criteria for solid tumors, a revised version of the WHO criteria published in June 1999 in the WHO Handbook for reporting results of cancer treatment. Overall survival (OS) and progression-free survival (PFS) were evaluated from the first vaccination, and were analyzed in order to investigate correlations between clinical benefits and immune responses. Kaplan-Meier curves were described, and survival times were compared using the Log-rank test.

Results

Patient Characteristics

Eleven PC patients were enrolled in this phase I clinical study of personalized peptide vaccination. The detailed characteristics are shown in Table 2. The mean age of the patients was 66.7 (range: 59-84). Six patients had undergone surgical resection of the primary lesion, and had histologically determined adenocarcinomas. For the remaining 5 inoperable patients, adenocarcinoma (n=4) and acinar cell carcinoma (n=1) were suspected by clinical evaluation and the laboratory findings, i.e., tumor markers, computer tomography (CT), and needle biopsy. Four patients had received chemotherapy, and two of them had received additional radiotherapy. The remaining 4 patients did not have any prior treatment. Ten advanced cases with confirmed recurrence entered into this trial, while the remaining one patient (case No. 11) received the vaccination 93 days after surgery as an adjuvant treatment without confirmed recurrence.

With regard to treatment after the vaccination, 9 patients did not receive any other combined form of therapy, but the remaining two patients received the vaccination combined with chemotherapy. Namely, cases No. 9 and 10 received GEM /5-fluorouracil /cisplatin after the 7th vaccination because of an elevation in tumor markers, and the 3rd vaccination because of progressive disease (PD), respectively.

Peptide screening, vaccination, and adverse events

Ten patients were HLA-A24 positive and were vaccinated with HLA-A24-binding peptides. Among the 14 peptides for HLA-A24$^+$ patients, lck-derived peptides were most frequently used for the vaccination (lck208 and lck488 for 6 patients, and lck486 for 5 patients)(Table 1). CypB84 and ART4-75 were not used for the vaccination into any patient due to an immediate-type hypersensitivity reaction. Representative results for peptide selection in 4 patients whose post-vaccination PBMCs showed increased responses are shown in Figure 1, and the summary is given in Table 3.

All 11 patients were evaluated for adverse events according to the NCI-CTC. The

vaccinations were generally well tolerated without hematological toxicity or symptoms of any autoimmune diseases. The most frequently observed toxicity was an inflammatory reaction at the injection site in 7 of 11 patients. Three patients showed a grade 1-level reaction, and three showed a grade 2-level reaction. No treatment was required for these six patients. The remaining 1 patient showed a grade 3 inflammatory reaction with leg edema at the 31st vaccination, after which a non-steroidal antiphlogistic agent was administrated. Fever (grades 2 and 1), anorexia (grade 1), and fatigue (grade 1) were observed in 3 (2 and 1), 1, and 1 patient, respectively (Table 4).

Cellular immune responses

No DTH reaction against the peptides was observed prior to vaccination in any patient. DTH reactions were observed in 7 patients (Nos. 1, 3-6, 9, and 11) until the 5th vaccination, the details of which are given in Table 3. For example, in case No. 3, a DTH reaction to lck208 was observed after the 2nd vaccination and reactions to SART1-690, SART2-161, and CypB91 were observed after the 5th vaccination.

Augmentation of CTL precursors reactive to at least one of the vaccinated peptides was observed in 4 of 8 patients tested (cases No. 1 and 2 for lck488, No. 8 for SART3-315, and No. 9 for SART2-93 and lck-486; Fig. 1). Because of the limited availability of blood samples, we kinetically evaluated the anti-tumor cytolytic activity of pre- and post-vaccination PBMCs by a ^{51}Cr-release assay in 7 patients in response to each of the four different cells of PC cell lines, but none of the post-vaccination PBMCs showed increased cytotoxicity against PC cells in an HLA-class I-restricted manner (data not shown). Significant and equal levels of cytotoxicity against PC cells were observed in an HLA-class I-non-restricted manner in both the pre- and post-vaccination PBMCs from 4 patients (cases No. 2, 6, 9, and 11). In contrast, such cytotoxicity decreased in the post-vaccination PBMCs from the remaining 3 patients (cases No. 1, 3, and 4).

Humoral immune responses

We also examined whether or not peptide-specific IgG could be detected in the vaccinated patients. Peptide-specific IgG to the vaccinated peptides was detected in the pre-vaccination sera of 4 of 10 patients tested (Table 3). Peptide vaccination increased the IgG levels in 1 of these 4 patients (case No. 11, IgG to SART3-109)(Fig. 2). Although no peptide-specific IgG was detected in the other 6 tested patients before the peptide vaccination, peptide vaccination resulted in the induction of peptide-specific IgG in 3 patients (case No 4, anti-ART1-170; No 6, anti-ART1-170, -lck208, and -SART3-315; No. 9, anti-SART2-93). The results are shown in Fig. 2. The peptide specificity of the IgG in

the sera of these patients was confirmed by an absorption test, although the data are not shown because the peptide specificity was reported in the previous reports.[14-19]

Clinical outcomes and prognostic factor analysis

It was difficult to draw any definitive results from this small-scale phase I study with regard to clinical responses and a prognostic factor analysis. Nevertheless, demonstration of the available results may be relevant from the point of view of developing a suitable peptide vaccine. In 10 patients who received more than 3 vaccinations and were eligible for clinical evaluation, stable disease (SD) of 3 patients (cases No. 2, 6, and 9) and PD of 6 patients were diagnosed at the time of 6th vaccination. The remaining one patient (case No. 11) with an adjuvant-setting vaccination had a recurrence in the bone marrow 336 days after the initial vaccination. The median time to progression (TTP) and the median survival time (MST) were 96±34.0 (±Standard Error) days and 232±32.4 days, respectively. Their 6-month and 1-year survival rates were 80% and 20%, respectively.

As regards the identification of a laboratory marker to predict long-term survival, the group of patients with increased peptide-specific IgG levels (n=4) showed prolonged survival compared to the group of patients with no increase in peptide-specific IgG levels (n=6)(MST 339 days vs.194 days, respectively; log-rank test p=0.0217)(Fig. 3). In contrast, neither the augmentation of a CTL precursor reactive to a peptide nor a positive DTH response influenced survival.

Discussion

This study was conducted in order to evaluate the safety and biological responses of the personalized peptide vaccination. Severe toxicity was rarely associated with the peptide vaccination, and this regimen can be recommended for further evaluation. Cellular and humoral responses to the vaccinated peptides were observed in 50% and 40% of the post-vaccination PBMCs and sera, respectively. Therefore, this regimen is also recommended for further evaluation of the immunological responses. However, from the clinical point of view, though clinical response was not a main objective, this regimen might not be recommended if used alone. It is because no significant clinical response was obtained, although the MST was not as short as those of the other clinical trials investigating patients with advanced PC.[3-5,21,22] Personalized peptide vaccination combined with chemotherapy might be recommended, since one such case had a partial response (PR). Nevertheless, clinical studies in a phase II setting are needed to address

this issue.

Increased rates of peptide-reactive cellular and humoral responses to the vaccinated peptides in the post-vaccination PBMCs and sera of advanced cancer patients other than PC patients were somewhat higher than those of the PC patients observed here.[14-19] In addition, no increment in HLA-class I-restricted CTL activity against PC cells was observed at all in the post-vaccination PBMCs from any of the 7 patients tested. In contrast, such an increment was observed in the post-vaccination PBMCs from patients with other types of cancer.[14-19] These results suggest that immunity in advanced PC is more depressed than that in other epithelial cancers. Alternatively, a more suitable peptide repertoire might be provided for PC patients. Tumor-associated antigens and peptides derived from PC cell lines might be more suitable in use for personalized peptide vaccinations administered to PC patients. From this point of view, the 14 peptides provided for HLA-$A24^+$ patients were primarily derived from esophageal cancers.[7-9] In contrast, the 16 peptides provided for HLA-$A2^+$ PC patients were primarily derived from PC9 tumor cell line, as reported previously,[10] but only one HLA-$A2^+$ patient was enrolled in this clinical study. Therefore, clinical study of additional HLA-$A2^+$ patients is needed to address this issue, which is now in progress.

We previously reported that the increase in IgG levels due to administration of a personalized peptide vaccination correlated well with long-term survival in patients with advanced stages of cancers other than PC.[19] The statistically significant difference ($p=0.0217$) was also observed among PC patients, although the number of patients in this study was small. Further studies are therefore needed to confirm this issue.

The biological role of peptide IgG in anti-tumor immunity should be clarified by future basic and clinical studies. The mechanisms of peptide-specific IgG production, including the involvement of CD4 T helper cells and HLA-restriction, also need to be elucidated.

Goydos *et al.* reported the data obtained from a phase I trial of a synthetic mucin MUC-1 peptide vaccine admixed BCG,[21] and only one of 24 patients (4.2%) had SD. The MST data were not given in that reported. Gjertsen *et al.* presented the data from a clinical phase I/II trial involving PC patients who were vaccinated by intradermal injection of synthetic mutant ras peptides in combination with granulocyte-macrophage colony-stimulating factor.[22] That report showed that, in the group with non-resectable cancer, 11 out 34 patients (32%) had SD and the 1-year survival rate was 6.3% (3 out of 48 cases). Our patients appeared to have better survival rates than those in previous clinical trials. However, as mentioned above, this regimen by itself may not be recommended for HLA-24^+ patients with advanced PC. Further basic and clinical studies shall be conducted for developing therapeutically effective peptide vaccinations

for advanced PC patients.

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Figure legends

Figure 1. Representative results of peptide-specific CTL precursors in PBMCs of the pre- and post (6th-vaccinations). Four sets of columns in each peptide indicate the results of quadruplicate cultures in the 4 wells. The value of each well was evaluated based on the following criteria. A level of armed response (Ar): $p \leq 0.01$ and $500 \leq$ net value (the amount of IFN-□ in response to the corresponding peptide minus that in response to HIV peptide); A level of response (A): $p \leq 0.05$ and $50 \leq$ net; B: $p \leq 0.05$ and $25 \leq$ net < 50; C: $0.05 < p \leq 0.1$ and $50 \leq$ net; D: $0.05 < p \leq 0.1$ and $25 \leq$ net < 50.

Figure 2. Kinetic study of IgG levels specific to the peptides administrated to each patient. Pre- and post (6th)-vaccination sera were serially diluted and the levels of peptide-specific IgG were measured using ELISA, as described in Materials and Methods. Results of 7 cases are shown in the figure. Horizontal lines indicated optical density (OD) and vertical lines indicate dilution of sera.

Figure 3. Antibody response and overall survival. Overall survival of 10 cases is given in Fig.3A. The group of patients with increased peptide-specific IgG levels (n=4) showed prolonged survival compared to the group of patients with no increase in peptide-specific IgG levels (n=6) (Fig. 3B). In contrast, neither the positive DTH response (Fig.3C) nor the augmentation of CTL precursor reactive to peptide (Fig.3D) influenced their survival.

Table 2. Patient's characteristics

No.	Age /Sex	HLA type	PS	Stage[1)]	Histlogy[2)]	Site of metastases[3)]	Previous treatments		
							Surgery[4)]	Chemo-therapy[5)]	Radio-therapy
1	60 M	A24	0	T4N1M0 III	adeno s/o	abdominal LNs	-	-	-
2	63 F	A24	1	recurrence	tubular adeno	neck LNs bone	PPPD	GEM	LNs bone
3	70 M	A24	0	recurrence	well diff. tubular adeno	liver local	PD	5Fu MTX	-
4	63 M	A24	0	T3NxM1(HEP) IV	adeno s/o	liver	-	5Fu CDDP	local
5	59 M	A2	0	recurrence	moderately diff. tubular adeno	peritoneum local	PD	-	-
6	69 M	A24	0	recurrence	well diff. tubular adeno	abdominal LNs	DP	-	-
7	84 F	A24	0	T4N1M0 III	adeno s/o	-	-	-	-
8	67 M	A24	0	T3N1M1 IV	adeno s/o	-	-	-	-
9	68 F	A24	0	recurrence	well diff. tubular adeno	abdominal LNs	PPPD	5Fu CDDP	-
10	60 M	A24	2	T3NxM1(HEP) IV	acinar cell carcinoma s/o	liver	-	-	-
11	71 F	A24	0	T3N0M0 IIA	moderately diff. tubular adeno	-	PD	-	-

1) UICC Classification of Pancreatic Cancer (6th Edition, 2002)
2) s/o, suspected of; diff., differentiated; adeno, adenocarcinoma
3) LNs, lymph nodes
4) PPPD, pylorus-preserving pancreaticoduodenectomy; PD, pancreaticoduodenectomy; DP, distal pancreatectomy
5) GEM, gemcitabine; 5FU, fluorouracil; MTX, methotrexate; CDDP, cisplatin

Table 1. Vaccinated peptide and immune responses

	Peptide Name	Sequence	No. of vaccinated patients[1)]	Increased immune responses[2)]		
				CTL	IgG	DTH
HLA-A24-binding	SART1 690	EYRGFTQDF	1	0/1	0/1	1
	SART2 93	DYSARWNEI	1	1/1	1/1	1
	SART2 161	AYDFLYNYL	2	0/1	0/1	1
	SART2 899	SYTRLFLIL	1	0/1	0/1	0
	SART3 109	VYDYNCHVDL	3	0/2	1/3	2
	SART3 315	AYIDFEMKI	2	1/2	1/2	1
	CypB84	KFHRVIKDF	0	-	-	-
	CypB91	DFMIQGGDF	4	0/3	0/3	2
	lck208	HYTNASDGL	6	0/6	1/6	4
	lck486	TFDYLRSVL	5	1/5	0/5	3
	lck488	DYLRSVLEDF	6	2/4	0/4	4
	ART1 170	EYCLKFTKL	3	0/2	2/2	2
	ART4 13	AFLRHAAL	1	0/1	0/1	0
	ART4 75	DYPSLSATDI	0	-	-	-
HLA-A2-binding	SART3 302	LLQAEAPRL	1	-	0/1	1
	SART3 309	RLAEYQAYI	0	-	-	-
	CypB129	KLKHYGPGWV	0	-	-	-
	CypB172	VLEGMEVV	0	-	-	-
	lck246	KLVERLGAA	1	-	0/1	1
	lck422	DVWSFGILL	1	-	0/1	1
	MAP294	GLLFLHTRT	0	-	-	-
	MAP432	DLLSHAFFA	1	-	0/1	1
	WHSC103	ASLDSDPWV	0	-	-	-
	WHSC141	ILGELREKV	0	-	-	-
	UBE43	RLQEWCSVI	0	-	-	-
	UBE85	LIADFLSGL	0	-	-	-
	UBE208	ILPRKHHRI	0	-	-	-
	HNRPL140	ALVEFEDVL	0	-	-	-
	HNRPL501	NVLHFFNAPL	0	-	-	-
	EIF51	RIIYDRKFL	0	-	-	-

1) HLA-A24-binding and -A2-binding peptides were vaccinated into 10 and 2 patients, respectively.

2) Immune responses to the vaccinated peptide were compared at pre- and post-vaccination. Interferon?? production of CTLs, Peptide-specific IgG antibody density, and DTH responses were tested.

Table 3. Immuno responses and clinical outcomes

No	Peptide	CTL response[1]		Antibody to peptide[2]		DTH[3]	Cyto-toxicity[4]	No. of vacci-nation	Clinical response at[5]		PFS / OS[6]
		Pre	Post	Pre	Post				6th	12th	days
1	lck 486	A	B	0.09	-	+(3)	Not increased	10	PD	-	59 / 165
	lck 208	C	C	0.05	0.03	+(3)					
	lck 488	C	ArB	-	-	+(3)					
2	ART1 170	AAA	AA	-	-	-	Not increased	7	SD	PD	102 / 271
	ART4 13	A	C	-	-	-					
	lck 488	CC	ArAC	-	-	-					
3	SART1 690	ArA	B	0.31	0.37	+(5)	Not increased	8	PD	-	43 / 194
	SART2 161	A	A	0.12	0.12	+(5)					
	CypB 91	A	-	0.14	0.23	+(5)					
	lck 208	A	A	0.27	0.22	+(2)					
4	SART3 109	C	NT	-	-	+(1)	Not increased	6	PD	-	57 / 232
	lck 488	D		-	-	+(1)					
	ART1 170	D		-	1.18	+(1)					
5	SART3 302	C	NT	-	-	+(2)	NT	5	PD	-	43 / 73
	lck 246	Ar		-	-	+(2)					
	lck 422	C		-	-	+(2)					
	MAP 432	C		-	-	+(2)					
6	ART1 170	ArBCC	-	-	2.06	+(3)	Not increased	32	SD	SD	898+ / 898+
	lck 488	AAA	-	-	-	+(3)					
	lck 208	AC	A	-	0.25	+(3)					
	SART3 315	C	-	-	0.03	+(3)					
7	SART2 899	AA	-	-	-	-	NT	8	PD	-	110 / 247
	SART3 109	A	-	0.10	0.09	-					
	lck 208	A	A	-	-	-					
	lck 486	A	B	-	-	-					
8	SART3 315	C	ArAC	-	-	-	NT	8	PD	PR chemo+	96 / 206
	CypB 91	C	A	-	-	-					
	lck 208	C	A	-	-	-					
	lck 486	C	-	-	-	-					
9	lck 488	ArAr	CC	-	-	+(4)	Not increased	7	SD	-	671+ / 671+
	SART2 93	ArCC	ArArArC	-	0.04	+(4)					
	lck 486	C	ACC	-	-	+(4)					
10	SART2 161	A	NT	NT	NT	-	NT	3	PD	-	49 / 617+
	lck 488	AB				-					
	CypB 91	Ar				-					
11	lck 486	A	B	-	-	+(3)	Not increased	12	no rec	no rec	336 / 339
	CypB 91	A	-	-	-	+(3)					
	SART3 109	D	C	0.03	1.51	+(3)					
	lck 208	D	-	-	-	+(3)					

1) The peptide-specific CTL precursor cells were evaluated by quadricate assay in pre- and post (6th)- vaccination. NT, not tested.

2) Values indicate the fluorescent intensity of sera (x100 dilution).

3) Number of the vaccination when DTH to the peptide was detected for the first time.

4) Cytotoxicity to HLA-matched cancer cells of pre- and post-PBMCs was evaluated by ^{51}Cr-relase assay.

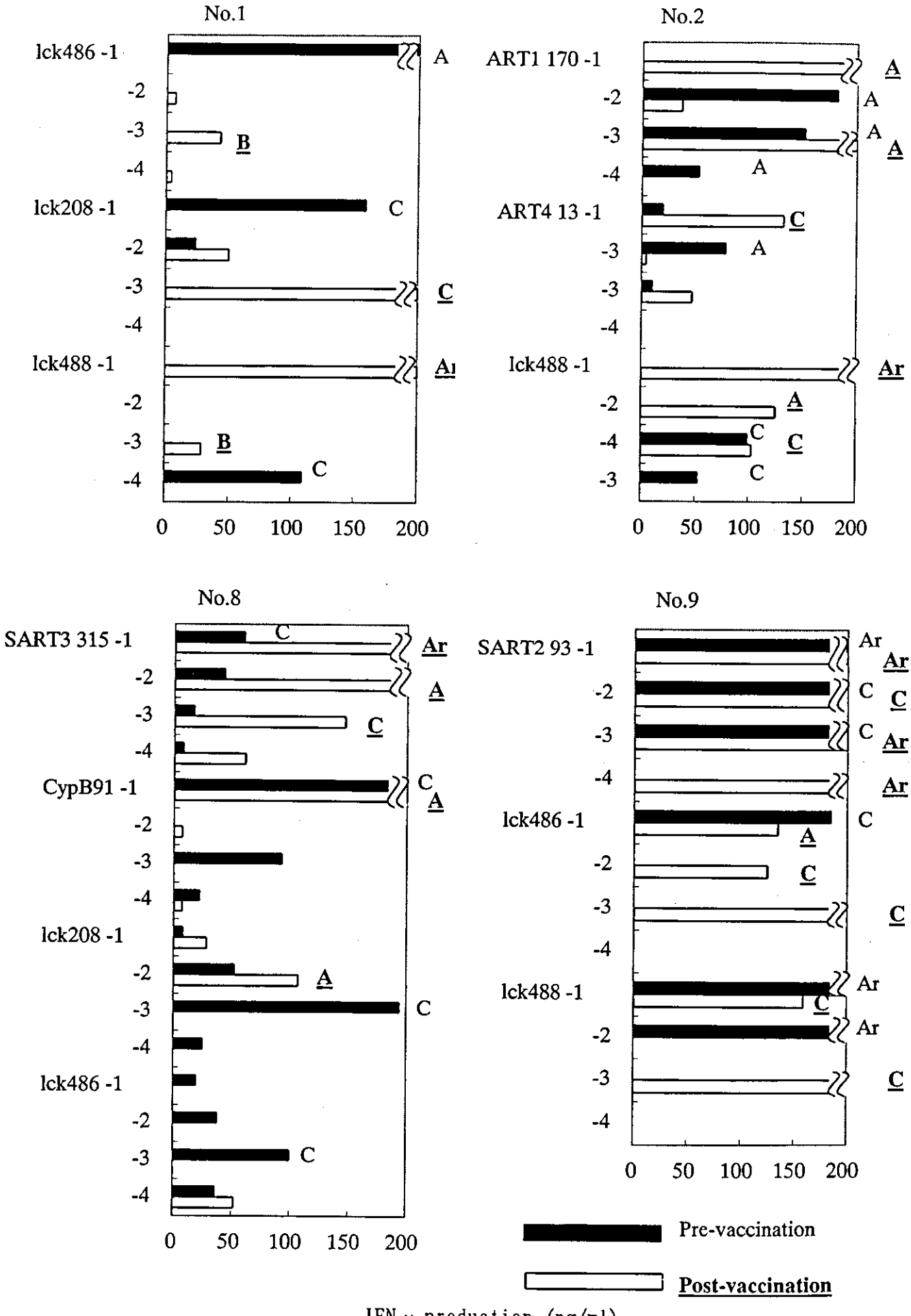
5) PD, progressive disease; SD, stable disease; PR, partial response; no rec, no recurrence

6) PFS, progression free survival; OS, overall survival; Pulse(+) mark, Patients are alive (2004.3.31).

Table 4. Adverse events

	Grade 1	Grade 2	Grade 3
Fever	1	2	
Inflammatory reactions at the vaccination site	3	3	1
Anorexia	1		
Fatigue	1		

According to NCI-CTC criteria version2.0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-2
-3
-4
lck208 -1
-2
-3
-4
lck488 -1
-2
-3
-4
A
B
C
C
A
B
C
0
50
100
150
200
No.2
ART1 170 -1
-2
-3
-4
ART4 13 -1
-3
-3
-4
lck488 -1
-2
-4
-3
A
A
A
A
C
A
A
C
C
Ar
No.8
SART3 315 -1
-2
-3
-4
CypB91 -1
-2
-3
-4
lck208 -1
-2
-3
-4
lck486 -1
-2
-3
-4
C
Ar
A
C
C
A
A
C
C
0
50
100
150
200
No.9
SART2 93 -1
-2
-3
-4
lck486 -1
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-3
-4
lck488 -1
-2
-3
-4
Ar
C
C
Ar
Ar
C
A
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C
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C
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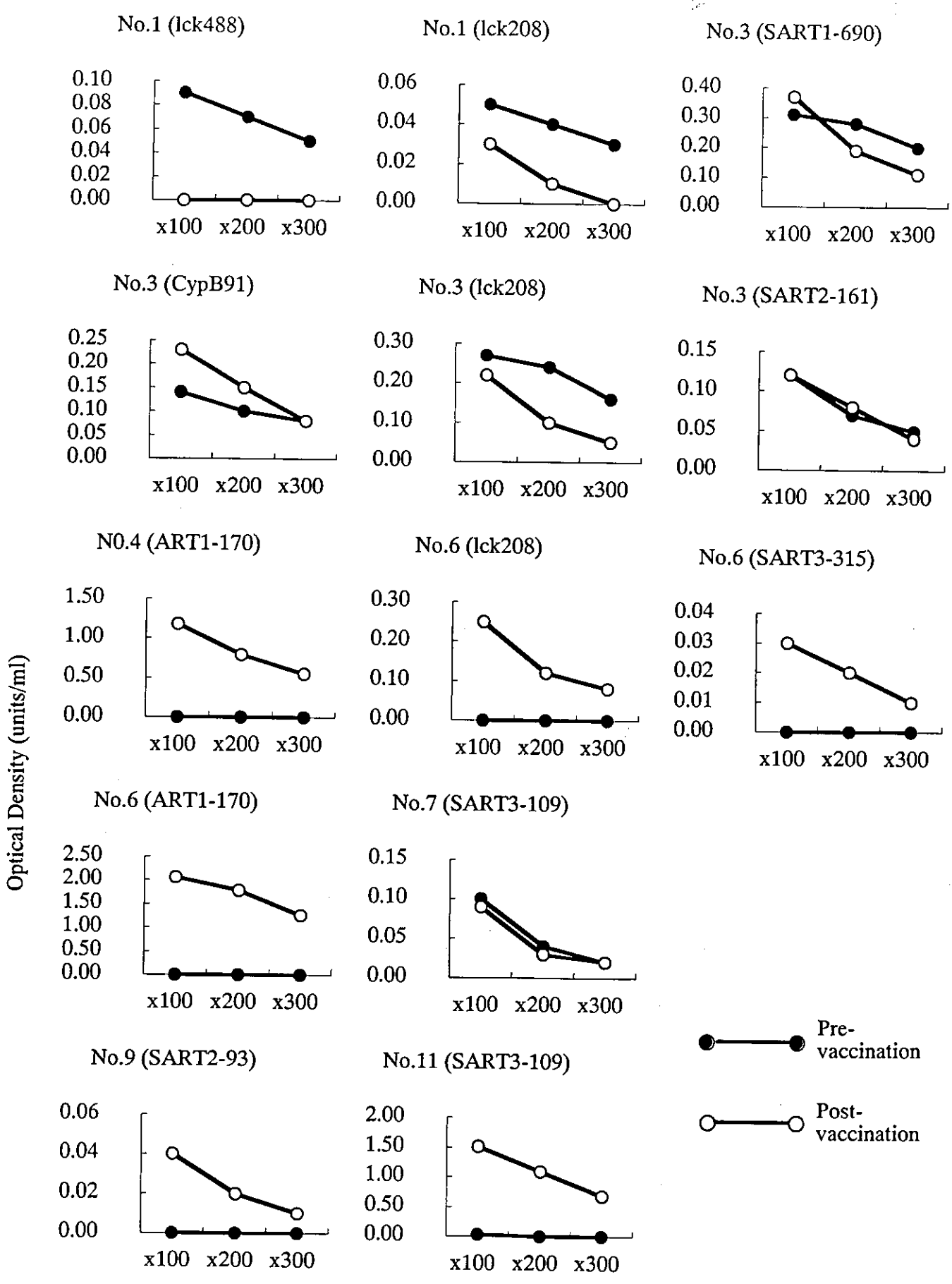
50
100
150
200
Pre-vaccination
Post-vaccination
IFN-γ production (ng/ml)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No.1 (lck208)
No.3 (SART1-690)
No.3 (CypB91)
No.3 (lck208)
No.3 (SART2-161)
N0.4 (ART1-170)
No.6 (lck208)
No.6 (SART3-315)
No.6 (ART1-170)
No.7 (SART3-109)
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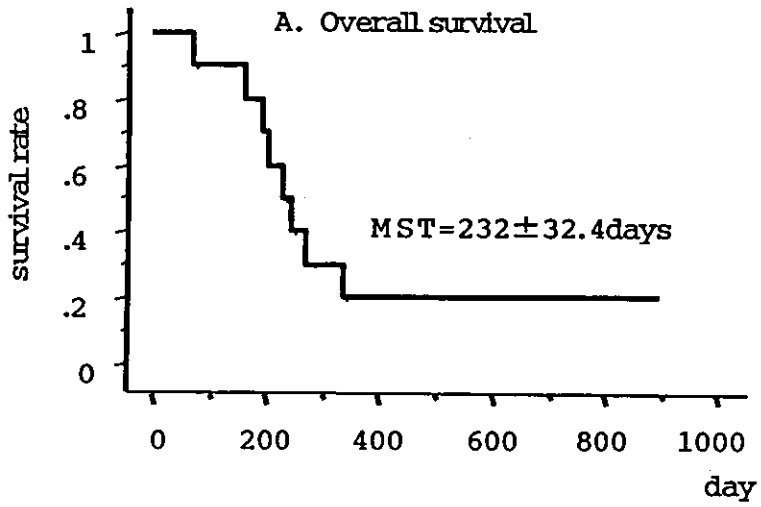
No.11 (SART3-109)
x100 x200 x300
Optical Density (units/ml)
Pre-vaccination
Post-vaccination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.6
.4
.2
0
MST=232±32.4days
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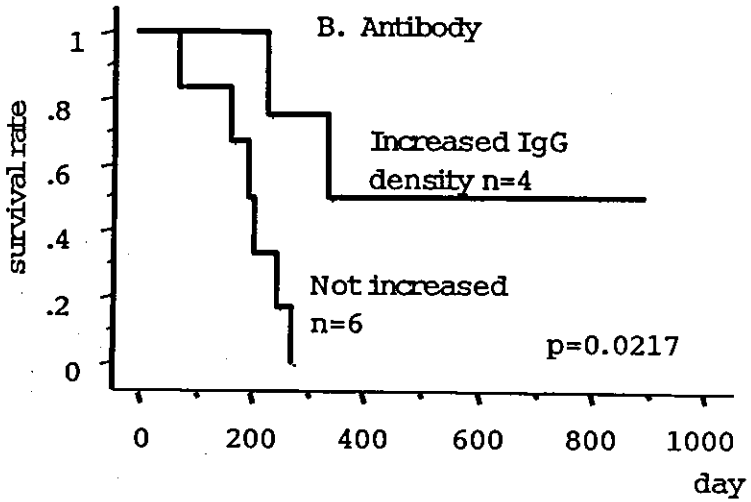
200
400
600
800
1000
day


B. Antibody
survival rate
1
.8
.6
.4
.2
0
Increased IgG
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Not increased
n=6
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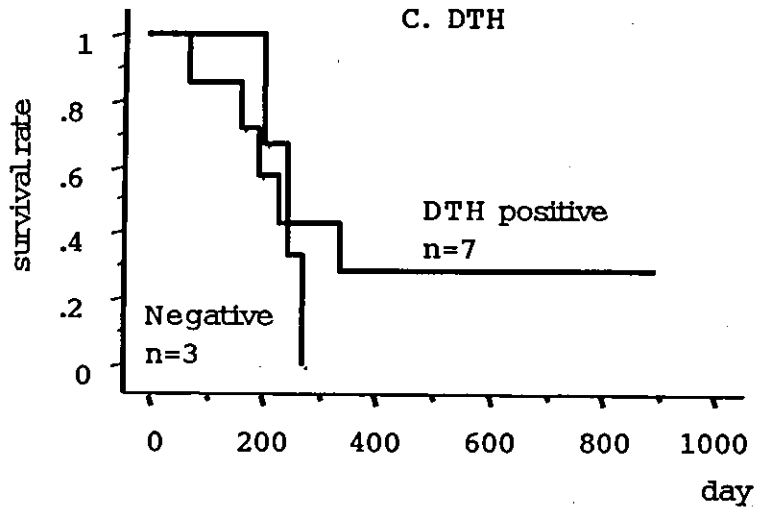
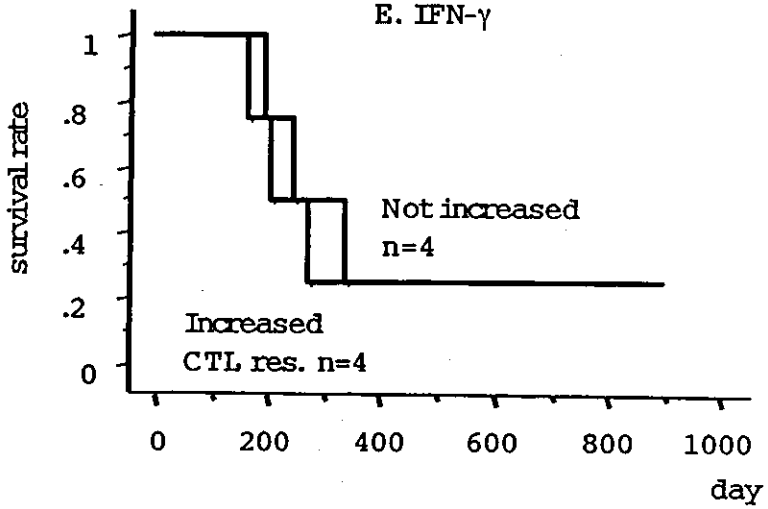
0
200
400
600
800
1000
day


C. DTH
survival rate
1
.8
.6
.4
.2
0
DTH positive
n=7
Negative
n=3
0
200
400
600
800
1000
day


E. IFN-γ
survival rate
1
.8
.6
.4
.2
0
Not increased
n=4
Increased
CTL res. n=4
0
200
400
600
800
1000
day

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Tumor HLA-DR expression linked to early intrahepatic recurrence of hepatocellular carcinoma

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The outcome of patients with hepatocellular carcinoma (HCC) remains poor because of the high frequency of intrahepatic recurrence (IHR), particularly early IHR within 1 year of hepatectomy. To search for genes involved in early IHR, we performed DNA microarray analysis in a training set of 33 HCCs and selected 46 genes linked to early IHR from approximately 6,000 genes by means of a supervised learning method. Gene selection was validated by a false discovery rate of 0.37%. The 46 genes included many immune response-related genes, which were all downregulated in HCCs with early IHR. Four of these genes (*HLA-DRA*, *HLA-DRB1*, *HLA-DG* and *HLA-DQA*), encoding MHC class II antigens, were coordinately downregulated in HCCs with early IHR compared to levels in HCCs with nonrecurrence. A cluster analysis reproduced expression patterns of the 4 MHC class II genes in 27 blinded HCC samples. To localize the major site of production of HLA-DR protein in the tumor, we used 50 frozen specimens from 50 HCCs. Immunofluorescence staining showed that HLA-DR protein levels in tumor cells, but not in stromal cells, were associated with the transcription levels of *HLA-DRA* determined by both DNA microarray analysis and real-time quantitative reverse transcription-PCR. Univariate analysis showed that tumor HLA-DR protein expression, pTNM stage and venous invasion were associated with early IHR. Multivariate analysis showed that tumor HLA-DR protein expression was one of the independent risk factors for early IHR, suggesting HLA-DR protein potential as a biomarker and a molecular target for therapeutic intervention.

Key words: HCC; microarray; MHC; HLA; recurrence

Hepatocellular carcinoma (HCC) is a common fatal cancer worldwide.[1–3] A major obstacle in the treatment of HCC is intrahepatic recurrence (IHR), which is observed in 30% to 50% of HCC patients who undergo curative surgery.[4,5] There are 2 representative modes of IHR after surgery, *i.e.*, early IHR and late IHR.[4,5] The former, most cases of which can be attributed to intrahepatic metastasis of cancer cells and detected within 1 year of surgery, limits the potential for surgical cure of HCC.[2,4,5] Thus, a better understanding of the molecular mechanisms of early IHR will enable us to develop novel therapeutic options for improving poor prognoses.

Since the initial description of DNA microarray analysis,[6] we have used this technology to profile gene expression patterns specific to many aspects of HCC.[5,7–10] Using a supervised learning method, we developed a DNA microarray-based system with only 12 genes for prediction of early IHR of HCC.[5] The 12-gene predictor detected accurately early IHR in HCC patients undergoing curative surgery; however, the 12 genes used are involved in a wide range of biological processes, and their roles in early IHR remain to be clarified.[5] To extend our previous findings,[5] we carried out a postplanned analysis of the DNA microarray datasets that were used to construct the 12-gene predictor.[5]

Our supervised learning procedure yielded 46 genes, which included many immune response-related genes in addition to the 12 genes mentioned above. Because 4 genes (*HLA-DRA*, *HLA-DRB1*, *HLA-DG* and *HLA-DQA*) of the same MHC class II antigen family were included in 46 genes, we focused our present investigation on the identification of primary sites of HLA-DR protein biosynthesis in HCC tissues and examined the relation of protein levels to clinical features of HCCs.

Material and methods

Selection of genes related to early IHR by DNA microarray data analysis

We have analyzed the levels of expression of approximately 6,000 genes in human HCC samples using high-density oligonucleotide arrays (HuGeneFL Array, Affymetrix, Santa Clara, CA).[5,7–10] In our study, to search for genes related to early IHR, we used DNA microarray data from a sample of 33 HCCs, termed training sample, that was used to construct the predictor for early IHR (http://surgery2.med.yamaguchi-u.ac.jp/research/DNAchip/: released according to MIAME by Brazma *et al.*[11]). Among the 33 HCCs, early IHR was found in 12 HCCs (HCV02T, HCV06T, HBV07T, HBV14T, nonBC15T, HCV20T, HCV31T, HCV45T, HBV55T, HCV80T, HCV89T and HCV90T). No recurrence within 1 year after surgery was found in 21 HCCs (nonBC03T, HBV05T, HCV08T, nonBC09T, HCV10T, HCV12T, HCV18T, HCV21T, HCV22T, HCV26T, HCV27T, HCV28T, HCV29T, HBV30T, nonBC32T, HCV37T, HCV42T, HCV46T, HBV48T, HBV57T and HCV59T).

From a pool of approximately 6,000 genes, we first investigated all genes whose mean average differences (ADs) (arbitrary units from Affymetrix) in the 12 HCCs with early IHR were 2-fold higher or 0.5-fold lower than those measured in the 21 HCCs with nonrecurrence. We subsequently selected genes that had mean ADs >20 in either group. This filtering yielded 332 genes. We then ranked the selected genes in order of decreasing magnitude of Fisher ratios.[7–10] To decide how many genes we should consider, we used a random permutation test as described previously.[7–10] From the distribution of the Fisher ratios from randomized data, the top-ranking 46 genes with Fisher ratios >0.92 were considered to show statistically significant ($p < 0.01$) differences in expression between the 2 groups (Fig. 1 and Table I). To validate our gene selection, we calculated the false discovery rate, the percentage of false positive genes, as previously described.[10]

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FIGURE 1 – Gene expression profiles linked to early intrahepatic recurrence (IHR). Color displays of expression of 35 downregulated genes (upper panel) and 11 upregulated genes (lower panel) in HCCs with early IHR compared to HCCs with nonrecurrence. Each gene was ranked in decreasing order of the Fisher ratio (see Material and methods) and was listed with an accession number and symbol. Accession numbers for each gene were obtained from PubMed (http://www3.ncbi.nlm.nih.gov/PubMed/) or TIGR databases (http://www.tigr.org/tdb/hgi/searching/reports.html). Symbols were obtained from LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink/).

To confirm reproducibility of the microarray data of 4 MHC class II genes (*HLA-DRA*, *HLA-DRB1*, *HLA-DG* and *HLA-DQA*), we investigated their expression patterns in 27 blinded HCC samples (http://surgery2.med.yamaguchi-u.ac.jp/research/DNAchip/:) using hierarchical cluster analysis with Cluster software and Tree View software.[12]

TABLE I – FORTY-SIX GENES RELATED TO EARLY INTRAHEPATIC RECURRENCE OF HCC[1]

Fisher ratio	Accession no.	Symbol	Function	Locus
Thirty-five genes downregulated in HCC with early IHR *vs.* HCC without recurrence				
2.6879	L13923	FBN1	Extracellular matrix	15q21.1
2.5592	**L08895**	**MEF2C**	**Transcription**	**5q14**
1.9674	**M59465**	**TNFAIP3**	**Immune system**	**6q23.1–q25.3**
1.7562	**U51240**	**LAPTM5**	**Lysosomal protein interacting with ubiquity**	**1p34**
1.7390	M17733	TMSB4X	Immune system/cell shape and size control	Xq21.3–q22
1.6647	U69546	CUGBP2	RNA binding and RNA processing	10p13
1.5794	**X00274**	**HLA-DRA**	**Immune system**	**6p21.3**
1.5165	**X75042**	**REL**	**Transcription**	**2p13–p12**
1.5095	L43579	clone110298	Unknown	Xq28
1.3955	Z37976	LTBP2	Miscellaneous	14q24
1.3814	**X82200**	**TRIM22**	**Transcription**	**11p15**
1.3591	M33600	HLA-DRBI	Immune system	6p21.3
1.2798	U13219	FOXF1	Transcription	16q24
1.2626	M34996	HLA-DQA	Immune system	6p21.3
1.2569	U19495	SDF-1	Immune system	10q11.1
1.2163	**U59321**	**DDX17**	**Nuclear process or RNA binding protein**	**22q13.1**
1.2156	**Y10032**	**SGK**	**Signal transduction**	**6q23**
1.2130	**M21574**	**PDGFRA**	**Signal transduction**	**4q11–q13**
1.2028	M62424	F2R	Blood coagulation	5q13
1.1963	M13560	HLADG	Immune system	
1.1837	D28915	MTAP44	Immune system	
1.1250	X82153	CTSK	Proteolysis and peptidolysis	1q21
1.1072	**Z19554**	**VIM**	**Cytoskelton**	**10p13**
1.0771	Z22534	ACVR1	Signal transduction	2q23–q24
1.0509	J03040	ON/SPARC	Ossification/extracellular matrix	5q31.3–q32
1.0354	M23178	SCYA3	Immune system	17q11–q21
1.0252	D13631	ARHGEF6	Apoptosis	Xq26
1.0195	**D13639**	**CCND2**	**Cell cycling**	**12p13**
0.9997	M55998	COL1A1	Extra cellular matrix	17q21.3–q22.1
0.9869	X64072	ITGB2/CD18	Immune system and cell adhesion	21q22.3
0.9858	Y00062	PTPRC	Miscellaneous	1q31–q32
0.9710	AB000409	MKNK1	Signal transduction	1pter–p31.3
0.9601	U20734	JUNB	Transcription	19p13.2
0.9364	Z84483	EST	Unknown function	13q12–q13
0.9202	U66075	GATA6	Transcription	18q11.1–q11.2
Eleven genes upregulated in HCC with early IHR *vs.* HCC without recurrence				
1.7441	U27326	FUT3	Metabolism	19p13.3
1.4150	L03411	RDBP	RNA binding and negative elongation factor	6p21.3
1.4096	X82693	E48	Cell adhesion	8q24-qter
1.3543	**AC000063**	**SEMA3F**	**Embryonic development**	**3p21.3**
1.3176	M20778	COL6A3	Extra cellular matrix	2q37
1.2370	J03060	GBAP	Metabolism	1q21
1.0222	M35531	FUT1	Metabolism	19p13.3
1.0018	U78190	GCHFR	Metabolism	15q15
0.9907	X13930	CYP2A	Detoxification and drug metabolism	19p13.2
0.9437	AF001359	MLH1	Mismatch repair	3p21.3
0.9263	U82306	EST	Unknown function	

[1]Bold face indicates the 12 genes used to construct the predictor.[5]

Immunofluorescence staining for HLA-DR protein

To identify the major location of HLA-DR protein in tumors, we used individual frozen specimens from 50 HCCs. All samples were immediately stored at −80°C in optimal cutting temperature (OCT) compound (Tissue-Tek®, Sakura Finetechnical Co., Ltd., Tokyo, Japan) after surgery. Of these 50 HCC samples, 21 and 9 were assigned to the training and blinded sets, respectively, in the previous study.[5] The remaining 20 were newly recruited in our study. Direct immunofluorescence staining was performed as previously reported.[13] Briefly, 5 μm-thin cryostat sections were fixed in 95% ethanol. Sections were stained for HLA-DR and control sections were stained with hematoxylin and eosin. Individual sections were incubated with fluorescein isothiocyanate-conjugated murine monoclonal antibody (1:100) (Becton Dickinson Immunocytometry Systems, Mountain View, CA) against a nonpolymorphic human HLA-DR antigen overnight at 4°C. The immunoreactivity was analyzed with the use of Olympus BX50 fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Positive HLA-DR immunoreactivity was found exclusively in kupffer cells and immune cells in nontumorous livers. Its positive frequency was less than 5% in all hepatocytes examined (data not shown). On the basis of this finding, tumor HLA-DR protein levels were scored as follows: 0, less than 5% of tumor cells were stained; 1, 5–30% of tumor cells were stained and 2, more than 30% of tumor cells were stained. Tumors scoring 1 or 2 were judged as overexpressed for HLA-DR protein. The scoring was performed independently by 2 pathologists blinded to clinical details. The scores assigned by the 2 pathologists were in agreement.

Real-time quantitative reverse transcription (RT)-PCR

To evaluate the relation between HLA-DR protein levels and *HLA-DRA* mRNA levels, we performed real-time quantitative RT-PCR in the 30 HCC samples mentioned above. Both the RT step and real-time quantitative PCR were performed as reported previously.[14,15] Standard plasmid (pAT153) containing the human *HLA-DRA* coding region was supplied by Health Science Research Resources Bank (Osaka, Japan). The plasmid was used as template DNA at numbers ranging from 10^3 to 10^8 copies to produce standard curves (data not shown). To detect human *HLA-DRA* mRNA, oligonucleotides 5′- CTCCCCTTCCTGCCCTCAAC-3′ (sense)

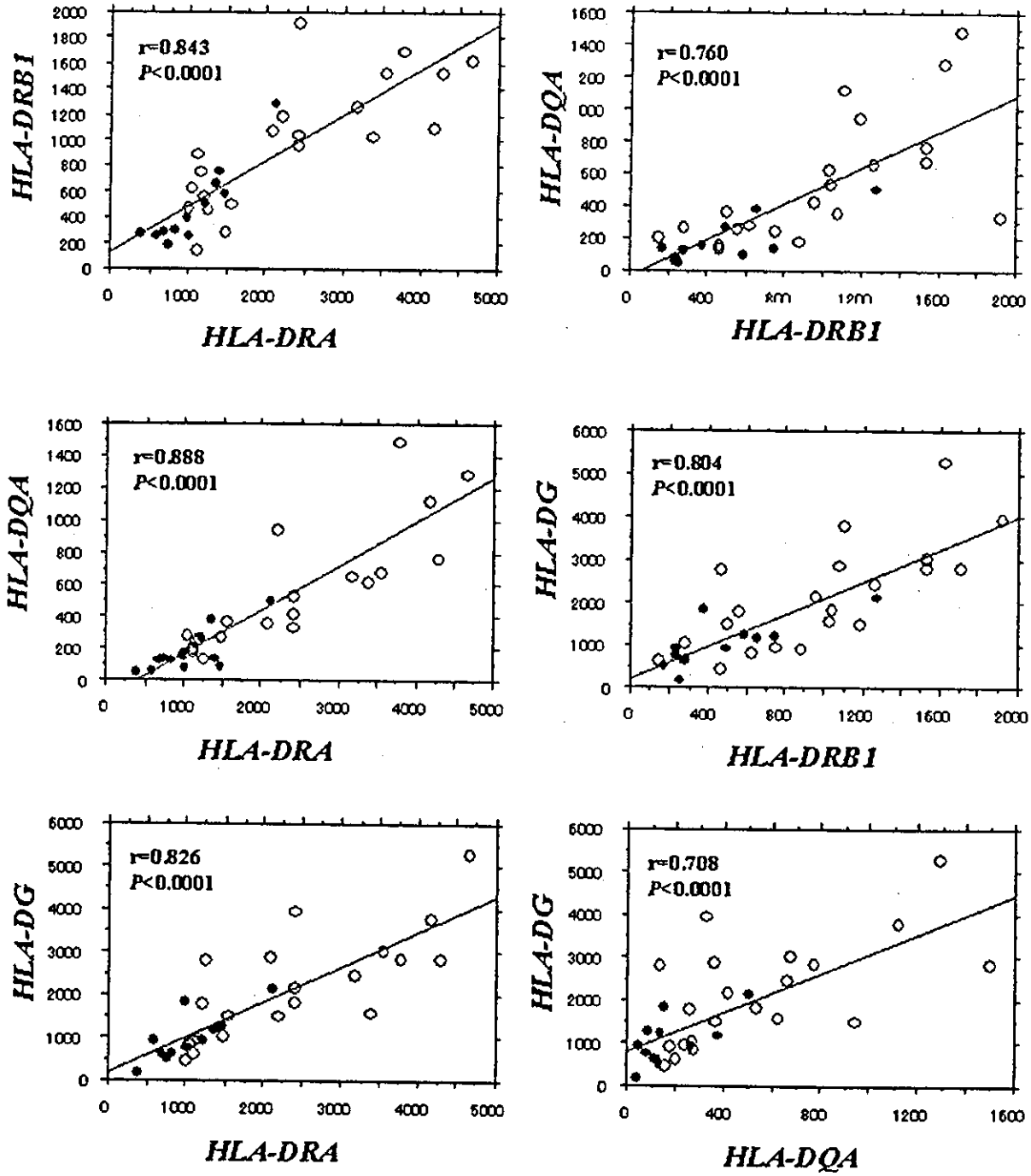

FIGURE 2 – Association among expression levels of 4 MHC class II genes determined by DNA microarray analysis. Pearson correlation coefficient was calculated using the software Statview. All values are average differences (arbitrary units from Affymetrix) of individual genes. All data are available at http://surgery2.med.yamaguchi-u.ac.jp/research/DNAchip/. Note that all 4 genes are coordinately expressed in HCC tissues and expression levels are relatively low in HCCs with early intrahepatic recurrence.

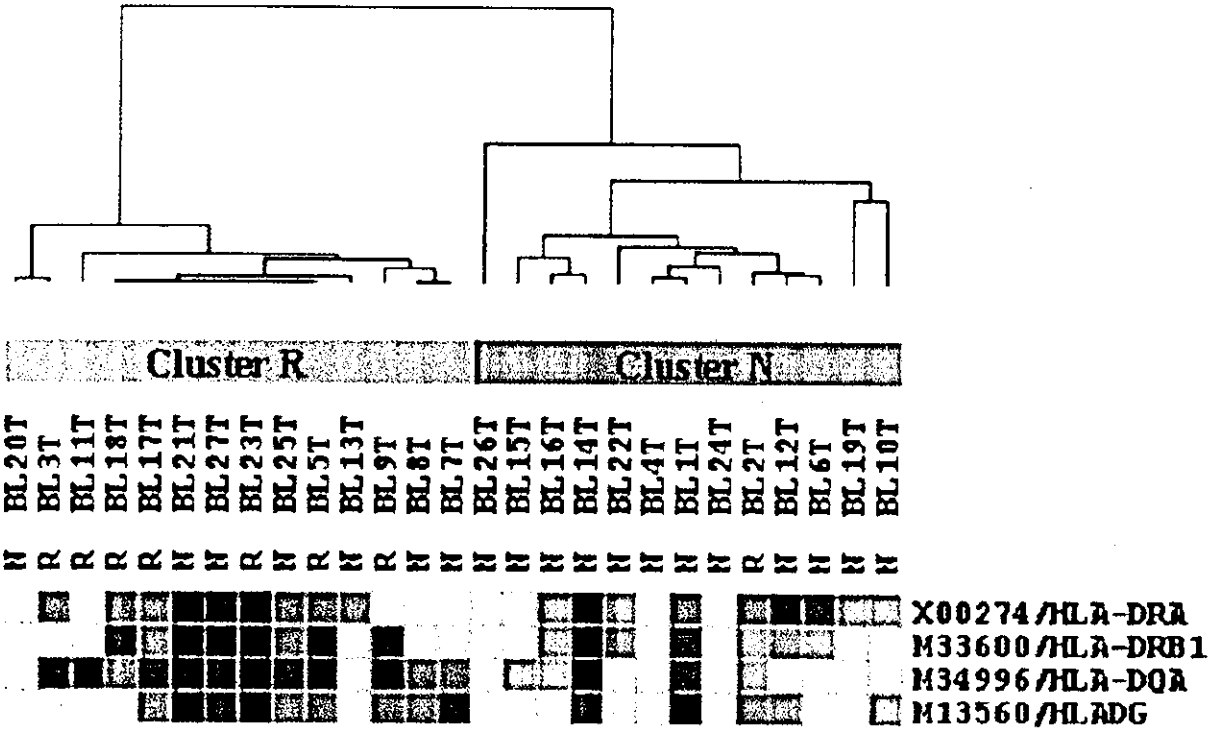

FIGURE 3 – Reproducibility of expression patterns of 4 MHC class II genes on an independent set of HCC samples. We investigated their expression patterns in 27 blinded HCC samples (http://surgery2.med.yamaguchi-u.ac.jp/research/DNAchip/:) using hierarchical cluster analysis with Cluster software and Tree View software.[12] R, HCC with early intrahepatic recurrence. N, HCC with nonrecurrence.

and 5′- ACCCACAGTCAGGCCCA AGG -3′ (antisense), which yield a 156 bp product were used as primers. Five microliters of cDNA solution (equivalent to cDNA from 100 ng of initial RNA) was subjected to real-time RT-PCR amplification. The reaction was performed on a LightCycler™ System Version 3 (Roche Diagnostics, Mannheim, Germany).[15] Products from PCR were quantified with a Lumi-Imager F1 (Roche Diagnostics) and analyzed with LightCycler Software (Roche Diagnostics). Each analysis was performed in duplicate. We calculated the abundance of each transcript as mean copy number per 100 ng RNA for each tissue.

Statistical analysis

Correlations between DNA microarray data of the 4 genes (*HLA-DRA*, *HLA-DRB1*, *HLA-DG* and *HLA-DQA*) and correlations between DNA microarray data and quantitative RT-PCR data for the *HLA-DRA* gene were determined by Pearson correlation coefficients. Reproducibility of expression patterns of the 4 MHC class II genes on 27 blinded samples were validated by Fisher's exact test. Relations between HLA-DR protein and *HLA-DRA* mRNA levels in tumor were evaluated by ANOVA. Relations between tumor HLA-DR protein levels and clinicopathologic factors and relations between early IHR and clinicopathologic factors were analyzed by ANOVA with Fisher's PLSD test, Fisher's exact test and the Mann-Whitney U test. We carried out multivariate analysis to assess independent factors for early IHR in the 50 HCC samples using the stepwise logistic regression model (SPSS 11.0J; SPSS, Inc., Chicago, IL). Seven variables (age, sex, tumor HLA-DR protein levels, tumor size, tumor differentiation, venous invasion and pTNM stage) were entered into a forward stepwise regression model. Each model was tested for goodness of fit by −2 log likelihood and chi-square in each step. $p < 0.05$ was accepted as statistically significant.

Results

We identified 46 genes linked to early IHR of HCC. This gene selection was validated by a false discovery rate of 0.37%. Of those 46 genes, expression levels increased in 11 (23.9%) and decreased in 35 (76.1%) of HCCs with early IHR compared to levels in HCCs without early IHR (Table I). The latter group included many immune response-related genes (Fig. 1 and Table I) including 4 genes (*HLA-DRA*, *HLA-DRB1*, *HLA-DG* and *HLA-DQA*) of the MHC class II antigen family whose expression levels were correlated (Fig. 2). Hierarchical cluster analysis with the 4 MHC-class II genes divided 27 blinded samples into 2 main clusters R and N. Seven of 14 had early IHR in cluster R; in contrast, 12 of 13 had nonrecurrence in cluster N (p=0.033 by Fisher's exact test) (Fig. 3). Thus, their expression patterns were maintained even in blinded HCC samples.

It is known that MHC-class I genes play a central role in host immune response in cancer patient.[16] There were many probes for MHC-class I genes or nonclassical genes on the array in our study; they did not survive in our filtering because their levels were markedly low in most HCCs examined (data not shown).

Among the 4 MHC class II family genes coordinately downregulated in HCC with early IHR, we chose to investigate the major location of HLA-DR protein in tumor. Using immunofluorescence staining, we found that HLA-DR protein was expressed in stromal cells in some cases, but its biosynthesis did not parallel the transcription levels determined by DNA microarray analysis (data not shown). For immunoreactivity of HLA-DR protein in tumor cells, 19 (38%) scored 0, 19 (38%) scored 1 and 12 (24%) scored 2 (Fig. 4). Levels of HLA-DR protein in tumor cells were associated positively with transcription levels of *HLA-DRA* gene by measured DNA microarray analysis and real-time quantitative RT-PCR ($p < 0.0001$ and $p = 0.0001$ by ANOVA) (Fig. 5). Tumor size in HCC scoring 0 was significantly larger than that in HCC scoring 1