a volume of 100 μL using a tuberculin syringe and a 27-gauge needle. Saline was injected as a negative control. Patients were examined 48 hours after the injection and were considered to be positive if they showed an at least 10-mm-diameter induration or erythema.

^{51}Cr-Release Assay and Targets

Cytotoxic activity was measured using a standard 6-hour ^{51}Cr-release assay.[21] In brief, cryopreserved PBMCs were thawed and cultured in the culture medium. On the 14th day of culture, the cells were harvested and used for the assay. Targets used for the ^{51}Cr-release assay were as follows: SKG-I (HLA-$A24^+$ cervical cancer cells), TOC-2 (HLA-$A2^+$ ovarian cancer cells), QG56 (HLA-$A24^-$ lung cancer cells), and HLA-$A2^+$ PHA-blastoid T cells. To minimize nonspecific killing, 20-fold unlabeled K562 cells were added to each well.

Kinetics of Peptide-Specific CTL Precursors

For kinetic analysis of peptide-specific CTL precursors, pre- and post-vaccination PBMCs were incubated at 1×10^5 cells per well in 96-well U-bottom microculture plates in the presence of a peptide. Cells from each well were harvested at the 14th day of culture and tested for their ability to produce IFN-γ by recognition of peptide-pulsed C1R-A24 or T2 cells. The criteria for positive wells are given in the legend for Table 2.

TABLE 1. Pre-vaccination Screening of Peptide-Specific CTL-Precursors

Peptide	Sequence	Reference	Patient						Positive	Vaccinated Case
<HLA-A24>			EBG-001	EBG-002	EBG-003	EBG-004	EBG-006	EBG-007		
SART1 690	EYRGFTQDF	6	● Ar	○ B	○ B				3/6	2/6
SART2 93	DYSARWNEI	7		○ C		○ AC			2/6	2/6
SART2 161	AYDFLYNYL	7	○ A			○ C	○ Ar		3/6	3/6
SART2 899	SYTRLFLIL	7	○ B		○ A			● E	3/6	2/6
SART3 109	VYDYNCHVDL	8	● A		○ A		○ C		3/6	2/6
SART3 315	AYIDFEMKI	8							0/6	0/6
CypB 84	KFHRVIKDF	9				● A			1/6	0/6
CypB 91	DFMIQGGDF	9			○ B	○ E	○ E		3/6	3/6
lck 208	HYTNASDGL	10	○ A	○ A				○ D	3/6	3/6
lck 486	TFDYLRSVL	10		○ A		○ CCC		○ D	3/6	3/6
lck 488	DYLRSVLEDF	10		● Ar					1/6	0/6
ART1 170	EYCLKFTKL	11					● ArA		1/6	0/6
ART4 13	AFLRHAAL	12			● B			● B	2/6	0/6
ART4 75	DYPSLSATDI	12	○ B				○ E		2/6	2/6
<HLA-A2>				EBG-101	EBG-102	EBG-103	EBG-194			
SART3 302	LLQAEAPRL	13			○ AA				1/4	1/4
SART3 309	RLAEYQAYI	13				● CC			1/4	0/4
CypB 172	VLEGMEVV	14		● Ar					1/4	0/4
CypB 129	KLKHYGPGWV	14							0/4	0/4
lck 246	KLVERLGAA	15				○ A			1/4	1/4
lck 422	DVWSFGILL	15			○ A		○ A		2/4	2/4
MAP 294	GLLFLHTRT	16		○ AC			○ AC		2/4	2/4
MAP 432	DLLSHAFFA	16		○ ABC		○ C	○ AAC		3/4	3/4
WHS 103	ASLDSDPWV	16		○ ArA					1/4	1/4
WHS 141	ILGELREKV	16				○ AC			1/4	1/4
UBE 43	RLQEWCSVI	16							0/4	0/4
UBE 85	LIADFLSGL	16							0/4	0/4
UBE 208	ILPRKHHRI	16							0/4	0/4
HNR 140	ALVEFEDVL	16							0/4	0/4
HNR 501	NVLHFFNAPL	16		○ CC	○ A	○ A	○ AA		4/4	4/4
EIF 51	RIIYDRKFL	16								

White circles indicate that the peptide was positive for the CTL-precursor induction assay and was vaccinated. Black circles indicate that the peptide was positive for the CTL-precursor induction assay but was not administered due to immediate-type hypersensitivity by skin test.

The assay was performed in quadruplicate and was evaluated by the criteria shown in Table 2.

The classification is shown as alphabet and each character represents the result of each well. For example, ABC means that three wells were judged as A, B, C and one well was negative quadruplicate wells.

Detection of Peptide-Specific Immunoglobulin G (IgG)

The serum levels of peptide-specific IgG were measured by ELISA as previously reported.[22] Briefly, a peptide (20 μL/well)-immobilized plate was blocked with BlockAce (Yukijirushi, Tokyo, Japan) and washed with 0.05% Tween 20-PBS. One hundred microliters per well of serum samples diluted with 0.05% Tween 20-BlockAce were added to the plate. After a 2-hour incubation at 37°C, the plate was washed and further incubated for two hours with a 1:1000-dilluted rabbit anti-human IgG (γ-chain-specific: DAKO, Glostrup, Denmark). The plate was washed again, 100 μL of 1:100-diluted goat anti-rabbit Ig-conjugated horseradish (En Vision, DAKO) was added to each well, and the plate was incubated for 40 minutes. The plate was washed once again, 100 μL/well of tetramethyl benzidine substrate solution (KPL, Guildford, UK) was added, and the reaction was stopped by the addition of one M phosphoric acid. To estimate peptide-specific IgG levels, the optical density (OD) values of each sample were compared with those of serially diluted standard samples, and

TABLE 2. Classification of CTL Response to Peptides

Classification	*P* Value*	IFN-γ Production†
Ar (armed response)	≤0.1	500 ≤ value
A	≤0.05	50 ≤ value
B	≤0.05	25 ≤ value
C	0.05 < *P* ≤ 0.01	50 < value
D	0.05 < *P* ≤ 0.01	25 ≤ value ≤ 50
E	0.1 < *P* ≤ 0.03	100 ≤ value

*The *P* value was determined by Student's *t* test.
†Specific IFN-γ production (pg/mL) was calculated by subtracting the response to HIV-derived irrelevant peptide.

the values were shown as OD units per milliliter. To confirm the specificity of IgG to the peptide, we cultured 100 μL of sample in the peptide-coated wells to absorb peptide-specific IgG, and determined the levels of peptide-specific IgG in the resultant sample.

Evaluation of Response to Treatment

All known sites of disease were evaluated every three months by CT scan or MRI, and/or x-ray examination before and after vaccinations. However, additional examinations were performed when the clinical conditions changed. Patients were assigned a response category according to the response evaluation criteria in solid tumors, based on the June 1999 revision of the WHO criteria published in the WHO Handbook for Reporting Results of Cancer Treatment. Tumor size was evaluated by the longest diameter, and tumor regression of more than 30% for four weeks was regarded as a partial response (PR). The levels of tumor markers, including CA125, CA19-9, carcinoembryonic antigen, and SCC, were measured at the Clinical Examination Laboratory at Kurume University.

RESULTS

Demographics of Patients

Four and 10 patients with gynecologic cancers were enrolled in two different vaccination regimens, respectively. The demographic details of the patients are shown in Table 3. The median age of these patients was 53.5 years (range, 38–68 years). Four HLA-A24$^+$ patients (2 with cervical cancer and 2 with ovarian cancer) were enrolled in the first vaccination regimen, in which predesignated peptides were vaccinated based on the finding that SART2 and ART4 antigens were identified using HLA-A24-restricted CTLs reactive to squamous cell carcinoma and adenocarcinoma, respectively.[7,12] The other 6 HLA-A24$^+$ and 4 HLA-A2$^+$ patients (five with cervical cancer, three with ovarian cancer, one with endometrial cancer,

TABLE 3. Patient Characteristics

Regime	Case No.	HLA	Age (yr)	PS*	Tumor	Stage	Site of Metastasis	Previous Treatment†	No. of Vaccination Received	Clinical Response
1	SART2-001	A24	67	0	Cervical cancer	IVa	Pelvic LN	C/R, C	9	PD (3M)
1	SART2-002	A24	52	1	Cervical cancer	IIb	Lung, para-aorta LN, pelvic LN, virchow LN	S, C, R	9	PD (5M)
1	ART4-001	A24	52	0	Ovarian cancer	IIc	Multiple liver, multiple LN	S, C	7	PD (1M)
1	ART4-002	A24	68	0	Ovarian cancer	IIIa	Lung, ischial bone	S, C, R	15	PD (3M)
2	EBG-001	A24	40	0	Cervical cancer	Ib	Para-aorta LN	C/R	31	SD (3M), PR (4M), PD (15M)
2	EBG-002	A24	67	0	Endometrial cancer	Ic	Lung	S, C	18	PD (2M)
2	EBG-003	A24	66	0	Cervical cancer	IIb	(−)	S, C	25	SD (3M), PD (6M)
2	EBG-004	A24	57	0	Ovarian cancer	IIIc	(−)	S, C	13	SD (8M)
2	EBG-006	A24	56	0	Uterine carcinosarcoma	III	(−)	S	8	SD (3M), PD (5M)
2	EBG-007	A24	38	0	Cervical cancer	IIIb	Lung	C/R, C	7	PD (2M)
2	EBG-101	A2 (A*0206)	67	0	Cervical cancer	IVa	(−)	C, R	10	SD (3M), PR (10M)
2	EBG-102	A2 (A*0206)	49	0	Ovarian cancer	IIIc	(−)	S	8	PD (3M)
2	EBG-103	A2 (A*0201)	63	0	Cervical cancer	IVa	Parametrium	R	14	SD (13M)
2	EBG-104	A2 (A*0201)	59	1	Ovarian cancer	IIIc	Spleen	S, C	5	PD (2M)

*Performance Status by ECOG score.
†S, surgery; C, chemotherapy; R, radiation therapy; C/R, chemoradiotherapy; LN, lymph node.

and one with uterine sarcoma) were enrolled in the second regimen, in which patients were vaccinated with peptides to which preexisting CTL precursors were confirmed before the peptide vaccination. Three patients (EBG-003, EBG-004, and EBG-006) had no measurable disease at the time of entry but were enrolled in this study because they had high risk of relapse (EBG-003, cervical cancer stage IIb post chemotherapy-radiotherapy; EBG-004, ovarian cancer stage IIIc recurrence postchemotherapy; and EBG-006, uterine carcinosarcoma stage III post simple total abdominal hysterectomy and bilateral salpingo-oophorectomy). No patient had received any concurrent treatments, or any steroids, or any other immunosuppressive drugs, for 4 weeks prior to the vaccination. All 10 patients completed the first three vaccinations within the protocol under informed consent, and all of them received additional vaccinations (5 to 31) after providing additional informed consent.

Toxicities

All patients were evaluated for toxicity levels. The overall toxicities are shown in Table 4. In the first regimen, local redness and swelling were observed in 3 of 4 patients, and no other toxicity was observed. In the second regimen, common adverse events were local redness and swelling (grade 1 or 2) at the injection sites. Fever was observed in 3 patients. Inguinal lymph node swelling (grade 1) was observed in one patient. Although rectal bleeding (grade 3) was observed in one patient (EBG-101) after the fifth vaccination, the correlation to the vaccination was unclear because this patient had radiation-induced colitis in the rectum before entry into this trial. In addition, there was no clinical evidence of autoimmune reactions as determined by symptoms, physical examination, or laboratory tests.

First Regimen

In the first regimen, patients were vaccinated with predesignated peptides. Although we had designated 3 SART2 peptides (SART2 93, SART2 161, and SART2 899) as peptides for two patients with cervical cancer (SART2-001 and SART2-002), the SART2 899 peptide was not vaccinated because this peptide elicited immediate-type hypersensitivity at the skin test (data not shown). The other two patients with ovarian cancer (ART4-001 and ART4-002) were vaccinated with only the ART4 75 peptide because the ART4 13 peptide also elicited immediate-type hypersensitivity at the skin test (data not shown).

Screening of Peptide-Specific CTL Precursors and the Second Regimen

In the second regimen, prevaccination PBMCs were used for screening of preexisting CTL precursors reactive to peptides. Fourteen peptides binding to HLA-A24 molecules and 16 peptides binding to HLA-A2 molecules were used for the screening. The results for each well were classified into 6 groups based on the P value and the amounts of IFN-γ, as shown in Table 2. Up to 4 peptides were selected as candidates for the peptide vaccination. Patients who showed immediate-type hypersensitivity by the skin test were vaccinated with the fifth-ranked peptide, provided that their skin test result for this peptide was negative. The results of prevaccination screening of peptide-specific CTL precursors are shown in Table 1. In HLA-A24$^+$ patients, the SART2 161, the CypB 91, the lck 208, and the lck 486 peptides were most frequently positive (3 of 6 patients) for CTL precursors without immediate-type hypersensitivity. In HLA-A2$^+$ patients, the HNR 501 peptide was positive in all patients, and the MAP 432 peptide showed the second highest rate of positivity (3 of 4 patients). It is of note that 8 patients were positive for at least 4 peptides, with the exception that EBG-007 and EBG-102 were positive for 2 and 3 peptides, respectively. After the 6th vaccination, peptide-specific CTL precursors were rescreened, and peptide candidates for additional vaccination were determined. In some cases, peptide-specific CTL precursors were screened a third time after the twelfth vaccination, and further peptide candidates for vaccination were determined. All data are summarized in Table 5. The peptide vaccination based on the second regimen augmented peptide-specific IFN-γ production in 7 patients. Unexpectedly, peptide vaccination seemed to induce CTLs reactive to irrelevant peptides. As typically observed in patient EBG-103, the first vaccination with the lck 246, the

TABLE 4. Toxicity Associated With the Peptide Vaccination

	Regimen 1					Regimen 2				
Toxicity	Grade 1	Grade 2	Grade 3	Grade 4	Total	Grade 1	Grade 2	Grade 3	Grade 4	Total
Dermatologic	3				3/4	4	1			5/10
Fever					0/4	3				3/10
Rectal bleeding					0/4			1		1/10
Inguinal lymph node swelling					0/4	1				1/10

Toxicities are based on NIH Common Toxicity Criteria.

TABLE 5. Summary of Responses of the Regimen 2

Case	Peptide	Peptide-specific CTL*			Ab to Peptides		DTH		Clinical Response/Time to Progression (Months)
		Pre	Post (6th)	Post (12th)	Pre	Post†	Pre	Post	
<HLA-A24>									
EBG-001	SART2 161	A‡	–	–	–	–	–	–	shrinkage of metastatic LN (42%)
	SART2 899	B	–	A	+	+ (6)	–	–	CEA; 207 → 105
	lck 208	A	Ar	AC	–	+ (9)	–	–	SD (3M), PR (4M), PD (15M)
	ART4 75	B	C	CCC	–	+ (21)	–	–	
	SARTI 690	Ar	C	ArAr	–	+ (12)	–	–	
	SART3 109	AA	Ar	–	–	+ (5)	–	–	
	lck 488	–	A	–	–	+ (5)	–	–	
	CypB 91	–	–	A	–	–	–	–	
EBG-002	SART1 690	B	–	–	–	–	–	–	PD/2M
	SART2 93	C	–	AA	–	–	–	–	
	lck 208	A	B	–	–	+ (9)	–	–	
	lck 486	A	–	A	–	+ (6)			
	ART1 170	–	–	–	–	–	–	–	
	lck 488	Ar	–	AAAE	–	–	–	–	
EBG-003	SART1 690	B	A	A	–	–	–	–	SD (3M), PD (6M)
	SART2 899	A	–	–	–	–	–	+ (3)	
	SART3 109	A	–	–	–	–	–	–	
	CypB 91	B	–	ArA	–	–	–	–	
	lck 488	–	AAA	–	–	–	–	+ (9)	
	SART2 93	–	BB	–	–	–	–	–	
	SART2 161	–	A	–	–	–	–	–	
	SART3 315	–	–	Ar	–	+ (10)	–	+ (14)	
EBG-004	SART2 93	AC	AC	A	–	–	–	–	SD (8M)
	SART2 161	C	AAA	AB	–	–	–	+ (3)	
	CypB 91	E	–	–	–	–	–	+ (3)	
	lck 486	CCC	–	AA	–	+ (6)	–	–	
	SART3 315	–	A	AAA	–	–	–	+ (7)	
EBG-006	SART2 161	Ar	ArAr	NT	–	–	–	–	SD (3M), PD (5M)
	SART3 109	C	ArArA	NT	–	+ (6)	–	–	
	CypB 91	E	–	NT	–	–	–	–	
	ART4 75	E	–	NT	–	–	–	–	
	SART2 93	–	ArAr	NT	–	–	–	–	
	lck 488	–	C	NT	–	–	–	–	
EBG-007	lck 208	D	–	NT	–	+ (6)	–	–	PD (2M)
	lck 486	D	–	NT	–	–	–	–	
	SART3 109	–	B	NT	–	+ (11)	–	–	
<HLA-A2>									
EBG-101	MAP 294	AC	–	NT	–	–	–	–	Shrinkage of tumor (48%)
	MAP 432	ABC	ArA	NT	–	+ (6)	–	–	SD (3M), PR (10M)
	WHS 103	ArA	A	NT	–	–	–	–	SCC 289 → 36
	HNR 501	CC	–	NT	–	–	–	–	CEA: 13.3 → 6.6
	lck 246	–	ArAr	NT	–	+ (4)	–	–	
	lck 422	–	ArArAr	NT	–	–	–	–	
	UBE 43	–	–	NT	–	+ (4)	–	–	
EBG-102	SART3 302	AA	A	NT	–	–	–	+ (2)	PD (3M)
	lck 422	A	NT	NT	–	–	–	+ (3)	CA 125: 24000 → 17000
	HNR 501	A	–	NT	–	–	–	–	CA 19-9: 113 → 29.3
	SART3 309	–	AA	NT	–	–	–	–	
	MAP 294	–	A	NT	–	–	–	–	
	MAP 432	–	B	NT	–	–	–	–	
EBG-103	lck 246	A	ArC	ArArA	–	+ (5)	–	–	SD (13M)
	MAP 432	C	–	AACC	–	+ (7)	–	–	
	WHS 141	AC	–	–	–	+ (4)	–	–	
	HNR 501	A	B	A	–	–	–	–	
	lck 422	–	ABCC	AAAA	–	–	–	–	
	UBE 43	–	AAAC	ABC	–	+ (2)	–	–	
EBG-104	lck 422	A	NT	NT	–	–	–	–	PD (2M)
	MAP 294	AC	NT	NT	–	+ (3)	–	–	
	WHS 432	AAc	NT	NT	–	–	–	–	
	HNR 501	AA	NT	NT	–	–	–	–	

NT: not tested.
*The criteria are shown in Table 2.
†The number in the parenthesis represents the vaccination when IgG to the peptide was detected for the first time.
‡Peptides shown as a bold letter were administered into patients.

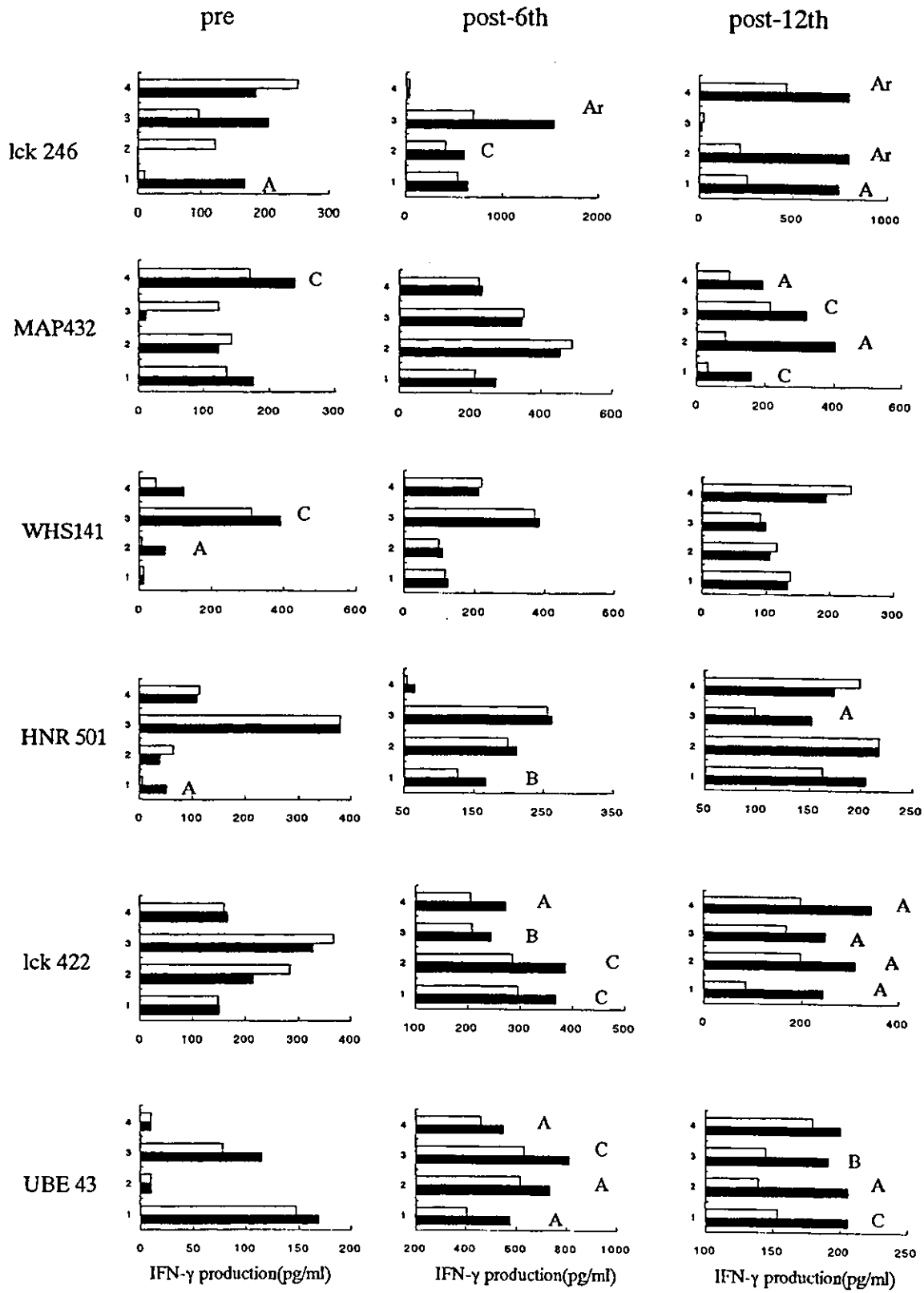

FIGURE 1. Detection of peptide-specific CTL precursors. Pre- and post- (6^{th} and 12^{th}) vaccination PBMCs from patient EBG-103 (HLA-$A2^+$) were applied for the screening of peptide-specific CTL precursors. Values represent IFN-γ production by the in vitro cultured PBMCs. Criteria for evaluation are shown in Table 2. Open and closed bars represent IFN-γ production in response to HIV peptide-pulsed and the corresponding peptide-pulsed stimulator cells, respectively. T2 cells were used as stimulator cells.