Comprehensive screening for antigens overexpressed on carcinomas via isolation of human mAbs that may be therapeutic

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Although several murine mAbs that have been humanized became useful therapeutic agents against a few malignancies, therapeutic Abs are not yet available for the majority of the human cancers because of our lack of knowledge of which antigens (Ags) can become useful targets. In the present study we established a procedure for comprehensive identification of such Ags through the extensive isolation of human mAbs that may become therapeutic. Using the phage-display Ab library we isolated a large number of human mAbs that bind to the surface of tumor cells. They were individually screened by immunostaining, and clones that preferentially and strongly stained the malignant cells were chosen. The Ags recognized by those clones were isolated by immunoprecipitation and identified by MS. We isolated 2,114 mAbs with unique sequences and identified 21 distinct Ags highly expressed on several carcinomas. Of those 2,114 mAbs 356 bound specifically to one of the 21 Ags. After preparing complete IgG_1 Abs the *in vitro* assay for Ab-dependent cell-mediated cytotoxicity (ADCC) and the *in vivo* assay in cancer-bearing athymic mice were performed to examine antitumor activity. The mAbs converted to IgG_1 revealed effective ADCC as well as antitumor activity *in vivo*. Because half of the 21 Ags showed distinct tumor-specific expression pattern and the mAbs isolated showed various characteristics with strong affinity to the Ag, it is likely that some of the Ags detected will become useful targets for the corresponding carcinoma therapy and that several mAbs will become therapeutic agents.

phage Ab library | therapeutic Ab | tumor-associated antigen

Since the discovery of a method to produce mAbs numerous scientists have been trying to identify and produce mAbs that could be used for immunotherapy against various malignancies. The success for example of alemtuzumab against CD52, trastuzumab against HER2, and rituximab against CD20 for treatment of chronic lymphocytic leukemia, breast cancer, and non-Hodgkins lymphoma, respectively (1–3), suggests that mAbs are likely to become very important therapeutic agents also against a wider range of cancers. However, for the majority of the human cancers useful therapeutic Abs are not yet available because of our lack of knowledge of which antigens (Ags) are likely to become useful targets (4). Therefore, several groups of investigators have been trying to identify other potential Ags as targets for immunotherapy using microarray technology (5, 6). Although many differences in transcripts have been revealed between malignant cells and the normal counterpart cells, it will take more time and laborious work to examine which Ags could be targets and to prepare therapeutic Abs against them. Furthermore, the presence of a large amount of transcripts does not always indicate expression of a large amount of the proteins.

Our experimental approach was designed in the opposite way to the strategy with the microarray technology mentioned above and was based on the phage-display technology (7). First we isolated a large number of mAbs that bind to the surface of cancer cells using a huge phage Ab library and many kinds of cancer-derived cell lines. Then using fresh tumor tissues we selected clones that gave significant staining of malignant cells but were negative or very weakly positive on the normal cells in the histological sections. At the third step the Ags recognized by the respective clones were isolated by immunoprecipitation and identified by MS analysis. Finally mAbs were converted to complete human IgG_1 and the antitumor activity was examined. Thus, the procedure adopted in our study enabled us to succeed in comprehensive identification of tumor-associated Ags (TAAs) and simultaneous isolation of mAbs against them.

Recently several groups of investigators have been using the phage-display method to screen for tumor-specific Ags according to a procedure similar to ours (8, 9), but the number of TAAs identified by them was limited, and to the best of our knowledge none converted their clones to complete Abs, which are essential for further studies to try to evaluate their potential therapeutic effects.

Results

Isolation of mAbs That Differentially Bound to Cancer Cells. Using 33 different tumor cell lines from seven carcinomas, hepatocarcinoma, renal carcinoma, pancreatic carcinoma, lung carcinoma, colonic carcinoma, gastric carcinoma, and ovarian carcinoma, the phage Ab library was screened 51 times for isolation of mAbs that bound to molecules present on the cell surface. The number

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Table 1. Summary of screenings

Cancer type	Screening no.	Cell	Isolated	Intact	Kinds	Select	Super Select
Hepatocarcinoma	035	HepG2	240	162	91		
	040	Nuk-1	286	254	100		
	041	OCTH-18	239	197	86		
	042	HepG2	96	21	20		
	044	Hep3B	190	120	112		
	045	HepG2	428	270	189		
	046	Clinical sample 0722*	190	156	14		
	047	Clinical sample 0722*	142	116	18		
	048	Clinical sample 0722*	142	137	8		
	049	Clinical sample 0722*	190	160	36		
	050	Clinical sample 0722*	190	138	51		
	051	HepG2	168	68	49		
	052	HepG2	208	187	94		
	053	HepG2	208	149	71		
	063	HLF	190	141	45		
	3172	Clinical sample 0317†	1	1	1		
	054	RBE	250	204	106		
	Total	17	3,358	2,481	1,091	967	
Renal carcinoma	057	Caki-1	190	168	90		
	059	CCF-RC1	190	148	80		
	061	Caki-1	190	146	53		
	062	CCF-RC1	190	140	111		
	060	ACHN	190	160	97		
	Total	5	950	762	431	341	
Pancreatic carcinoma	055	PANC-1	286	181	62		
	058	MIA PaCa-2	190	159	50		
	085	BxPC-3	190	145	61		
	087	Capan-1	190	44	27		
	Total	4	856	529	200	180	
Lung carcinoma	064	A549	189	172	56		
	065	PC-14	379	349	60		
	066	NCI-H441	190	167	71		
	068	Calu-3	48	34	22		
	067	EBC-1	285	210	107		
	079	RERF-LC-AI	190	172	73		
	080	LK-2	190	158	86		
	086	VMRC-LCP	190	177	69		
	Total	8	1,661	1,439	544	437	
Colonic cancer	028	Caco-2	190	170	102		
	029	CW-2	190	153	92		
	082	SW480	190	175	46		
	084	HT-29	190	177	70		
	Total	4	760	675	310	279	
Gastric cancer	031	MKN-45	190	159	90		
	075	NCI-N87	190	145	50		
	077	SNU-5	190	143	65		
	081	KATO III	190	162	79		
	Total	4	760	609	284	240	
Ovarian cancer	015	SKOv3	240	183	81		
	021	SKOv3	48	10	9		
	022	SKOv3	48	15	10		
	025	SKOv3	48	10	6		
	026	SKOv3	48	20	8		
	039	SKOv3	48	43	36		
	074	KF28	190	143	60		
	076	RMG-1	190	177	76		
	078	RMG-2	190	176	79		
	Total	9	1,050	777	365	287	
Total		51	9,395	7,272	3,225	2,731	2,114

*Clinical sample 0722 was derived from a male patient with hepato cell carcinoma HCV (+) stage II.
†Clinical sample 0317 was derived from a male patient with hepato cell carcinoma HBV (+) stage IV-B.

of clones that were picked up in each screening is indicated in the column "Isolated" in Table 1. A total of 9,395 clones were picked up. Those clones were then screened by ELISA using anti-cp3 Ab to examine expression of the intact single-chain Fv (scFv) molecules on the phage because scFv fused with a truncated cp3 was expressed in our system. The number of clones that were

judged to express the intact molecule is indicated in the column "Intact" in Table 1. A total of 7,272 clones turned out to express intact scFv molecules on the phage. Each one of those 7,272 clones was sequenced. The number of clones with different sequences isolated in respective screenings is indicated in the column "Kinds" in Table 1. Because the same clones were redundantly isolated from different screenings, the total number of different clones against the same carcinoma is shown in the column "Select" in Table 1. Because the same clones were also isolated from screenings against different types of carcinoma, 7,272 clones were composed of 2,114 different clones indicated as "Super Select" in Table 1. Of those 2,114 clones 406 were redundantly present in the 3,225 clones summed up in the column "Kinds" in Table 1, and 1,708 were isolated only once in all of the 51-time screenings performed in the present work. The number of times such redundant clones were isolated ranged from two to 27.

Those 2,114 mAbs were individually screened using at least three different fresh tumor tissues for each assay. They were classified into four groups based on the immunostaining patterns in the histological sections. When mAbs significantly stained only the surface of tumor cells but negatively or very weakly stained the other normal cells, they were classified to group A. When the strong staining by mAbs was localized on the surface of malignant cells but a part of the other normal tissue was also stained, they were classified to group B. When mAbs showed positive staining patterns both on malignant cells and on normal cells nonspecifically, they were classified into group C. The clones that did not give any positive signal were classified into group D. Of 2,114 mAbs 281 were classified to group A and 384 were classified to group B.

Identification of 21 TAAs. Of the 665 clones, 300 that strongly stained the malignant cells were chosen for further studies. Each of the 300 clones was screened against six different tumor cell lines by using flow cytometry (FCM). They were grouped according to their staining pattern on the basis of the following principle. In FCM analysis, the degree of peak shift should reflect the amount of Ag, the accessibility of Ab, and the strength of binding. The width and shape of peak should reflect the degree of homogeneity of expressed Ags in the cell population. Therefore, if the staining patterns against the six cell lines were identical or very similar among the clones examined, they were grouped together. It led to 40 groups made up of 150 clones. The other 150 clones could not be grouped because of a weak signal in the FCM.

The cell membrane proteins of carcinoma-derived cell lines were biotinylated and then individually immunoprecipitated by the mAbs of the same group and analyzed by SDS/PAGE. When several mAbs in each group gave rise to the same band on the gel the bands were cut out and subjected to MS analysis. This enabled us to identify 21 distinct membrane Ags, which are listed in Table 2. Those 21 Ags are recognized by 84 of the 300 mAbs that we studied.

We also synthesized the extracellular portions of nine of the 21 Ags. Using ELISA we tested the 2,114 mAb clones against those nine synthetic Ags. Of those, 272 clones gave a positive reading in addition to the 84 clones that had been already identified by the MS analysis. We are now in the process of synthesizing the remaining 12 Ags for further screening. To date 356 clones of the 2,114 mAbs isolated in the present study were revealed to specifically bind to one of the 21 TAAs. Of those 356 clones 156 belonged to the redundantly isolated 406 mAbs.

Expression of Fresh Cancer Tissues. Using representative clones that specifically bound to 18 TAAs except for three TAAs PTK7, CD9, and CDCP1, which were recently identified, the immunostaining analysis was performed against 24 fresh lung carcinomas. Table 3 summarizes the results of eight TAAs that gave simple patterns showing one of the following two cases: +, overexpression on malignant cells but no or very weak expression on the other normal cells; and −, no expression on either malignant or normal cells. In the case of the other 10 TAAs, although differential expression on malignant cells was distinct, expression on a part of the normal tissue was also observed. From these analyses we concluded the following. (*i*) TAAs identified in our study were overexpressed in fresh tumors at some frequency, but there is no TAA that was overexpressed in all of the fresh tumors. (*ii*) All of the fresh malignant cells analyzed to date overexpressed some of 18 TAAs in various combinations. (*iii*) Approximately half of them showed distinct tumor-specific expression pattern.

Table 2. Cell-surface Ags identified by the mAbs

Ag	MS*	ELISA†
Growth factor receptor		
EGFR	3	6
HER2	1	15
HGFR	3	84
PTK7/CCK-4	1	ND
Transmembrane protein-tyrosine phosphatase		
PTP-LAR	5	ND
Adhesion molecule		
Ig superfamily		
IGSF4	10	13
ALCAM	3	8
ICAM-1	5	17
Lu/BCAM	1	48
CEACAM6	1	ND
Non-Ig family		
CD44	3	ND
EpCAM	2	ND
Tetraspanin		
CD9	1	ND
Adenosine metabolism		
Ecto-5′-nucleotidase	1	ND
Complement inhibitor		
MCP	8	81
Protease inducer		
EMMPRIN	1	0
Iron metabolism		
TfR	6	ND
Anoikis regulator		
CDCP1	2	ND
Integrin family		
$\alpha 3\beta 1$	14	ND
$\alpha v\beta 3$	8	ND
$\alpha 6\beta 4$	5	ND

*Number indicates that of different clones identified by MS analysis.
†Clones identified by MS analysis are not included.

Antitumor Activity of Complete Human IgG mAbs. After preparing IgG_1 mAbs we performed an *in vitro* assay for Ab-dependent cell-mediated cytotoxicity (ADCC) using 22 clones against 10 Ags (EGFR, ALCAM, ICAM-1, EpCAM, HGFR, TfR, ITGA3, EMMPRIN, PTP-LAR, and CD44) using the cell lines listed in Table 4. As can be seen, those mAbs gave a positive reading that ranged between 5% and 95% for cell killing. The details using anti-EGFR Abs and anti-EpCAM Ab are in Fig. 1 *a* and *e*, respectively. The degree of ADCC by clone 059-152 was strong compared with that by cetuximab. Clone 067-153 showed ADCC activity even at an extremely low concentration such as lower than picomolar. We also performed an *in vivo* assay using three

Table 3. Hisotological analysis of lung carcinomas with mAbs against TAAs

		A			B	C	D											E						
Clinical sample		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Antigen	Stage Clone Ab	IA	IA	IIIB	IB	IIIB	IA	IA	IA	IB	IB	IB	IB	IB	IIB	IIIA	IIIA	IIIB	IB	IIB	IIIA	IIIA	IIIA	IIIB	IIIB
ITGA6	029-023	−	−	−	−	+	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
ITGAV	064-139	−	+	−	+	+	−	+	−	−	−	−	−	−	−	−	+	−	+	−	−	+	−	−	−
CD147	059-053	−	−	−	−	−	+	−	−	−	−	−	−	−	+	+	−	+	+	−	+	+	−	−	+
LAR	064-044	+	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	+	−
IgSF4	076-048	−	−	−	−	+	+	−	−	+	+	−	+	−	−	+	−	+	−	−	−	−	+	−	−
EGFR	048-006	+	+	−	+	−	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	−	−
HER2	015-126	−	−	−	+	−	+	+	−	−	−	−	−	+	+	+	+	+	−	+	+	−	−	−	−
HGFR	067-133	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−	−

A, squamous cell carcinoma; B, adenosquamous carcinoma; C, bronchioloalveolar carcinoma; D, adenocarcinoma; E, large-cell carcinoma.

mAbs against two of the Ags (EGFR and EpCAM) in cancer-bearing athymic mice. As can be seen in Fig. 1*d* the anti-EGFR Abs showed a strong antitumor activity against tumor cell line A431. When we compared our mAbs (048-006 and 059-152) against EGFR with cetuximab it appeared that they had a very similar level of antitumor activity. The anti-EpCAM Ab also prevented the growth of HT29 (Fig. 1*f*).

The two anti-EGFR mAbs were used to analyze the mechanism of their antitumor activities. As can be seen in Fig. 1*b* mAb 048-006 was very effective in inhibiting the binding of EGF to the EGFR, whereas mAb 059-152 only partially prevented the binding reaction. The phosphorylation assay (Fig. 1*c*) showed that mAb 048-006 was effective in the inhibition of phosphorylation. The mAb 059-152 also gave inhibitory effects on phosphorylation, although less effective than 048-006. It suggests that mechanisms of antitumor activity mediated by these two mAbs might be different from each other.

Discussion

In the present study we used a phage Ab library that had been constructed from human B cells (10). It has been suspected that majority of the clones isolated from phage Ab libraries may not show high affinity to the Ags because they should be naïve to the Ags (11). However, as shown in Fig. 1 two anti-EGFR Abs and one anti-EpCAM Ab showed strong ADCC activity at the concentration of 0.01–0.1 μg/ml, which corresponds to 0.06–0.6 nM. This strength appeared to be practically strong enough to be therapeutic agents whereas comparison of nucleotide sequences of V_H genes encoding theses three Abs with those of germ-line genes indicated that mutations had not been introduced (data not shown). In the screenings of Ab library there were many cases where the same clones were redundantly isolated from different screenings against the same carcinoma as well as against different types of carcinoma. The reason why specific clones were redundantly isolated might be as follows: (*i*) amounts of Ags recognized by them were relatively abundant on the cells used in the screenings, and (*ii*) the binding activity to the Ags was stronger than that of other clones. This interpretation could be at least partly correct. For example, while anti-EGFR Ab 048-006 was isolated by six time screenings the dissociation constant (K_d) of the Ag/Ab complex measured by the BIAcore instrument was 0.025 nM (data not shown).

Table 4. ADCC activities of mAbs that have been converted to IgG

Ag	Clone	Target cells	Cell killing,* %
EGFR	048-006	NCI-H1373, CCF-RC1, A431, ACHN	36–80
	055-147	CCF-RC1, HT-29, A431	25–95
	059-152	NCI-H1373, CCF-RC1, A431, ACHN	35–75
	059-173	CCF-RC1, HT-29, A431	35–85
ALCAM	035-234	NCI-H1373, SKOv3, CW-2	8–19
	041-118	NCI-H1373, EBC-1	14–18
	066-174	NCI-H1373, SKOv3, CW-2	45–59
	083-040	NCI-H1373	10
ICAM1	053-042	NCI-H1373	16
	053-051	NCI-H1373, NCI-H441, HepG2	5–31
	053-059	NCI-H1373, NCI-H441, HepG2	8–39
	053-085	NCI-H1373, NCI-H441, HepG2	7–26
EpCAM	067-153	NCI-H1373, MKN45, HT-29, EBC-1	23–80
HGFR	067-133	NCI-H1373, MKN45, EBC-1	19–42
TfR	028-178	MIA Paca2	65
	052-138	MIA Paca2	80
	041-288	MIA Paca2	30
ITGA3	015-003	ACHN	20
EMMPRIN	059-053	CCF-RC1, ACHN	40
PTP-LAR	064-044	PC14	10
	079-085	PC14	32
CD44	064-003	PC14	84

*Percentage of cell killing increased dose-dependently. When it reached plateau, the percentage for cell killing was indicated.

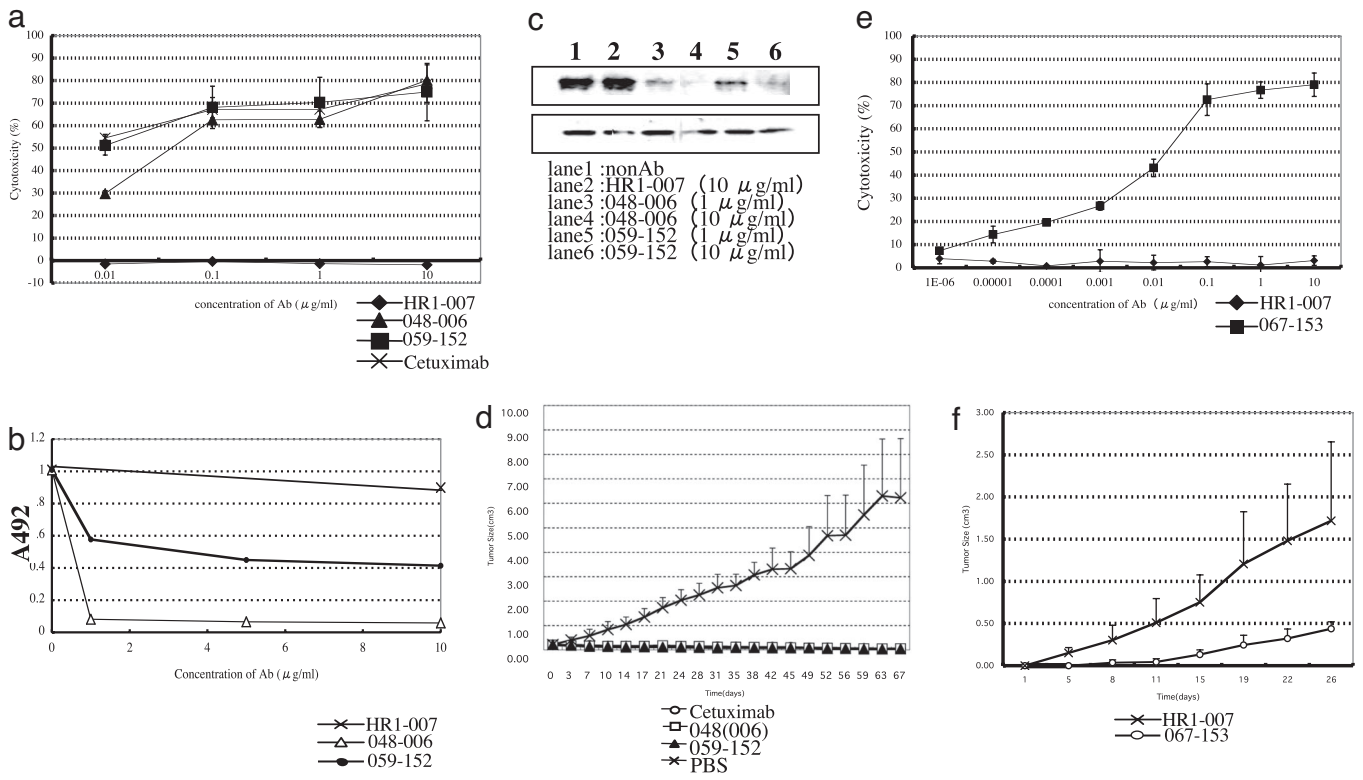

Fig. 1. Antitumor activities of two anti-EGFR mAbs (clone 048-006 and clone 059-152) and an anti-EpCAM mAb (clone 067-153). (*a*) ADCC. Target cells: NCI-1373. Ab: HR1-007 (negative control), mAb 048-006, 059-152, and cetuximab (positive control). (*b*) Inhibitory effects of mAbs on the binding of EGF to EGFR on cell line A431. Ab: HR1-007 (negative control), mAb 048-006, and 059-152. (*c*) Inhibitory effects of mAbs on phosphorylation of EGFR induced by EGF. Upper bands: Western blot by anti-phosphotyrosine mouse mAb. Lanes 1–6: incubated for 30 min after addition of EGF at 1 μg/ml; lane 1, without Ab; lanes 2–6, incubated with Ab for 30 min, then EGF was added; lane 2, HR1-007 (negative control) at 10 μg/ml; lanes 3 and 4, 048-006; lanes 5 and 6, 059-152; lanes 3 and 5, 1 μg/ml; lanes 4 and 6, 10 μg/ml. Lower bands: control Western blot with rabbit antiserum against β-actin. (*d*) Inhibitory effects of mAbs on the growth of tumor cell line A431 in athymic nude mice assayed by the first method described in *Antitumor Activity in Vivo*. Ab: HR1-007 (negative control), mAb 048-006, 059-152, and cetuximab. (*e*) ADCC. Target cells: HT-29. Ab: HR1-007 (negative control) and mAb 067-153. (*f*) Inhibitory effects of mAb on the growth of a tumor cell line in athymic nude mice assayed by the alternative method described in *Materials and Methods*. Ab: HR1-007 (negative control) and mAb 067-153.

In this study we isolated 2,114 mAbs with unique sequences that bound to molecules on the surface of tumor-derived cells and selected 665 clones that gave tumor-specific immunostaining patterns. To identify TAAs recognized by the tumor-specific mAbs we developed two strategies. The grouping of mAbs by FCM enabled us to achieve efficient identification of TAAs by the following reasons. (*i*) FCM analyses against several cell lines taught us which cell expressed most abundantly the target molecules. (*ii*) Because multiple mAbs in each group turned out to bind to the same Ag in most cases, many clones have been treated very efficiently by a limited number of experiments. (*iii*) Detergent for solubilization of membrane proteins may destroy the structural integrity with the results of losing the antigenic structure. When several clones classified into the same group were analyzed together, some of them could bind to a relatively detergent-resistant epitope. Screenings of all of the 2,114 mAbs by ELISA with the polypeptides that correspond to the extracellular portions of the TAAs already identified by MS analysis also enabled us to efficiently identify the Ags recognized by respective Abs. Now it is likely that we have already revealed more than half of TAAs potentially identified according to this procedure.

We characterized two anti-EGFR mAbs, clone 048-006 and clone 059-152. Whereas clone 048-006 inhibited both the binding of EGF to EGFR and the phosphorylation of EGFR, clone 059-152 partly inhibited the binding of EGF to EGFR and gave inhibitory effects on the phosphorylation of EGFR less effectively. However, both clones showed a strong antitumor activity in cancer-bearing athymic mice. The characteristics of clone 048-006 appeared to be similar to that of cetuximab (12). However, to the best of our knowledge there has been no report describing mAb whose characteristics was similar to that of 059-152. In the present study multiple Abs have been isolated against respective TAAs. It is possible that these mAbs may have various characteristics as shown by anti-EGFR mAbs.

As indicated in Table 3, half of the TAAs identified in this study gave a tumor-specific staining pattern that is overexpression on malignant cells but no or very weak expression on the other normal cells in the histological sections. These TAAs could be candidates to be useful targets for therapeutic Abs. Furthermore, as indicated in Table 4 and Fig. 1, IgG form of mAbs that bound to them showed strong antitumor activity *in vitro* and *in vivo*. Therefore, we believe that some Ags detected will be useful targets for cancer therapy and that several mAbs will become useful therapeutic agents in the foreseeable future.

Materials and Methods

Ab Library and Screening. AIMS-5 library constructed by using a phage-display system was employed (10). Screenings were performed by a method similar to the one developed by Giordano *et al.* (13). In brief, the phages (2×10^{13} cfu) were mixed with cells ($0.2–1 \times 10^{8}$) in 1.6 ml of solution A (1% BSA, MEM, and 0.1% NaN_3), and Ag–Ab complexes on the cell surface were formed. The cell and phage suspension was overlaid on the organic solution in an Eppendorf tube. After the tube was centrifuged, water and organic layers were discarded. The collected cells were suspended in solution A. This process was repeated three times. Finally, the cells were suspended in PBS and frozen in liquid nitrogen. The frozen cells were thawed and mixed with *Escherichia coli*

DH12S. The phages were prepared. This screening round was performed repeatedly three times. After three rounds of screenings, *E. coli* DH12S infected with recovered phages was spread on plates. Approximately 200 colonies were picked up. Thirty-three cancer cell lines listed in Table 1 were used as Ags.

Immunostaining of Fresh Tumors. Tumor tissues and the neighboring normal tissues resected by operation were used for immunostaining. They were fixed with 4% paraformaldehyde in 0.1 M cacodylic buffer (pH 7.4) by microwave irradiation as described previously (14).

Identification of Ag. Membrane protein analysis was performed according to Zhao *et al.* (15). Proteins present on the cell surface were biotinylated according to the manufacturer's instruction by using the EZ-Link Sulfo-NH-LC Biotinylation kit (Pierce). After the cells were homogenized with a Dounce homogenizer, the protein–membrane complexes were banded between 0.25 M and 1.25 M sucrose layers by centrifugation. The complexes were dissolved in a detergent mixture: 50 mM Hepes (pH 7.6), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 1% β octyl glucoside. scFv-C_L fused with cp3 was converted to scFv-C_L fused with protein A domains (scFv-C_L-PP) (16). scFv-PP form was covalently bound to beads that were CNBr-activated Sepharose 4B (GE Health Care Bioscience). Ab-bound beads were used for immunoprecipitation as described by David *et al.* (17). MS analysis was performed according to Geuijen *et al.* (8).

Preparation of IgG_1. ScFv was converted to IgG_1 and prepared by using a high-level expression vector (18). Using IgG_1 mAbs we examined ADCC, effects on binding of EGF to EGFR, effects on phosphorylation of EGFR, and antitumor activity in athymic nude mice.

ADCC. The enzymatic activity of lactic dehydrogenase released from the target cells was measured for estimation of ADCC (19). Various cell lines were used as targets for the mAbs. Cells were derived from the following cancers: NCI-H1373, lung adenocarcinoma; CCF-RC1, renal clear cell carcinoma; A431, vulva epidermoid carcinoma; ACHN, renal adenocarcinoma; HT-29, colorectal adenocarcinoma; SKOv3, ovarian adenocarcinoma; CW-2, colorectal adenocarcinoma; EBC-1, lung squamous cell carcinoma; NCI-H441, lung papillary adenocarcinoma; HepG2, hepatocellular carcinoma; MKN45, gastric adenocarcinoma; MIAPaca-2, pancreatic carcinoma; PC14, ling carcinoma. Effector cells were prepared from blood of healthy volunteers and used in a ratio of 100:1 (10^6 to 10^4 in 200 μl) (20).

Effects of Anti-EGFR mAbs on the Function of EGFR. Binding of EGF to EGFR on the cell surface was estimated according to Yang *et al.* (21). Phosphorylation of EGFR induced by EGF was measured according to Matar *et al.* (22).

Antitumor Activity *in Vivo*. Two different methods were adopted. In the first method each mouse was injected with 5×10^6 cells, and when the tumor grew to 0.2 cm^3 mAb therapy was initiated. Treatments consisted of twice-weekly i.p. injections of mAb for 3 weeks. One milligram in 0.5 ml of PBS was used in each injection. Control animals received injection of PBS. Six mice were used for each treatment. The alternative method: 1 day after injection of cells mAb therapy was started. Treatments consisted of twice-weekly i.v. injections of mAb for 2 weeks. A total of 50 μg was used in each injection.

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