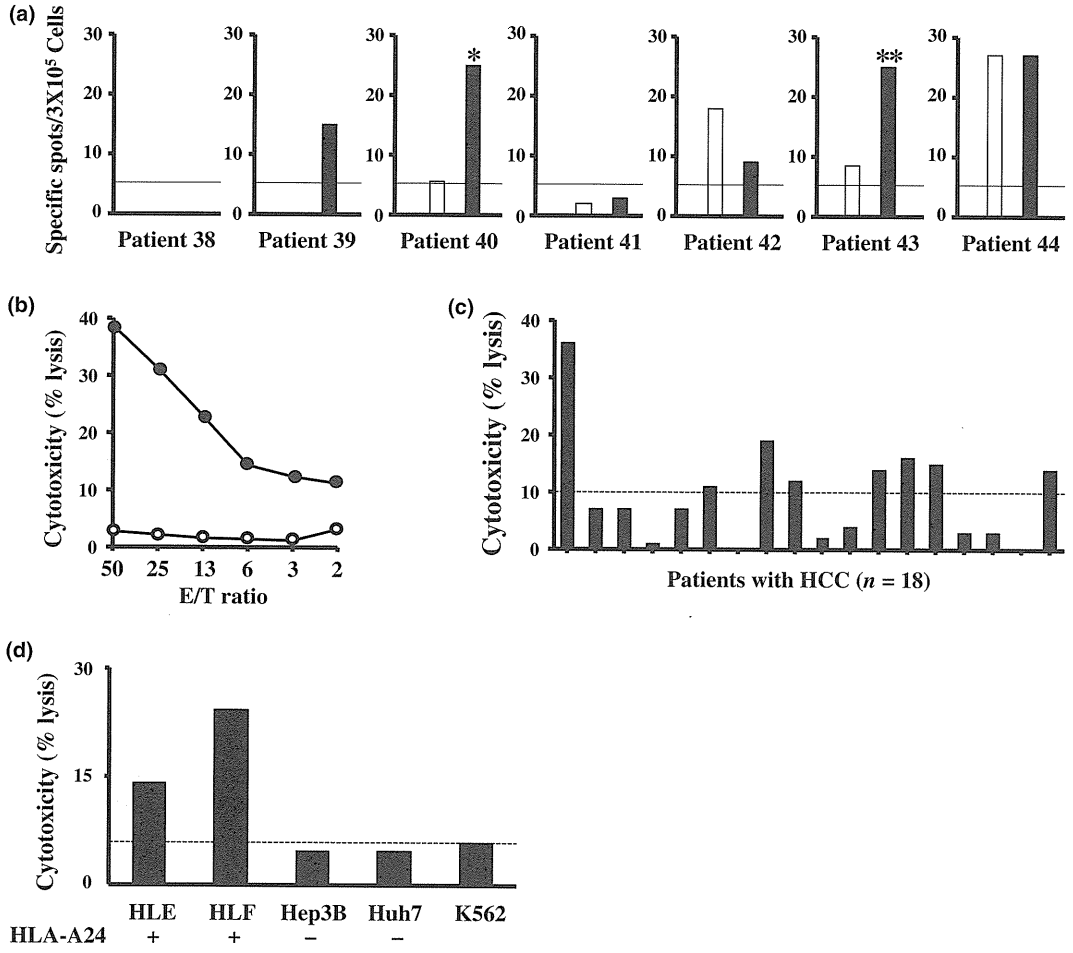

Fig. 3. Characteristics of chondroitin-glucuronate C5-epimerase-specific CTLs. (a) IFN-γ ELISPOT assay of PBMCs and TILs to one of the chondroitin-glucuronate C5-epimerase-derived peptide (peptide 3) in seven HCC patients. Open and solid bars show the frequency of chondroitin-glucuronate C5-epimerase-specific T cells in PBMCs and TILs respectively. *denotes 114 specific spots. **denotes 42 specific spots. (b) Representative results of the CTL assay. The closed and open circles show the cytotoxicity against C1R-A*2402 cells pulsed with and without a peptide respectively. (c) CTL assays (E/T ratio of 50:1) were performed in 18 HCC patients. Solid bars show the result for one patient. The results are shown as specific cytotoxic activity, which was calculated as follows: (cytotoxic activity in the presence of peptide) - (cytotoxic activity in the absence of peptide) and considered positive when higher than 10%. (d) Cytotoxicity of chondroitin-glucuronate C5-epimerase-specific T-cell lines derived with peptides was also measured against hepatoma cell lines. The cytotoxicity was considered positive when it was higher than that against K562 which shows non-specific lysis (E/T ratio of 50:1).

patients who showed immune responses to each peptide was 0–19% (10). In the present study, the frequency of chondroitin-glucuronate C5-epimerase-derived peptide-specific CTLs in HCC patients was 0–30 cells/ 3×10^5 PBMCs and the frequency of the patients who showed immune responses to the peptides was 11–27%. These results show that the frequencies of chondroitin-glucuronate C5-epimerase-specific CTLs in PBMCs and the patients with CTLs responsive to the TAA are very similar to those of previously identified immunogenic TAA-derived epitopes and suggest that the antigen and its CTL epitope are immunogenic. In addition, the CTLs were generated even in the early stages of HCC. These results suggest the advantages of using chondroitin-glucuronate C5-epimerase-derived peptides as a vaccine for immunotherapy of HCC.

For the next step to investigate the usefulness of chondroitin-glucuronate C5-epimerase as an immunotherapeutic target in HCC, we examined the safety and efficacy of chondroitin-glucuronate C5-epimerase-derived peptide as a cancer vaccine. In previous studies using chondroitin-glucuronate C5-epimerase-derived peptides for several cancers, they were reported to be safe. However, most patients with HCC have chronic liver disease. Therefore, safety of the peptide vaccine should be confirmed in the patients with chronic hepatitis or cirrhosis. The present vaccination study included nine patients with chronic liver diseases (four chronic hepatitis and five cirrhotic patients) confirmed by histological examination and there was no severe adverse event in all patients vaccinated. The induction of chondroitin-glucuronate C5-epimerase-specific CTLs

Table 3. Patient characteristics

Patient	Peptide Dose (mg)	Age	gender	Aetiology	Stage of HCC	ALT (IU/L)	AFP (ng/ml)	Child-Pugh (A/B/C)	Histology of liver	Treatment	Immune response	Toxicity (grade)
A1	0.03	73	F	HCV	I	26	12	A	F4A2	RFA	–	Pa(1)
A2	0.03	78	F	HCV	I	45	10	B	F4A2	RFA	–	P(1)
A3	0.03	59	M	NBNC	II	30	10	A	ND	RFA	–	None
B1	0.3	79	M	HCV	I	40	61	A	F3A1	RFA	–	R(1), S(1)
B2	0.3	72	M	NBNC	II	24	66	A	ND	RFA	–	R(1), S(1), P(1), H(1)
B3	0.3	78	M	HCV	II	45	10	A	F3A2	RFA	–	P(1)
C1	3.0	67	M	HCV	I	111	49	A	F3A1	RFA	+	P(1), S(1)
C2	3.0	73	M	NBNC	I	30	5	A	ND	RFA	–	None
C3	3.0	78	F	HCV	I	23	24	A	F4A2	RFA	+	P(1)
C4	3.0	75	M	HBV	I	21	15	A	F3A1	RFA	+	R(1), P(1)
C5	3.0	49	M	HBV	I	18	14	A	F4A1	RFA	+	None
C6	3.0	69	F	HBV	II	42	84	A	F4A2	RFA	–	Pa(1)

H, headache; Pa, pain; P, pruritus; R, rubor; S, skin induration.

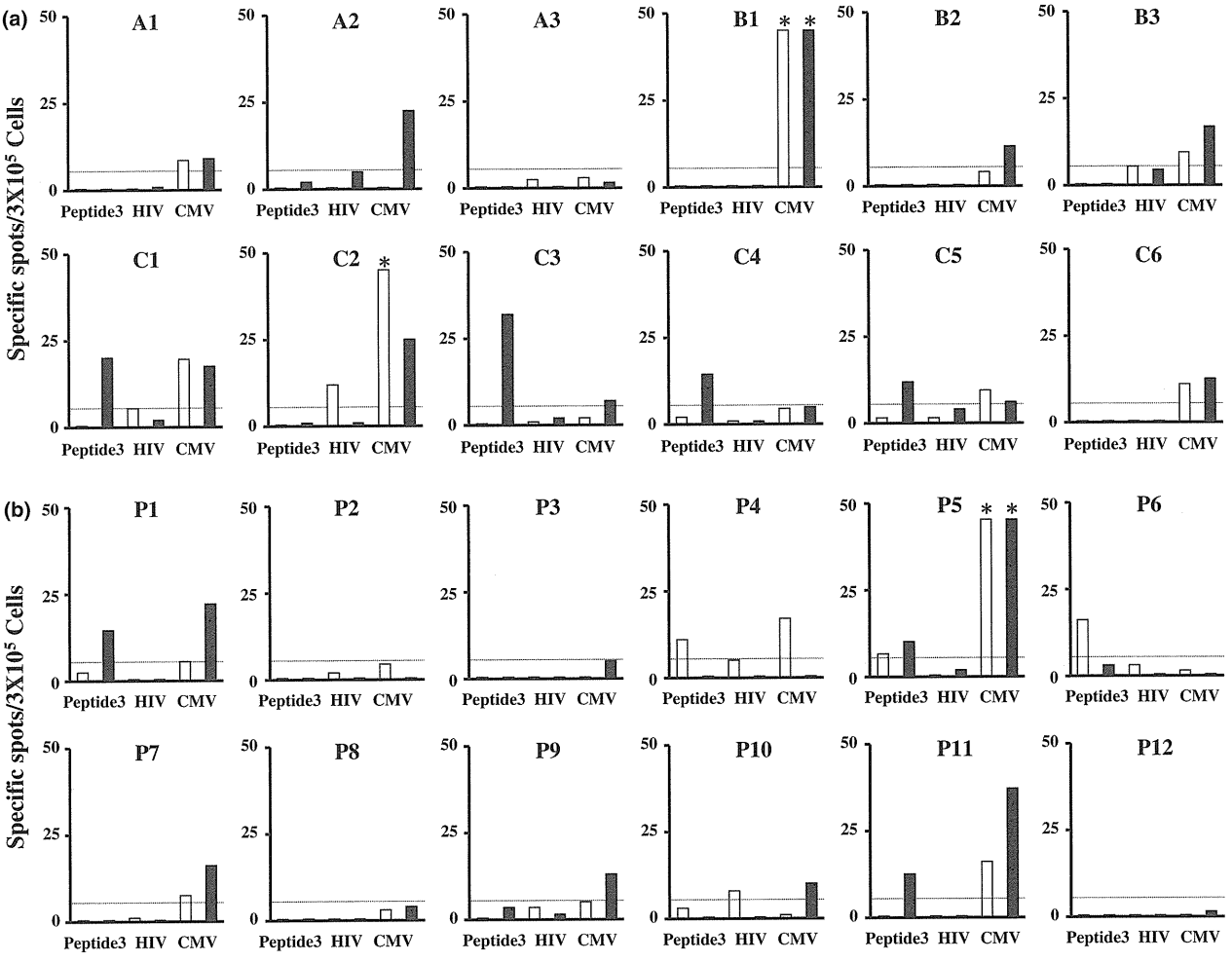

Fig. 4. IFN-γ ELISPOT assays of PBMCs to chondroitin-glucuronate C5-epimerase-derived peptide (peptide 3) or control peptides (peptides $HIVenv_{584}$ and $CMVpp65_{328}$) in HCC patients with RFA. (a) The assays were performed in the patients with peptide 3 vaccination. White and black bars show the T-cell responses before and after vaccination respectively. (b) The assays were also performed in the patients without vaccination. White and black bars show the T-cell responses before and after RFA respectively. *denotes more than 50 specific spots.

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was observed in four of six (66.7%) patients vaccinated with 3 mg of peptide, which is similar to the frequency of responded patients reported in other peptide vaccination studies (11, 20).

Apart from induction of CTLs, the efficacy of chondroitin-glucuronate C5-epimerase-derived peptides as a vaccine for advanced HCC is still unclear. In previous vaccine studies for advanced HCC, AFP, hTERT and glypican-3 have been targeted as tumour-associated antigens for the treatment (25, 30–32). In these studies, peptide-specific CTLs were reported to be induced in 10–80% of vaccinated patients. However, in spite of the induction of peptide-specific CTLs, it has been reported that the anti-tumour effect was very limited. Recent studies have shown that the frequency of myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) is increased in HCC patients and the cells inhibit the function of T cells (33, 34). Therefore, controlling their function might be important to develop more effective vaccination for advanced HCC.

In contrast, other recent studies using chondroitin-glucuronate C5-epimerase-derived peptides for other advanced cancers have shown the induction of cellular immune responses and clinical responses for certain patients (9, 11). In the analysis of the prognosis of patients with RFA and chondroitin-glucuronate C5-epimerase-derived peptide vaccination in the present study, the recurrence rate in the patients with an increase in the peptide-specific CTLs after vaccination was lower than that in the patients without immune response. Although further studies are necessary to evaluate the efficacy of chondroitin-glucuronate C5-epimerase-derived peptides for HCC, the results of our study suggest that chondroitin-glucuronate C5-epimerase is a potential candidate for a target of HCC immunotherapy.

In conclusion, chondroitin-glucuronate C5-epimerase is a potential candidate for a tumour antigen with immunogenicity, and peptides derived from the protein would be useful for immunotherapy in cases of HCC.

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