Expression profiling of esophageal squamous cell carcinoma patients treated with definitive chemoradiotherapy: Clinical implications

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Abstract. In esophageal squamous cell carcinoma (ESCC), chemoradiotherapy (CRT) has a curative potential even in cases of locally advanced carcinoma. However, only about half of the patients benefit from CRT, and an accurate prediction of sensitivity to CRT is eagerly awaited. Using microarrays, we analyzed gene-expression patterns of pretreatment biopsy specimens from 33 patients with CRT alone including long-term survivors, more than 3 years (14 cases) and short-term survivors, less than 1 year (11 cases). The expression patterns of about 12,600 genes were used to identify genes correlated with survival terms. Fifty-seven genes correlating with short-term survival and 120 genes with long-term survival were identified. The genes involved in the immune response were characteristically upregulated in the long-term survivors, and an immunohistochemical staining confirmed an increased CD8-positive T cell number in the long-term survivors over that in the short-term survivors. In the short-term survivors, on the other hand, increased expression of the genes involved in drug resistance was observed. Our gene list should contribute to the elucidation of the mechanisms of CRT response and contains useful markers for predicting the prognosis of individual ESCC patients treated with CRT alone.

Introduction

Esophageal cancer in East Asian countries including Japan and China, and in some parts of Europe consists mainly of squamous cell carcinomas located mostly in the thoracic esophagus, while adenocarcinoma in the distal part of the esophagus has increasingly become the major pathological type found in Europe and North America. Overall, esophageal squamous cell carcinoma (ESCC) is the sixth most frequent cancer in the world, and is a highly virulent disease. Although surgery is the standard therapy for locally confined ESCC, results of surgery alone remain poor, with 5-year survival rates of 6-24% in Western countries (1). Recent improvement in surgical results following radical node dissection has been reported at some Japanese institutions, with 5-year survival rates of 31-55% (2,3). In a Japanese prospective randomized study that compared surgery alone with surgery followed by adjuvant chemotherapy, the group that received surgery with radical dissection alone showed a 5-year survival rate of 45%, although the study did not show a survival advantage over treatment with adjuvant chemotherapy (4). The success of radical surgery led many Japanese surgeons to extend the indications of surgery to locally advanced carcinoma. On the other hand, a combination of 5-fluorouracil (5-FU) and cisplatin (CDDP) has become a standard world regimen, not only because of its clinical outcome, but also because of the synergism between the two agents and their radiosensitizing effects (5-7). Recent studies clearly indicated that definitive chemoradiotherapy (CRT) seemed to have a curative potential even in cases of locally advanced carcinoma (8), although the treatment may be associated with significant toxicity (9). The reported 5-year survival rate is 27% or 29% (9,10). Despite recent progress in the molecular genetics of ESCC, little is known about the mechanisms of sensitivity to CRT, and a prediction of the outcome of CRT by clinicopathological terms still remains very difficult. We conducted gene expression profiling using an oligonucleotide microarray of pretreatment biopsy samples from 33 ESCC patients treated with CRT alone. The purpose of the study was to explore the underlying mechanisms of CRT responses and to contribute to the development of the markers, which aid to select the best therapeutic modality prior to the initiation of therapy.

Materials and methods

RNA isolation from biopsy specimens. Patients received protracted infusion of 5-FU 400 mg/m^2/24 h on days 1-5 and

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8-12, 2-h infusion of CDDP 40 mg/m^2 on days 1 and 8, and concurrent radiation therapy at a dose of 30 Gy in 15 fractions over 3 weeks. Filgrastim was prophylactically administered to all patients. This schedule was repeated twice every 5 weeks, for a total radiation dose of 60 Gy, followed by two courses of 5-FU (800 mg/m^2/24 h for 5 days) and CDDP (80 mg/m^2 on day 1). Biopsy samples, which were obtained from patients before CRT, were immediately frozen at -80˚C until use. The samples were homogenized in Isogen lysis buffer (Nippon Gene Co., Ltd., Toyama, Japan) at room temperature, extracted with chloroform, and precipitated with 20 μg glycogen in isopropanol. The RNA pellet was resuspended in RNase-free water, and treated with RNase-free DNase I in the presence of RNase inhibitor followed by phenol extraction and precipitation in isopropanol. The pellet was resuspended in 15 μl of RNase-free water; one-third of the total RNA from each sample was used for quality analysis of RNA by RNA LabChip (Caliper Technologies Corp., CA), and an appropriate amount from the remaining two-thirds was used for oligonucleotide microarray analysis. This study with biopsy specimens was approved by our institutional review boards.

Microarray analysis. We used human U95A oligonucleotide probe arrays (Affymetrix, Santa Clara, CA) for analysis of mRNA expression levels corresponding to 12,600 transcripts. The procedures were conducted according to the supplier protocols, and are thus described briefly. Each 5 μg of total RNA was used to generate a cRNA probe. Ten microgram of fragmented cRNA was hybridized to the microarrays in 200 μl of a hybridization cocktail at 37˚C for 16 h in a rotisserie oven set at 60 rpm. The arrays were then washed with a low stringent wash buffer (6X SSPE) at 25˚C, followed by stringent wash buffer [100 mM MES (pH 6.7), 0.1 M NaCl, and 0.01% Tween-20] at 50˚C, stained with streptavidin phycoerythrin (Molecular Probes), washed again with 6X SSPE, stained with biotinylated anti-streptavidin IgG, followed by a second staining with streptavidin phycoerythrin and a third wash with 6X SSPE. The arrays were scanned using a GeneArray scanner (Affymetrix) at 3-μm resolution, and quantitatively the canned image analyzed with computer software Microarray Suite 4.0 (Affymetrix). For normalization of the data to compare mRNA expression levels among the samples, we unified 1000 as an average of AD scores corresponding to signal intensities of all probe sets in each sample. We selected genes whose expression levels were higher than an average of signal intensity in one group (14 long-term survivors or 11 short-term survivors) plus its standard deviation (SD) in more than 60% of another group. The two-dimensional hierarchical clustering analysis of the 177 selected genes differentially expressed in long- and short-term survivors was performed by the Cluster and Treeview programs (11).

RNA slot blot analysis. To verify differences in expression indicated by microarray analysis, we performed slot blot analysis of cRNAs produced from total RNA extracted from 14 long-term survivors and 12 short-term survivors. A SuperScript Choice System (Invitrogen Corp., CA) was used for cDNA synthesis prior to generating cRNA. A MEGA-script *in vitro* Transcription Kit (Ambion Inc., TX) was used for cRNA production by *in vitro* transcription using T7 RNA polymerase. Each 0.5 μg cRNA was denatured and blotted to a NitroPlus membrane (Micron Separations, Inc.). The filter was hybridized with radiolabeled probes. Hybridization was carried out in 50% formamide, 5X standard saline citrate (SSC) (1X standard saline citrate = 0.15 M NaCl, 0.015 M sodium citrate), 5X Denhardt's solution, 5 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, and 100 μg/ml denatured salmon sperm DNA at 42˚C for 16 h. The filter was washed twice in 0.1X SSC and 0.1% SDS at room temperature for 10 min each and then washed at 65˚C for 30 min, and exposed to Kodak XAR film at -80˚C.

Immunohistochemical analysis. Biopsy specimens were embedded in paraffin, sectioned, and treated with phosphate-buffered saline (PBS). The specimen was then treated with a blocking reagent containing 10% goat serum and incubated with an anti-CD8 antibody in PBS also containing 3% goat serum. After incubation with the secondary antibody, a streptavidin-peroxidase solution was applied to the specimen followed by a development solution containing diamino-benzidine.

Results

Classification of ESCC patients who received CRT alone and microarray analysis. The clinicopathological characteristics of 33 esophageal carcinoma patients who received CRT alone are summarized in Table I. All cases were diagnosed as squamous cell carcinoma, and all patients were treated according to the same protocol as described in Materials and methods. Twenty-five of the 33 patients were classified into two groups on the basis of the duration of survival: long-term survivors, more than 3 years (14 cases, A-1-1 to A-1-14); short-term survivors, less than 1 year (11 cases, D-1-1 to D-1-11). The two groups showed no significant differences with respect to clinicopathological features. Of the remaining 8 patients, 6 (A-2-1 to A-2-4, D-2-1, and D-2-3) survived for 2-3 years until the end of the follow-up period, and 2 patients (D-2-2 and D-2-4) survived for 1-2 years. Approximately 10-30 μg of total RNA was isolated from each biopsy sample of these 33 ESCC patients, and all RNA samples were confirmed to be of good quality by visualization of ribosomal RNAs using the capillary electrophoresis method as described in Materials and methods and considered sufficient for microarray analysis. The 33 RNA samples were converted to cRNA, labeled by biotin and hybridized to Affymetrix Human Genome U95Av2 Array according to the protocol recommended by the manufacturer (Affymetrix, CA, USA). The array analyzed mRNA expression levels corresponding to 12,600 transcripts. First, we conducted an unsupervised clustering analysis using the 316 genes expressed (i.e., gave 'Presence' call by data analysis) in all the 33 samples. Three patient clusters appeared (Fig. 1). Cluster 1 (7 cases) consisted of 5 long-term survivors (A-1-1, A-1-7, A-1-9, A-1-11, and A-1-13), and 6 of the 7 cases (86%) in this cluster survived for more than 2 years. Cluster 2 (15 cases) consisted of 9 short-term survivors (D-1-1 to D-1-5, and D-1-7 to D-1-10), and 73% of the cases of this cluster (11 of 15) survived for less than 2 years. Cluster 3 (11 cases) consisted of 6 long-term survivors (A-1-2, A-1-5, A-1-6, A-1-10, A-1-11, and A-1-14), and 10 of the 11 cases (91%) of

Table I. Clinical outcome and disease stage of 33 patients.

No.	Age	Sex	T	N	M	TNM stage	CR[a]	Outcome[b]
A-1-1	56	M	3	1	0	3	CR	A (>2171)
A-1-2	61	M	3	1	1	4	CR	A (>2233)
A-1-3	68	M	3	1	0	3	CR	A (>2012)
A-1-4	48	M	3	0	0	2A	CR	A (>1891)
A-1-5	55	M	3	0	0	2A	CR	A (>2076)
A-1-6	54	M	2	1	0	2B	CR	A (>1857)
A-1-7	67	M	3	0	0	2A	CR	A (>1901)
A-1-8	68	M	3	1	0	3	CR	A (>1733)
A-1-9	50	M	3	1	0	3	CR	A (>1527)
A-1-10	65	M	3	1	0	3	CR	A (>1367)
A-1-11	72	M	3	0	0	2A	CR	A (>1366)
A-1-12	64	F	3	1	0	3	CR	A (>1346)
A-1-13	58	F	3	1	0	3	CR	A (>1233)
A-1-14	72	M	3	0	0	2A	CR	A (>1136)
D-1-1	72	F	3	1	0	3	Non-CR	D (379)
D-1-2	60	M	3	1	0	3	Non-CR	D (251)
D-1-3	61	M	3	1	1	4	Non-CR	D (227)
D-1-4	49	M	3	1	1	4	Non-CR	D (239)
D-1-5	53	M	3	0	0	2A	Non-CR	D (364)
D-1-6	50	M	3	1	1	4	Non-CR	D (226)
D-1-7	55	M	2	1	0	2B	Non-CR	D (280)
D-1-8	70	M	3	0	1	4	Non-CR	D (299)
D-1-9	72	M	3	1	1	4	Non-CR	D (156)
D-1-10	75	M	3	0	0	2A	Non-CR	D (309)
D-1-11	67	M	3	1	0	3	Non-CR	D (236)
A-2-1	71	M	3	0	0	2A	CR	A (>912)
A-2-2	70	M	3	1	0	3	CR	A (>1018)
A-2-3	44	M	3	0	0	2A	CR	A (>919)
A-2-4	55	M	3	0	0	2A	CR	A (>959)
D-2-1	71	M	3	1	0	3	CR	D (664)
D-2-2	64	M	2	1	0	2B	CR	D (299)
D-2-3	61	M	3	1	0	3	CR	D (968)
D-2-4	59	F	3	0	0	2A	CR	D (521)

[a]A CR was defined as the complete disappearance of all measurable and assessable disease for a minimum of 4-weeks. [b]Disease-free survival (days).

this cluster survived for more than 2 years. In summary, 11 (79%) of the 14 long-term survivors (A-1-1 to A-1-14) or 16 (80%) of the 20 cases that survived for more than 2 years were grouped into clusters 1 and 3, whereas cluster 2 was characterized by a poorer prognosis, gathering 9 (82%) of the 11 short-term survivors (D-1-1 to D-1-11) or 10 (77%) of 13 cases that survived for less than 2 years. These unsupervised data on expression profiling suggested the presence of distinct subclasses within the 33 patient groups, and the subclasses may be associated with the survival term.

To compile a gene list for prognostic marker candidates for CRT, we selected genes by comparing expression levels between the two groups, 14 long-term survivors and 11 short-term survivors. A gene was selected if more than 60% of the samples in one group expressed the gene at a higher level than an average of signal intensity plus its standard deviation (SD) in the other group. By this procedure, 57 genes associated with short-term survival and 120 genes with long-term survival were identified (Table II). To investigate whether the subclass exists among long-term survivors or short-term survivors, we

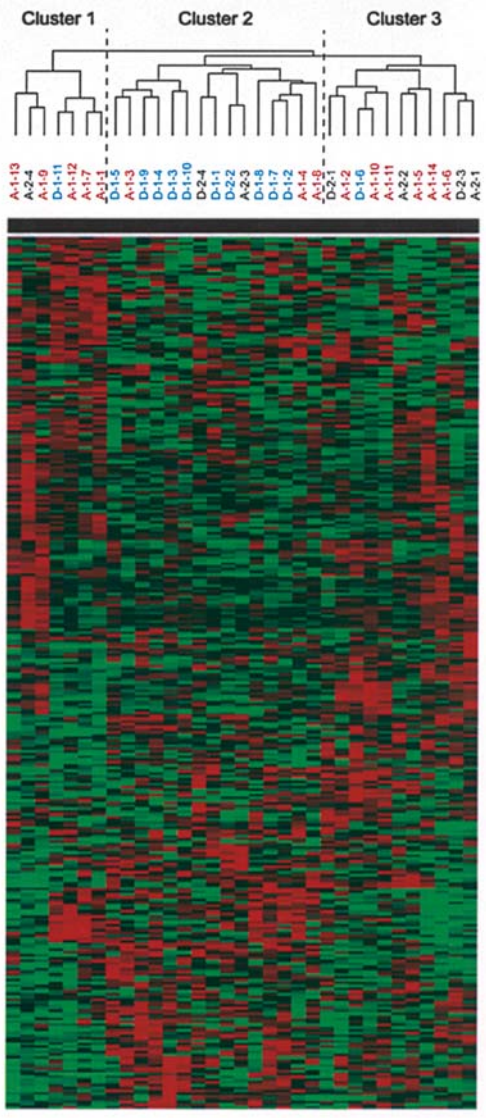

Figure 1. Unsupervised clustering analysis with expression data of the 316 genes in the 33 ESCC patients treated with CRT alone. Expression data of the 316 genes expressed in all the 33 samples were analyzed by the Cluster and Treeview programs (11). The results showed a significant association between the expression profile and the survival term. The long-term survivors (red), the short-term survivors (blue).

carried out a two-dimensional hierarchical clustering analysis of the 177 selected genes. A dendrogram of the clustering analysis was generated, and is shown in Fig. 2. Two patient clusters (vertical bar) and three gene clusters (horizontal bar) were recognized. One of the two patient clusters consisted of 10 of 11 short-term survivors, and the other patient cluster included all of the 14 long-term survivors. Only one case (D-1-11) was found in an inappropriate cluster. Among the gene clusters, the 11 short-term survivors appear to compose a single gene cluster (A in Fig. 2), whereas two major gene clusters, B and C, exist for the 14 long-term survivors.

Genes differentially expressed between long- and short-term survivors. We next analyzed the known functional annotations of 177 genes identified as differentially expressed genes between the long- and short-term survivors. More than 20 out of 120 genes over-expressed in the long-term survivors were found to have some functions in the immune response (Table II).

Table II. Over-expressed genes in long- or short-term survivors.

No.	GenBank	Gene symbol
Long-term survivor		
1	U19142	GAGE1
2	L76191	IRAK1
3	X53777	RPL17
4	AF023612	DIM1
5	M96860	DPP6
6	AF031416	IKBKB
7	Z48482	MMP15
8	S61953	ERBB3
9	Y18448	BSN
10	W26628	MRPL9
11	M24194	GNB2L1
12	D63478	NICE-4
13	AF054187	NACA
14	U84570	C21orf2
15	J03592	SLC25A6
16	U87954	PA2G4
17	Z48481	MMP14
18	AB016902	MLLT4
19	AI381790	APM2
20	M24194	GNB2L1
21	M62982	ALOX12
22	Y00764	UQCRH
23	X78710	MTF1
24	H16917	KIAA0563
25	AB028959	KIAA1036
26	M86667	NAP1L1
27	M32334	ICAM2
28	L05148	ZAP70
29	M31767	MGMT
30	M54914	FSHB
31	M60483	PPP2CA
32	M90356	BTF3L3
33	X96969	SLC14A2
34	Z50853	CLPP
35	X75252	PBP
36	AB029036	TRIM33
37	AB014596	FBXW1B
38	M58458	RPS4X
39	Z25749	RPS7
40	AF070638	CGI-57
41	Z46973	PIK3C3
42	L33842	IMPDH2
43	J04132	CD3Z
44	S72869	D10S170
45	M31315	F12
46	U70451	MYD88
47	L19185	PRDX2
48	D11466	PIGA
49	D89667	MM-1
50	D21089	XPC
51	U19144	GAGE3
52	AL050135	RFX5
53	AB018344	DDX46
54	AF052155	SEC13L1
55	U19145	GAGE5
56	X15940	RPL31
57	AF039555	VSNL1
58	S79522	RPS27A
59	AC004523	CYP4F12
60	X78926	ZNF268

Table II. Continued.

No.	GenBank	Gene symbol
Long-term survivor		
61	U91329	KIF1C
62	AB002332	CLOCK
63	AF054174	H2AFY
64	Z11692	EEF2
65	AA846749	APOM
66	D83702	CRY1
67	U22526	LSS
68	AB007960	SH3GLB1
69	J02625	CYP2E1
70	AL080119	PAI-RBP1
71	M24398	PTMS
72	AI816034	NOLA2
73	M32313	SRD5A1
74	L18960	EIF1A
75	D87002	IGL
76	S76992	VAV2
77	M95678	PLCB2
78	X69391	RPL6
79	M86546	PBX1
80	AB002533	KPNA4
81	M58378	SYN1
82	X53505	RPS12
83	Y09445	TBX5
84	AF112472	CAMK2B
85	AF025654	RNGTT
86	AB007447	FLN29
87	AF006621	SLC30A9
88	U35139	NDN
89	M23323	CD3E
90	U79259	DJ159A19.3
91	X04828	GNAI2
92	Z22865	DPT
93	AF037195	RGS14
94	W72733	KIAA1536
95	AL096744	REV3L
96	AB005047	SH3BP5
97	AB007896	KIAA0436
98	L24564	RRAD
99	W26677	FLJ35827
100	Z22555	SCARB1
101	M64174	JAK1
102	U88629	ELL2
103	S77812	FLT1
104	U26455	ATM
105	X72631	NR1D1
106	M83088	PGM1
107	M28393	PRF1
108	AF010242	SYNPO
109	M81141	HLA-DQB1
110	M17016	GZMB
111	U69645	ZNF32
112	X54871	RAB5B
113	U15552	HSU15552
114	AB014597	ANKRD17
115	M64322	PTPN7
116	AB018333	SASH1
117	M98539	PTGDS
118	AI526079	RPL22
119	AF062341	CTNND1
120	AF030234	SFRS2IP

Table II. Continued.

No.	GenBank	Gene symbol
Short-term survivor		
1	AF030428	T1A-2
2	Z80782	HIST1H2BI
3	AF035287	SDFR1
4	Y08614	XPO1
5	AL049944	DKFZP564G2022
6	Z83738	HIST1H2BM
7	U35451	CBX1
8	AL050089	BAZ1A
9	AF009615	ADAM10
10	X59892	WARS
11	U51920	SRP54
12	D13629	KTN1
13	Z80779	HIST1H2BF
14	AB023187	KIAA0970
15	U53204	PLEC1
16	D38551	RAD21
17	AI888084	GPR107
18	X63679	TRAM1
19	D83485	GRP58
20	AL120559	ARPP-19
21	U29332	FHL2
22	AF013759	CALU
23	X82103	COPB
24	AA142964	LOC285148
25	AL049851	CARD10
26	U76421	ADARB1
27	AI365215	RRAS2
28	AF084523	CREG
29	M35878	IGFBP3
30	AF085692	ABCC3
31	D38521	PSME4
32	L02426	PSMC1
33	U81006	TM9SF2
34	AJ223352	HIST1H2BK
35	AF043324	NMT1
36	AB014562	PHF2
37	AA176780	TRIM44
38	AJ010901	MUC4
39	L04282	ZNF148
40	M57730	EFNA1
41	AF038844	DUSP14
42	AB014529	AKAP11
43	J03060	GBA
44	M13194	ERCC1
45	X55885	KDELR1
46	AF006010	DD5
47	AF012072	EIF4G3
48	AB002357	KIF3B
49	L24123	NFE2L1
50	X76534	GPNMB
51	AF011468	STK6
52	X13466	APP
53	U28249	FXYD3
54	U49020	MEF2A
55	U82328	PDHX
56	AL022162	OCRL
57	AI018098	MGC15523

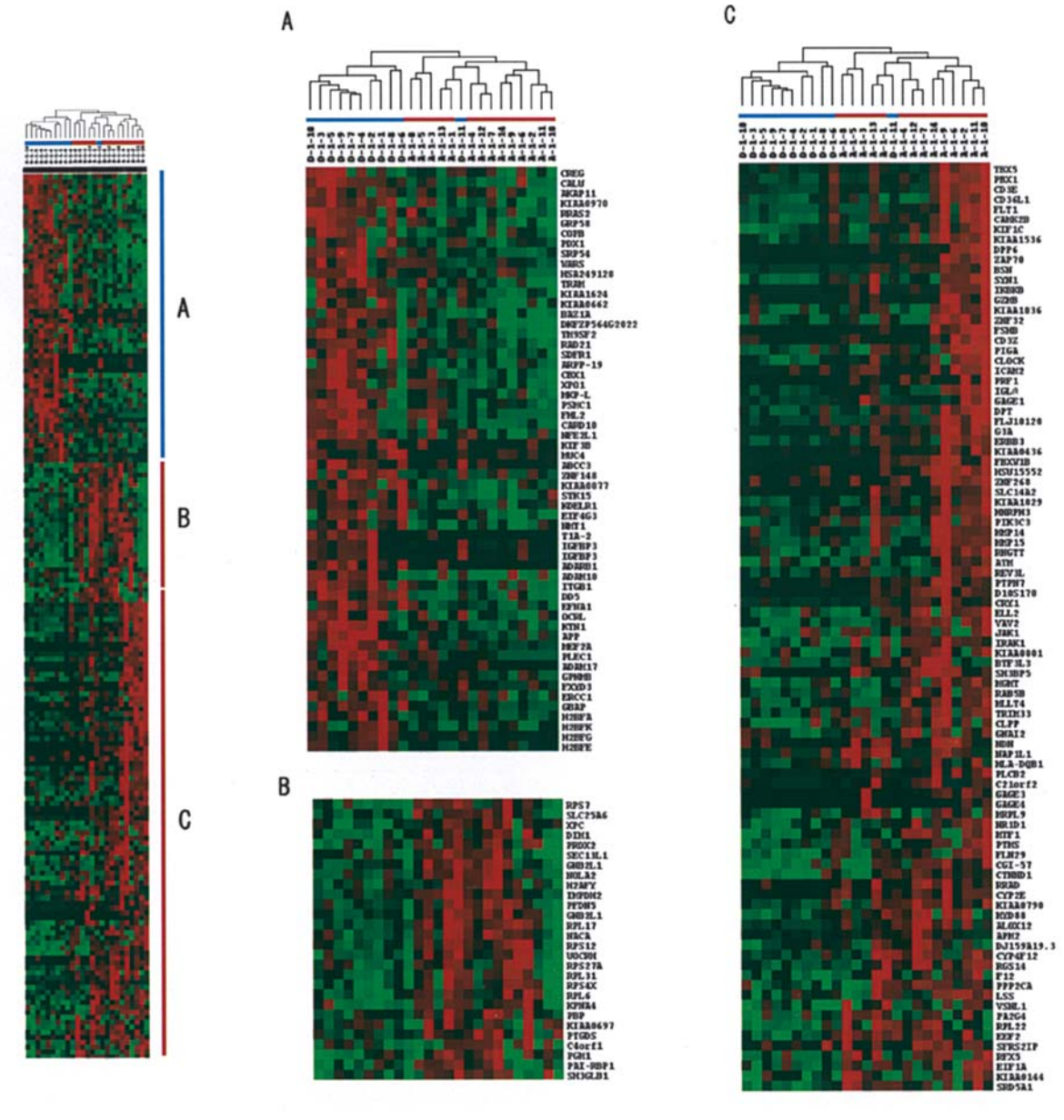

Figure 2. Supervised clustering analysis with expression data of the 177 genes differentially expressed between the 14 long-term survivors and the 11 short-term survivors. A dendrogram of the clustering analysis was generated with the same programs as used in Fig. 1. Two patient clusters (vertical bar) and the three major gene clusters (horizontal bar) are present. Three gene clusters are indicated as an insert. The long-term survivors (red), the short-term survivors (blue).

For example, *GZMB/Granzyme B*, *Perforin1* and *ICAM2* encoding crucial effectors for cytolysis by natural killer cells and cytotoxic T cells or for recruitment of those cells were found over-expressed in 14, 10, and 9 of the 14 long-term survivors, respectively. Similarly, of the 14 long-term survivors, upregulations were noted for *GAGE1, 3* and *5* encoding tumor antigen mRNAs in 11, 12, and 10 patients, *IRAK1, MyD88* and *IKBKB* encoding factors for IL-1 receptor-mediated NFκB activation in 11, 9, and 9 patients, *VAV2* and *ZAP70* encoding crucial effectors for T cell activation by APC-T cell interaction in 9 and 9 patients, and the *MHC class II* gene in 9 patients, respectively. These data suggest that cytotoxic T cell activation occurred preferentially in the long-term survivors. It is also noted that a vascular endothelial cell receptor gene *FLT1* was over-expressed in 7 of the 14 long-term survivors.

In contrast, there was only a limited number of genes involved in T cell activation in the 11 short-term survivors. As shown in Table II, 57 genes associated with the short-term survivors were identified. *ABCC3/MRP3* and *ERCC1* which are involved in drug resistance were found over-expressed in 9 and 8 of the 11 short-term survivors. Among the other over-expressed genes in the short-term survivors, *BAZ1A* and *SRP54*, which were located at chromosome 14q12-13, have been reported amplified in ESCCs (12).

To verify the differences in expression indicated by microarray analysis, we performed slot blot analysis of cRNAs produced from total RNA extracted from 8 of the 14 long-term survivors and 8 of the 11 short-term survivors, whose RNAs were available in both microarray and slot blot analyses. Microarray data and the slot blot data of some of the above-mentioned genes are shown in Fig. 3. By slot blot analysis, over-expression

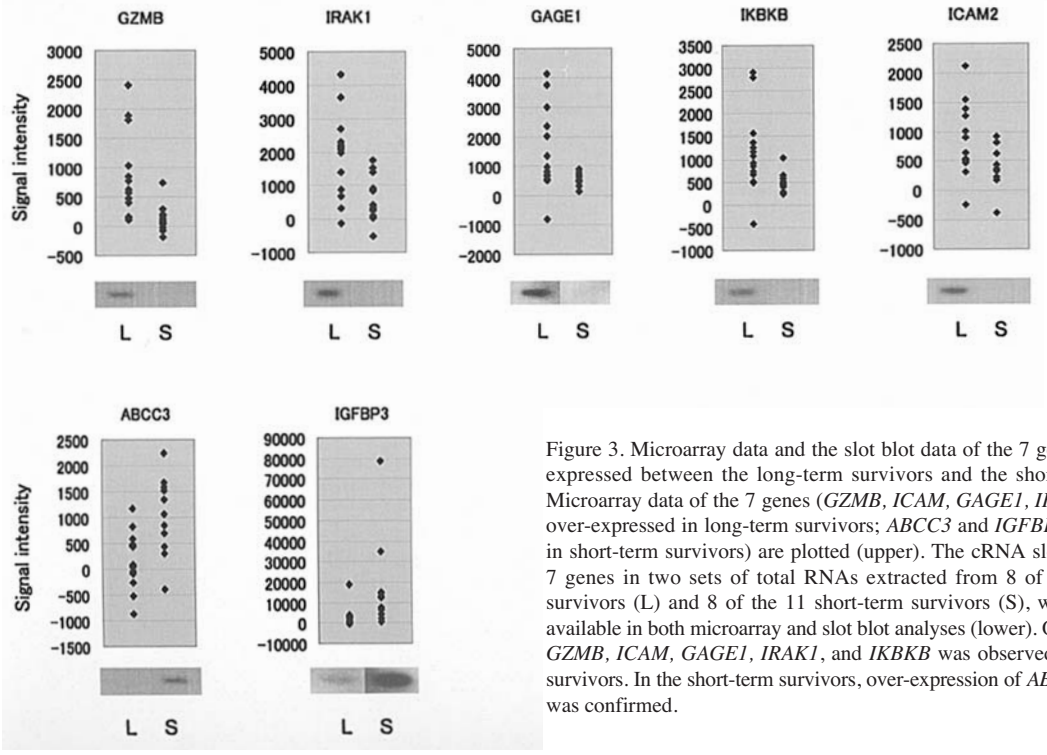

Figure 3. Microarray data and the slot blot data of the 7 genes differentially expressed between the long-term survivors and the short-term survivors. Microarray data of the 7 genes (*GZMB, ICAM, GAGE1, IRAK1*, and *IKBKB* over-expressed in long-term survivors; *ABCC3* and *IGFBP*3 over-expressed in short-term survivors) are plotted (upper). The cRNA slot blot data of the 7 genes in two sets of total RNAs extracted from 8 of the 14 long-term survivors (L) and 8 of the 11 short-term survivors (S), whose RNAs were available in both microarray and slot blot analyses (lower). Over-expression of *GZMB, ICAM, GAGE1, IRAK1*, and *IKBKB* was observed in the long-term survivors. In the short-term survivors, over-expression of *ABCC3* and *IGFBP3* was confirmed.

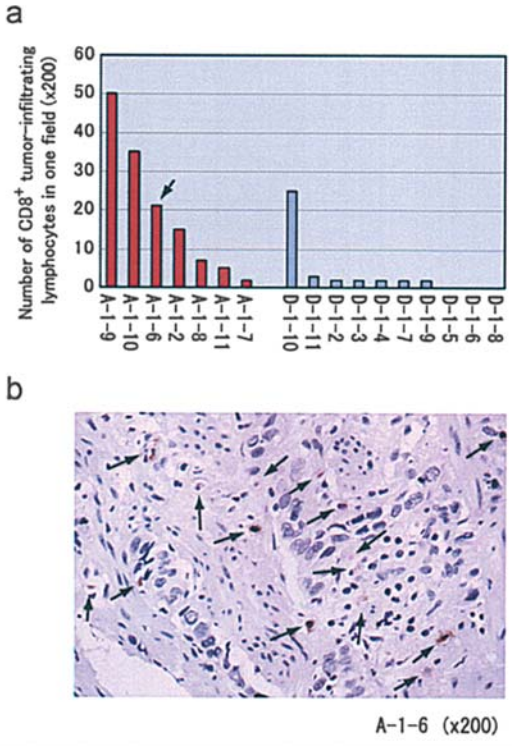

Figure 4. Immunohistochemical analysis of tumor infiltrating T-lymphocytes in long- and short-term survivors. (a), The CD8-positive T cell number in a field (x200) in 7 long-term survivors were 50, 35, 21, 15, 7, 5 and 2, respectively, whereas those in 11 short-term survivors were 25, 3, 2, 2, 2, 2, 2, 0, 0, 0, and 0, respectively. (b), A representative result showing presence of tumor infiltrating CD8-positive T cells in long-term survivors is shown.

of *GZMB, ICAM, GAGE1, IRAK1*, and *IKBKB* was observed in the long-term survivors. In the short-term survivors, over-expression of *ABCC3* and *IGFBP3* was confirmed.

Comparison of the number of tumor infiltrating T-lymphocytes between long- and short-term survivors. Next, using paraffin-embedded tissues of 7 biopsy samples of the 14 long-term survivors and all of the 11 short-term survivors, which were available for immunohistochemistry, we compared the number of tumor infiltrating lymphocytes between the long-term survivors and the short-term survivors. As shown in Fig. 4, the CD8-positive T cell numbers in a field (x200) in 7 long-term survivors were 50, 35, 21, 15, 7, 5 and 2, respectively, whereas those in 11 short-term survivors were 25, 3, 2, 2, 2, 2, 2, 0, 0, 0, and 0, respectively. The CD8-positive T cell number in the long-term survivors was significantly higher than that in the short-term survivors, although A-1-7 and D-1-10 were exceptions. However, D-1-10, the short-term survivor with 25 CD8-positive T cells in a field, showed no over-expression of any of the 14 above-mentioned genes for T cell activation, whereas the genes were over-expressed in all of the long-term survivors except A-1-7. These data suggested that the increased number of activated T cells in a tumor is significantly associated with a good prognosis for patients with ESCC treated by CRT.

Discussion

The clustering analysis on gene expression profiles (Fig. 2) together with the known functions of the genes selected as prognostic marker candidates (Table II) suggested that multiple factors are involved in the sensitivity and resistance to CRT in ESCC. The early studies in the basic radiation biology of tumor tissues, both *in vitro* and *in vivo* showed that well-oxygenated cells are radiosensitive (13-15). Recently, it has been reported that microvessel density in laryngeal SCCs and ESCCs is a useful factor for predicting radio-sensitivity (16,17). These vascularity-oxygenation-radio-

sensitivity relationships seem to be supported also by our analysis, in which we noted over-expression of a tumor vessel marker gene *FLT1* in the long-term survivors.

Second, it has been recognized that doses of radiation, lower than or equal to those that cause direct cytolysis, may alter the phenotype of target tissue by up-regulating gene products that may make tumor cells more susceptible to T-cell-mediated immune attack (18,19). Our present results (Fig. 4) suggest that the effect of CRT is correlated with the number of CD8-positive T cells in a tumor in each patient. Therefore, genes for T cell activation and for tumor vessel formation may become good markers for identifying potentially long-term survivors.

On the other hand, identification of poor-responders to CRT is also clinically important in the decision process for treatment modality. The genes which might be associated with the short-term survival include those for drug resistance or apoptosis resistance and some genes such as *BAZ1A* and *SRP54* showing gene amplification in ESCC.

Establishment of a CRT response prediction algorithm requires ascertainment of another set of validation samples. However, our analysis suggests the feasibility of such class prediction, and the gene list may contribute to the understanding of the underlying molecular architecture of the heterogeneity of CRT responses and the selection of useful markers for predicting the prognosis of individual ESCC patients treated with CRT alone.

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