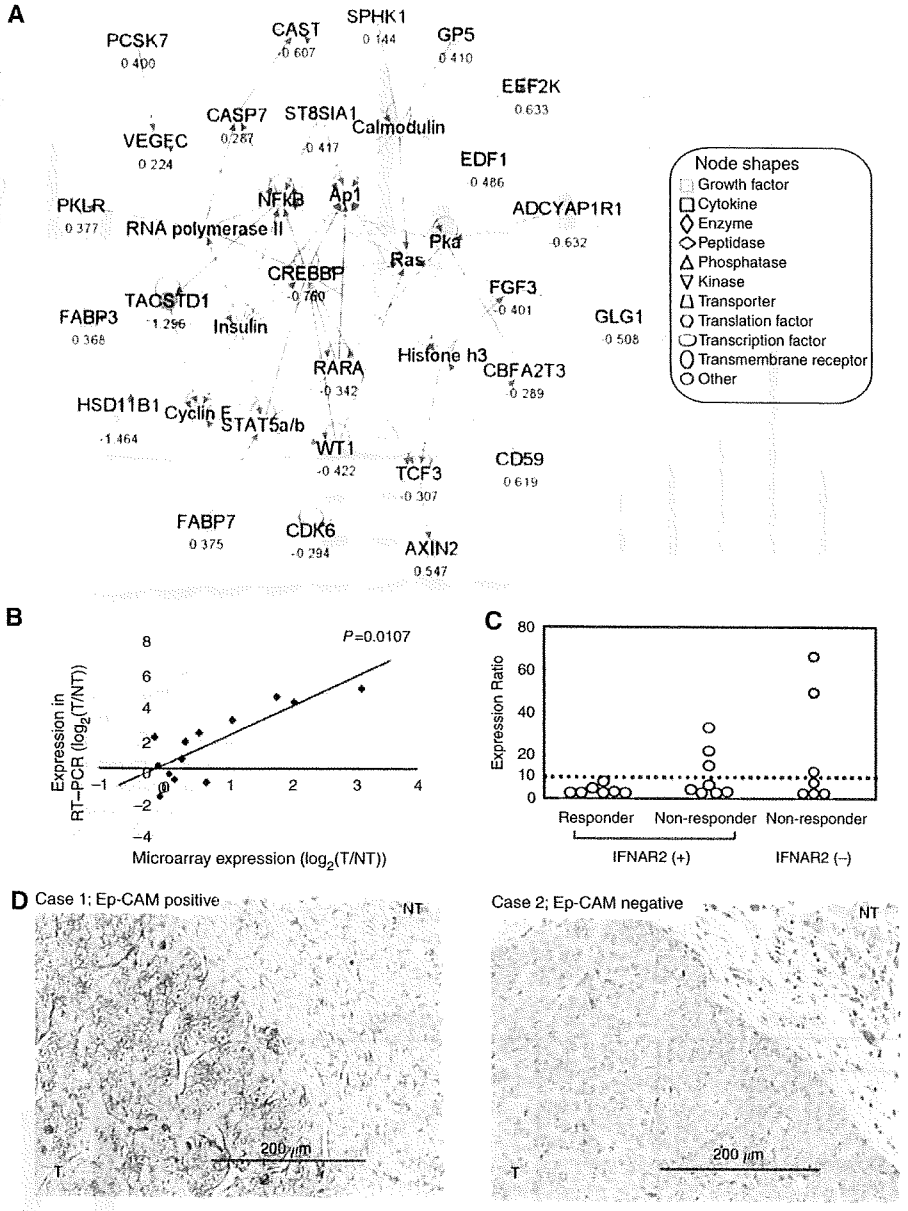

Figure 1 (**A**) Gene network of genes related to resistance to IFN-α/5-FU combination therapy. This network with the highest score consisted of 35 molecules in 19 focus molecules (red or green colour) and 16 interconnecting molecules (not coloured). The network included AXIN2, TCF3, RARA, CREBBP and TACSTD1, which are all associated with Wnt/β-catenin signalling. Each value of gene expression correlated directly with the intensity of the node colour. Red: upregulation in non-responders, green: downregulation in non-responders. The ratio of expression of each gene (non-responders/responders) is indicated below each node. (**B**) The expression levels determined by quantitative RT–PCR analysis correlated significantly with the microarray data. The Pearson correlation coefficient (*P*-value) for TACSTD1 were 0.668 ($P = 0.0107$) (**C**) Among non-responders with IFNAR2-positive HCC or IFNAR2-negative HCC, the TACSTD1 expression ratio was higher in several cases than that in responders with IFNAR2-positive HCC. (**D**) Immunohistochemical staining for Ep-CAM in representative cases. Left panel: the majority of tumour cells showing staining for Ep-CAM on the plasma membrane. Right panel: tumour cells were negative for Ep-CAM. T, tumour lesion; NT, non-tumour lesion. (Magnification, × 200). The colour reproduction of this figure is available on the html full text version of the paper.

Table 4 Immunohistochemical analysis of Ep-CAM expression

	Ep-CAM expression		
	Negative	**Positive**	***P*-value**
Responders	10	0	0.0528
Non-responders	14	6	

combination of BIO and 5-FU alone and BIO and IFN-α exhibited reduced anti-proliferative effects (data not shown).

Activation of Wnt/β-catenin signalling interferes with the inhibitory effect of IFN-α/5-FU on DNA synthesis

To investigate whether activation of Wnt/β-catenin signalling is involved in the reduction of the growth inhibitory effects of

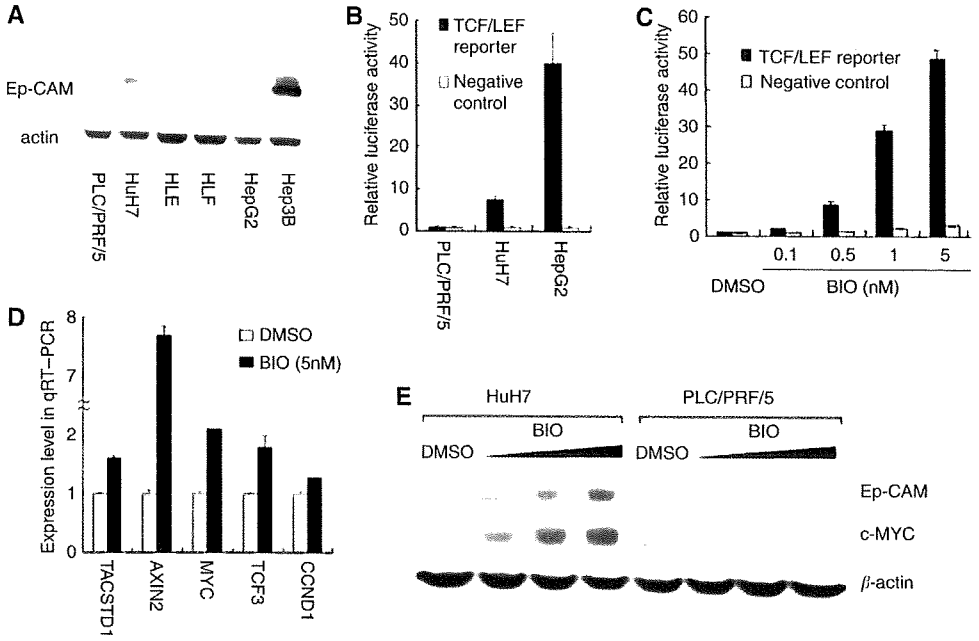

Figure 2 Changes in Ep-CAM expression and TCF/LEF transcription activity after treatment with BIO. (**A**) Western blot analysis of Ep-CAM in human hepatoma cell lines. Expression of Ep-CAM was positive in HuH7, HepG2 and Hep3B cell lines, but not in PLC/PRF/5, HLE and HLF. (**B**) Luciferase reporter assay of PLC/PRF/5, HuH7 and HepG2 cells. Relative luciferase activities were high in both Ep-CAM-positive HuH7 cells and HepG2 cells, whereas very low in Ep-CAM-negative PLC/PRF/5 cells. The assay was conducted in triplicate and results are shown as mean ± s.d. (**C**) Luciferase reporter assay of HuH7 cells treated with various concentrations of BIO for 24 h. Treatment with 5 nM induced 48.6-fold increase in relative luciferase activity compared with DMSO. The assay was conducted in triplicate and results are shown as mean ± s.d. (**D**) qRT–PCR analysis of HuH7 cells treated with BIO for 24 h. BIO increased the expression levels of TACSTD1, AXIN2, MYC, TCF3 and CCND1 compared with DMSO. Data are mean ± s.d. values of gene expression measured in duplicate. (**E**) Western blot analysis of HCC cell lines treated with BIO for 48 h. The expression of Ep-CAM and c-MYC increased in a BIO dose-dependent manner in HuH7 cells, but not in PLC/PRF/5 cells.

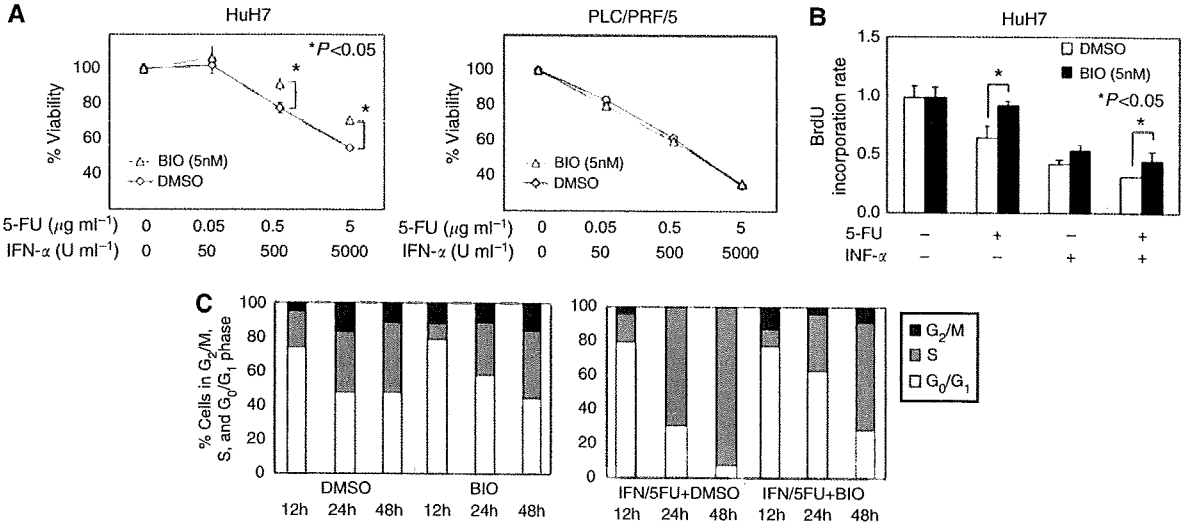

Figure 3 (**A**) Changes in susceptibility to the combination of 5-FU and IFN-α was measured by MTT assay. All cells were incubated with various concentrations of 5-FU and IFN-α and with BIO (5 nM) or DMSO. When BIO was combined with IFN-α/5-FU, it significantly reduced the growth inhibitory effects of IFN-α/5-FU in HuH7 cells, but not PLC/PRF/5 cells. The viability of cells incubated without drugs was defined as 100% and data are shown as mean ± s.d. (**B**) DNA synthesis-inhibition assay of HuH7 cell was assessed by BrdU incorporation rate. Cells were incubated with 5-FU and/or IFN-α and with BIO (5 nM) or DMSO. In both cell lines, the addition of BIO with 5-FU alone and IFN-α/5-FU significantly reduced the inhibitory effects of IFN-α/5-FU on DNA synthesis. Data was measured in triplicate and are shown as mean ± s.d. (**C**) Results of flow cytometric analysis of HuH7 cells treated with BIO and/or IFN-α/5-FU combination. Data represent percentages of cells in G_2/M, S and G_0/G_1 phases of the cell cycle. Concurrent use of BIO with IFN-α/5-FU delayed the accumulation of S-phase fraction.

IFN-α/5-FU, we evaluated the effects of BIO and IFN-α/5-FU on DNA synthesis using a BrdU-based cell proliferation ELISA. In HuH7 cells, the BrdU incorporation rates (representing DNA synthesis) in cultures treated with 5-FU alone and IFN-α/5-FU were 0.649 ± 0.052 and 0.312 ± 0.004, respectively. Activation of Wnt/β-catenin signalling by BIO resulted in a significant interference with the inhibitory effect of IFN-α/5-FU on DNA synthesis; the BrdU incorporation rates in cells cultured with BIO and 5-FU alone and with BIO and IFN-α/5-FU were significantly increased to 0.928 ± 0.020 ($P = 0.002$) and 0.458 ± 0.037 ($P = 0.007$) (Figure 3B).

Cell cycle assay

Finally, we used flow cytometric analysis to examine changes in cell cycle progression in cultures treated with BIO and IFN-α/5-FU. In cultures refed with serum plus 5-FU and IFN-α, the distribution of cells at different cell cycles was similar to that of cells treated with DMSO at 12 h. Thereafter, HuH7 cell lines treated with 5-FU and IFN-α showed accumulation of cells in S-phase and a gradual increase in S-phase fraction from 24 to 48 h. Addition of BIO and IFN-α/5-FU to the cell cultures delayed the accumulation of S-phase fraction. Marked accumulation of cells in S-phase (24 h; 69.4% and 48 h; 92.9%) was noted in cultures of cells treated with IFN-α/5-FU, whereas the percentage of cells in S-phase in cultures of BIO and IFN-α/5-FU decreased to 34.9% and 62.9% at the respective time points (Figure 3C).

DISCUSSION

Gene expression profiling analyses represent a high-throughput approach to dissect the biology underlining resistance to anticancer drugs in malignancies. We earlier identified a 63-gene set that could predict the response to IFN-α/5-FU combination therapy using a small-scale PCR array system of a total of 2666 genes (Kurokawa *et al*, 2004a). In this study, we used advanced technology with human whole genes analysis covering 30,336 human probes compared with the PCR array system. This comprehensive analysis allowed us to identify the biological actions of IFN-α/5-FU combination therapy. Furthermore, creating biological networks from comprehensive gene expression profiling could be useful for discovering certain targeted molecules and pathways. In fact, we reported recently genome-wide expression profiling of 100 HCC tissues using this network analysis, Ingenuity Pathway Analysis and identified novel targeted molecules related to specific signalling pathways (Kittaka *et al*, 2008).

In this study, gene expression profiling and pathway analysis identified Wnt/β-catenin signalling as a significant canonical pathway. The Wnt/β-catenin-signalling pathway plays an important role in the development of various malignancies, as well as cell proliferation and differentiation in several adult stem cells (Barker and Clevers, 2006; Klaus and Birchmeier, 2008). It has been also shown that anti-cancer drugs or irradiation often kill tumour cells, yet putative cancer stem/progenitor cells are resistant to these agents (Jamieson *et al*, 2004; Woodward *et al*, 2007; Klaus and Birchmeier, 2008). Cancer stem/progenitor cells provide an attractive explanation for chemotherapy-induced tumour remission as well as relapse. Analysis of the molecular and signalling mechanism of resistance of cancer stem/progenitor cells should be important for the development of new therapeutic strategies. Recent studies showed that the Wnt/β-catenin pathway plays a role in radiation and/or chemotherapy resistance of various malignancies such as leukaemia, head and neck tumours, prostate cancer and HCC (Jamieson *et al*, 2004; Ohigashi *et al*, 2005; Chang *et al*, 2008; Yang *et al*, 2008). In this study, we also showed that activation of Wnt/β-catenin signalling by a specific GSK-3 inhibitor in hepatoma cell lines decreased the susceptibility to IFN-α/5-FU through a reduction in their DNA synthesis inhibitory effects and regulation of cell cycle progression. We have already reported the mechanisms of the anti-proliferative effects of IFN-α/5-FU combination therapy, including regulation of cell cycle progression by increasing S-phase fraction (Eguchi *et al*, 2000), induction of apoptosis through IFNAR2, by downregulating Bcl-xl and by Fas/FasL pathway (Kondo *et al*, 2005; Damdinsuren *et al*, 2007; Nakamura *et al*, 2007; Nagano *et al*, 2007a), modulation of the immune response by inducing the TRAIL/TRAIL-receptor pathway (Yamamoto *et al*, 2004) and inhibition of tumour angiogenesis (Wada *et al*, 2007). In addition to the above mechanisms related to their anti-proliferative effects, this study showed that activation of Wnt/β-catenin signalling resulted in reduction of the inhibitory effects of IFN-α/5-FU on DNA synthesis, by decreasing the accumulation of cells in S-phase. With regard to the apoptotic effect of the combination therapy, it is reported that Wnt/β-catenin signalling is closely linked to JAK–STAT signalling (Yamashina *et al*, 2006), and regulates STAT3 expression, thus enhancing cell growth and anti-apoptotic activity of various cancer cells (Kusaba *et al*, 2007). We earlier reported that IFN-α/5-FU combination therapy increased the frequency of apoptosis in PLC/PRF/5 cells, but only minimally in HuH7 cells (<1%) (Eguchi *et al*, 2000). We also analysed the influence of activation of Wnt/β-catenin signalling on the apoptotic effects of IFN-α/5-FU combination therapy, but no significant change was observed in HuH7 cells probably because of the low rate of apoptosis. Further studies are needed to examine the molecular mechanisms of Wnt/β-catenin signalling-related enhancement of resistance to IFN-α/5-FU combination therapy.

Activation of the Wnt/β-catenin signalling pathway is reported in various diseases including many malignancies (Moon *et al*, 2004; Reya and Clevers, 2005; Branda and Wands, 2006). The ideal method for detecting the signalling activity in human tissues remains controversial (Giles *et al*, 1980). A recent study identified Ep-CAM as a novel Wnt/β-catenin signalling target gene in HCC cell lines, which could also serve as a biomarker (Yamashita *et al*, 2007). Ep-CAM is a first tumour-associated antigen and encoded by the TACSTD1 gene (Herlyn *et al*, 1979; Litvinov *et al*, 1994). In liver neoplasia, Ep-CAM is expressed in almost all cholangiocarcinomas, whereas 14% of HCCs manifested the expression, which seems to be more pronounced in poorly differentiated HCCs (Breuhahn *et al*, 2006). Ep-CAM-positive HCC displayed a molecular signature with features of hepatic progenitor cells, including the presence of known stem/progenitor markers such as c-kit, cytokeratin 19. In earlier studies, we showed that the expression of IFNAR2 is the only significant predictor of clinical outcome of IFN-α/5-FU combination therapy (Ota *et al*, 2005; Nagano *et al*, 2007a). On the basis of the present results on 30 HCC tissue samples, Ep-CAM seems to be another predictor of IFN-α/5-FU combination therapy. Further studies are needed to validate this result using larger sample numbers to establish the precise clinical response to IFN-α/5-FU combination therapy.

In summary, we showed that activation of Wnt/β-catenin signalling enhanced the resistance to IFN-α/5-FU therapy by reducing the inhibitory effects of these drugs on DNA synthesis and regulation of cell cycle progression *in vitro*. Furthermore, the results identified Ep-CAM expression in HCC tissue specimen as a potential biological marker for resistance to IFN-α/5-FU combination therapy.

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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Dickkopf-1 Expression as a Marker for Predicting Clinical Outcome in Esophageal Squamous Cell Carcinoma

Tomoki Makino, MD[1], Makoto Yamasaki, MD[1], Ichiro Takemasa, MD[1], Atsushi Takeno, MD[1], Yurika Nakamura, PhD[1], Hiroshi Miyata, MD[1], Shuji Takiguchi, MD[1], Yoshiyuki Fujiwara, MD[1], Nariaki Matsuura, MD[2], Masaki Mori, MD[1], and Yuichiro Doki, MD[1]

[1]Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan; [2]Department of Molecular Pathology, School of Allied Health Science, Faculty of Medicine, Osaka University, Suita, Osaka, Japan

ABSTRACT

Background and Objectives. Dickkopf-1 (DKK1) is the inhibitor of the canonical Wnt signaling pathway, however it is highly transactivated in various cancers, suggesting the presence of unknown mechanism. Its implication in human esophageal squamous cell carcinoma (ESCC) has not been sufficiently investigated.

Patients and Methods. We evaluated DKK1 protein expression in resected specimens from 170 patients with ESCC by immunohistochemistry. Tumors were categorized as positive or negative for DKK1. The relationships between DKK1 expression in ESCC and various clinicopathological parameters and prognosis (disease-free survival; DFS) were analyzed separately.

Results. Immunohistochemically, 72 (42.4%) tumors were DKK1 positive while no significant staining was observed in the normal squamous epithelium except for few basal cells. There was no significant relationship between DKK1 expression in ESCC and any of the clinicopathological parameters tested in this study. Patients with DKK1-positive tumors had poorer DFS than those with negative ESCC (5-year DFS; 31.5% versus 53.6%, $P = 0.0062$). Univariate analysis showed a significant relationship between pT [hazard ratio (HR) = 2.944, 95% confidence interval (CI) = 1.713–5.059, $P < 0.0001$], number of pN (HR = 2.836, 95% CI = 1.866–4.309, $P < 0.0001$), lymphatic invasion (HR = 2.892, 95% CI = 1.336–6.262, $P = 0.0070$), and DKK1 expression (HR = 1.763, 95% CI = 1.167–2.663, $P = 0.0071$) and DFS. Multivariate analysis including the above four parameters identified pT (HR = 2.053, 95% CI = 1.157–3.645, $P = 0.0140$), pN number (HR = 2.107, 95% CI = 1.362–3.260, $P = 0.0008$), and DKK1 expression (HR = 1.813, 95% CI = 1.195–2.751, $P = 0.0052$) as independent and significant prognostic factors for DFS.

Conclusion. Our data suggest the usefulness of DKK1 as a novel predictor of poor prognosis of patients with ESCC after curative resection and also as a therapeutic target for future tailored therapies against ESCC.

Esophageal squamous cell carcinoma (ESCC), the major histological form of esophageal cancer in East Asian countries, is one of the most lethal malignancies of the digestive tract and, in most cases, the initial diagnosis is made when malignancy is in the advanced stage.[1] In spite of recent improvements in multitreatment modalities, including surgical techniques, radiotherapy, and chemotherapy, the prognosis of patients with ESCC is still unsatisfactory.[2] Assessment of prognosis through clinicopathological features remains inadequate even when using the staging systems of tumor–node–metastasis (TNM) classification because of the considerable variability and heterogeneity within the same stage.[3] Therefore, there is a need to identify novel biological markers that allow a more accurate identification of high-risk population of recurrent disease and help in the design of appropriate treatment strategies for ESCC patients.

Dickkopf-1 (German for "big head, stubborn"), also known as DKK-1, is a secreted protein involved in embryonic development and known as a potent inhibitor of the Wnt signaling pathway, which enables appropriate

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M. Yamasaki, MD
e-mail: myamasaki@gesurg.med.osaka-u.ac.jp

positioning and development of the embryonic brain and other organ structures.[4–6] Specifically, Wnt-1 protein binds to the frizzled receptor (Fz) and the low-density lipoprotein receptor-related protein-5/6 (LRP5/6), triggering signals important for proliferation via β-catenin.[7] DKK1 binds to LRP5/6 and blocks interaction with Wnt-1, resulting in β-catenin degradation and retardation of proliferation.[8] In contrast to other Wnt/β-catenin signaling antagonists, DKK1 is reported to be overexpressed in many malignant tissues including breast cancer, lung cancer, esophageal carcinomas, ovarian endometrioid adenocarcinomas, multiple myeloma, Wilms' tumor, hepatoblastoma, and hepatocellular carcinoma (HCC), indicating negative feedback of Wnt/β-catenin signaling or the presence of unknown mechanism for DKK1, other than its association with chemo- or hormone sensitivity.[9–15] On the other hand, in human colon tumors, DKK1 expression decreases and DKK1 acts as a tumor suppressor gene where the DKK1 promoter is selectively hypermethylated, resulting in epigenetic silencing as observed in leukemia.[16–18] Thus, the expression and role of DKK1 might vary according to cancer location. The direct mechanisms in each type of cancer are in fact under investigation at present. Notwithstanding the above previous studies, there is little or no information on the clinical significance of DKK1 in ESCC, especially prognosis of patients with ESCC.

In the present retrospective study, we first conducted immunohistochemical (IHC) analysis of DKK1 protein expression in 170 resected specimens of ESCC and then determined its association with prognosis of patients with ESCC.

MATERIALS AND METHODS

Patients and Treatments

The present study involved 170 patients with histopathologically confirmed primary thoracic esophageal cancer who underwent surgical resection at our hospital between 1998 and 2007. They included 18 female and 152 male patients, with age ranging from 38 to 82 years (median 63.3 years). Subtotal esophagectomy via right thoracotomy with two- or three-field lymphadenectomy was performed in all patients. Curative resection (R0) was achieved in 162 patients (95.3%), while the outcome in the remaining 8 (4.7%) patients was noncurative resection (R1, 2). None of the patients died of postoperative complications. Ninety-five patients with lymph node metastasis at initial diagnosis received neoadjuvant chemotherapy (NACT), which consisted of two courses of 5-fluorouracil (5-FU), cisplatin (CDDP), and adriamycin (ADM).[19–22] We provided adjuvant chemotherapy (docetaxel or CDDP plus 5-FU regimen) to 11 patients with larger numbers of pathologically positive lymph nodes.[23]

After surgery, the patients were surveyed every 3 months by physical examination and serum tumor markers [squamous cell carcinoma (SCC) antigen, carcinoembryonic antigen (CEA)], every 6 months by computed tomography (CT) scan and abdominal ultrasonography, and every year by endoscopy until tumor recurrence was evident. Patients with tumor recurrence received chemotherapy or chemoradiotherapy, as long as their systemic condition permitted. Mean overall survival was 32.0 months and mean disease-free survival was 27.8 months. Mean follow-up period after surgery was 44.7 months.

Immunohistochemical Analysis

DKK1 protein accumulation was examined by IHC staining of formalin-fixed and paraffin-embedded ESCC tissue sections. One representative slide with the deepest tumor invasion was selected from each patient and subjected to immunohistochemistry as follows. Briefly, after deparaffinization in xylene and dehydration in graded ethanol solutions, endogenous peroxidase activity was blocked by incubation with 30 mL/L hydrogen peroxide for 20 min. Then tissue sections were heated at 95°C for 40 min in citrate buffer (0.05 mol/L, pH 6.0) for antigen retrieval. After incubation with rabbit polyclonal primary antibody DKK1 (Santa Cruz Biotechnology, Santa Cruz, CA, dilution 1:60) for 2 h at room air temperature, staining was performed by labeled streptavidin–biotin (LSAB) method. Negative controls of immunohistochemical reactions included omission of the primary antibody. Normal human placenta was used as a positive control. DKK1 staining for each ESCC sample was judged positive when more than 10% of the cancer cells in the section were immunoreactive to DKK1, and otherwise negative when 10% or less of the cells were positive. All slides were assessed independently by two pathologists and then by conference in case of disagreement. Both pathologists were blinded to the clinicopathological data.

Statistical Analysis

Correlations between DKK1 expression and various clinicopathological parameters were each evaluated by the χ^2 test and Fisher's exact probability test. The association between continuous parameters was evaluated by Mann–Whitney's *U*-test. Prognostic variables were assessed by log-lank test, and disease-free survival (DFS) was analyzed by the method of Kaplan and Meier. Cox's proportional hazard regression model with stepwise comparisons was used to analyze the independent prognostic factors. With

TABLE 1 Patient characteristics (n = 170)

Parameters	Number of patients (%)
Age (year)	63.3 [38–82][a]
Gender (male/female)	152(89.4)/18(10.6)
Histology (well/mod/poor)[b]	41(24.1)/85(50.0)/44(25.9)
Location (upper/middle/lower)[c]	18(10.6)/81(47.6)/71(41.8)
Neoadjuvant chemotherapy (yes/no)	95(55.9)/75(44.1)
pT (0/1/2/3/4)[d]	0(0)/24(14.1)/29(17.1)/ 103(60.6)/14(8.2)
pN (N0/N1/M1lym)	53(31.2)/117(68.8)/42(24.7)
Number of pN (0/1–3/4–7/≥ 8)	50(29.4)/64(37.7)/ 24(14.1)/32(18.8)
pStage (0/I/II/III/IV)	0(0)/13(7.6)/60(35.3)/ 55(32.4)/42(24.7)

[a] Average and range

[b] Well-, moderately, and poorly differentiated squamous cell carcinoma

[c] Middle, lower, and upper thoracic esophagus

[d] pN, pT, pStage (pathological classification) and M1lym (distant lymph node metastasis) according to TNM classification

respect to survival analysis, we adopted four lymph nodes as the cutoff value based on the guidelines of the Japanese Society for Esophageal Diseases (JSED).[24] These analyses were carried out using SPSS version 10 (SPSS, Inc., Chicago, IL) for Windows. A P value of less than 0.05 denoted the presence of statistical significance.

RESULTS

DKK1 Expression in ESCC

A total of 170 samples (Table 1) that contained both cancerous and noncancerous lesions were evaluated for DKK1 protein expression by immunohistochemical analysis. Of these, 72 (42.4%) showed positive DKK1 expression, mainly in the cytoplasm of tumor cells, with faint nuclear staining (Fig. 1a), while the remaining 98 (57.6%) were negative (Fig. 1b). The positive staining was almost homogeneous at single cancer nest and among different areas (surface, central, and deepest areas) of the cancer lesion. In contrast, none of the normal squamous epithelium showed significant level of immunohistochemical staining, although some basal cells showed faint nuclear immunostaining (Fig. 1c). The grading of immunostained sections was almost identical by the two pathologists, with interobserver variation of less than 10%.

Correlation Between DKK1 Expression and Clinicopathological Parameters

Table 2 lists the correlations between DKK1 expression and various clinicopathological parameters. DKK1-positive tumors tended to be associated with larger number of pathologically positive lymph nodes compared with DKK1-

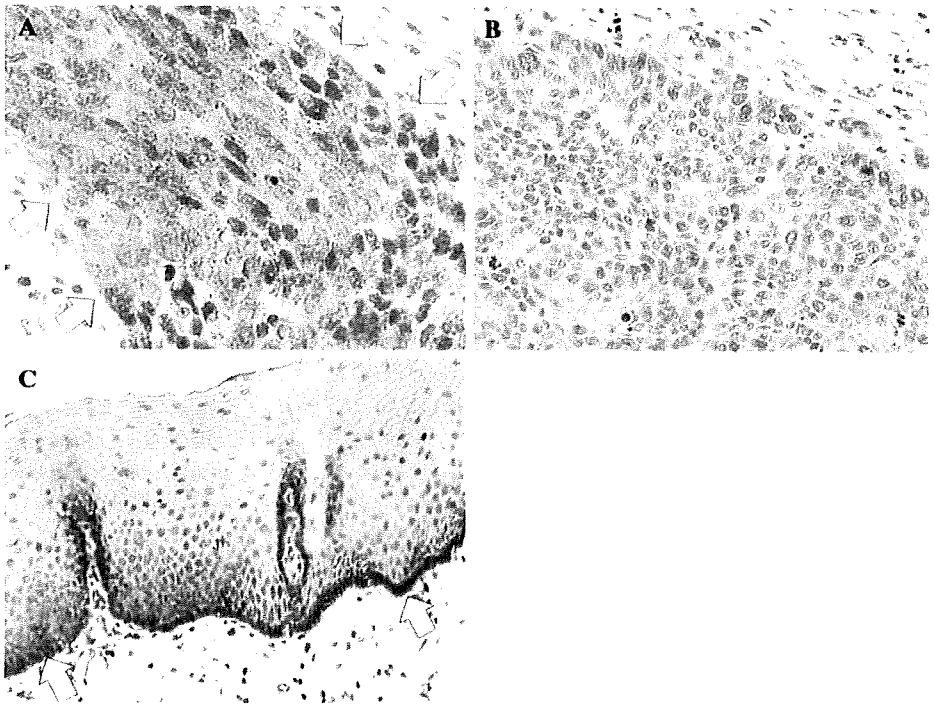

FIG. 1 DKK1 expression by immunohistochemical staining. **a** Representative DKK1-positive esophageal squamous cell carcinoma showing staining mainly in the cytoplasm of tumor cells (arrows) (magnification ×400). **b** Representative DKK1-negative esophageal squamous cell carcinoma showing almost no appreciable staining of tumor cells (magnification ×200). **c** Representative normal squamous epithelium negative for DKK1 expression except in a few basal cells (arrows) (magnification ×200)

negative tumors, although not statistically significantly so (3.4 versus 6.3, $P = 0.2199$). No significant correlations were observed with other parameters, including age, gender, tumor location, use of neoadjuvant chemotherapy, and pT and pStage (Table 2).

Correlation Between DKK1 Expression and Survival

Disease recurrence after curative resection was diagnosed in 83 (51.2%) of 162 patients with curative resection (R0) and the mean time to recurrence was 9.5 months. The total 5-year disease-free survival (5-year DFS) rate was 44.4%. Patients with DKK1-positive tumors showed poorer DFS than those with negative tumors (5-year DFS; 31.5% versus 53.6%, $P = 0.0062$) (Fig. 2a). Analysis of each subgroup showed that this trend was apparent in the advanced pT stage group (pT3/4) (5-year DFS; 19.1% versus 44.4%, $P = 0.0063$) and pN1 group (5-year DFS; 18.5% versus 46.7%, $P = 0.0021$), but not in early pT stage group (pT1/2) (5-year DFS; 63.4% versus 71.9%, $P = 0.5404$) or pN0 group (5-year DFS; 62.3% versus 66.7%, $P = 0.9673$). Similarly, there was a significant difference in prognosis of patients with DKK1-positive and DKK1-negative tumors, especially at pStage III (5-year DFS; 17.6% versus 55.1%, $P = 0.0122$), but not pStage I (5-year DFS; 100% versus 83.3%, P value not applicable), pStage II (5-year DFS; 37.7% versus 65.8%, $P = 0.1019$) or pStage IV (5-year DFS; 11.8% versus 27.8%, $P = 0.1140$) (Fig. 2b).

On univariate analysis, the relationships between pT (HR = 2.944, 95% CI = 1.713–5.059, $P < 0.0001$), number of pathologically positive lymph nodes (HR = 2.836, 95% CI = 1.866–4.309, $P < 0.0001$), lymphatic invasion (HR = 2.892, 95% CI = 1.336–6.262, $P = 0.0070$), and DKK1 expression (HR = 1.763, 95% CI = 1.167–2.663, $P = 0.0071$) and DFS were significant, but not between all other parameters tested (e.g., age, gender, histology, tumor location, and venous invasion) (Table 3). Neoadjuvant chemotherapy group, which mainly consisted of cN1 patients, tended to show unfavorable prognosis but without significance ($P = 0.0521$). Furthermore, the number of patients who received adjuvant chemotherapy ($n = 11$, 6.5%) was too small to evaluate its prognostic significance. Multivariate analysis using the above four parameters with statistical significance ($P < 0.05$) in univariate analysis identified that four or more pathological lymph node metastases (pN $\geq$ 4) was the poorest prognostic factor (HR = 2.107, 95% CI = 1.362–3.260, $P = 0.0008$), followed by pathological tumor invasion (pT3,4) (HR = 2.053, 95% CI = 1.157–3.645, $P = 0.0140$) and positive DKK1 expression (HR = 1.813, 95% CI = 1.195–2.751, $P = 0.0052$) (Table 4).

TABLE 2 Correlation between DKK1 and various clinicopathological parameters

Parameters	DKK1 expression		
	Positive (%)	Negative (%)	P value
Age (years)			
<65	43 (45.3)	52 (54.7)	0.4359
≥65	29 (38.7)	46 (61.3)	
Gender			
Male	63 (41.4)	89 (58.6)	0.6151
Female	9 (50.0)	9 (50.0)	
Histopathology			
Well[a]	13 (31.7)	28 (68.3)	0.1467
Mod, poor	59 (45.7)	70 (54.3)	
Location			
Upper, middle[b]	44 (44.4)	55 (55.6)	0.5331
Lower	28 (39.4)	43 (60.6)	
Neoadjuvant chemotherapy			
Yes	40 (42.1)	55 (57.9)	>0.9999
No	32 (42.7)	43 (57.3)	
pT[c]			
T1	12 (50.0)	12 (50.0)	0.1748
T2	9 (31.0)	20 (69.0)	
T3	42 (40.8)	61 (59.2)	
T4	9 (64.3)	5 (35.7)	
Number of pN[d]	6.3	3.4	0.2199
pStage			
Stage I	7 (53.8)	6 (46.2)	0.3742
Stage II	21 (35.0)	39 (65.0)	
Stage III	27 (49.1)	28 (50.9)	
Stage IV	17 (40.5)	25 (59.5)	

[a] Well-, moderately, and poorly differentiated squamous cell carcinoma

[b] Middle, lower, and upper thoracic esophagus

[c] pT, pN, pStage (pathological classification) according to TNM classification

[d] Average

DISCUSSION

The present IHC study of DKK1 expression in 170 ESCC resected specimens showed positive cancer-cell DKK1 expression in 42.4% of the specimens with negative immunostaining in the normal squamous epithelium, apart from basal cells. DKK1 protein expression did not correlate with various clinicopathological parameters. With respect to prognosis, immunostaining of ESCC for DKK1 was a significant determinant of unfavorable prognosis, especially in patients with pStage III disease. In addition to DKK1-positive expression, multivariate analysis also identified pT and the numbers of pN as independent prognostic predictors for DFS. Therefore, immunostaining

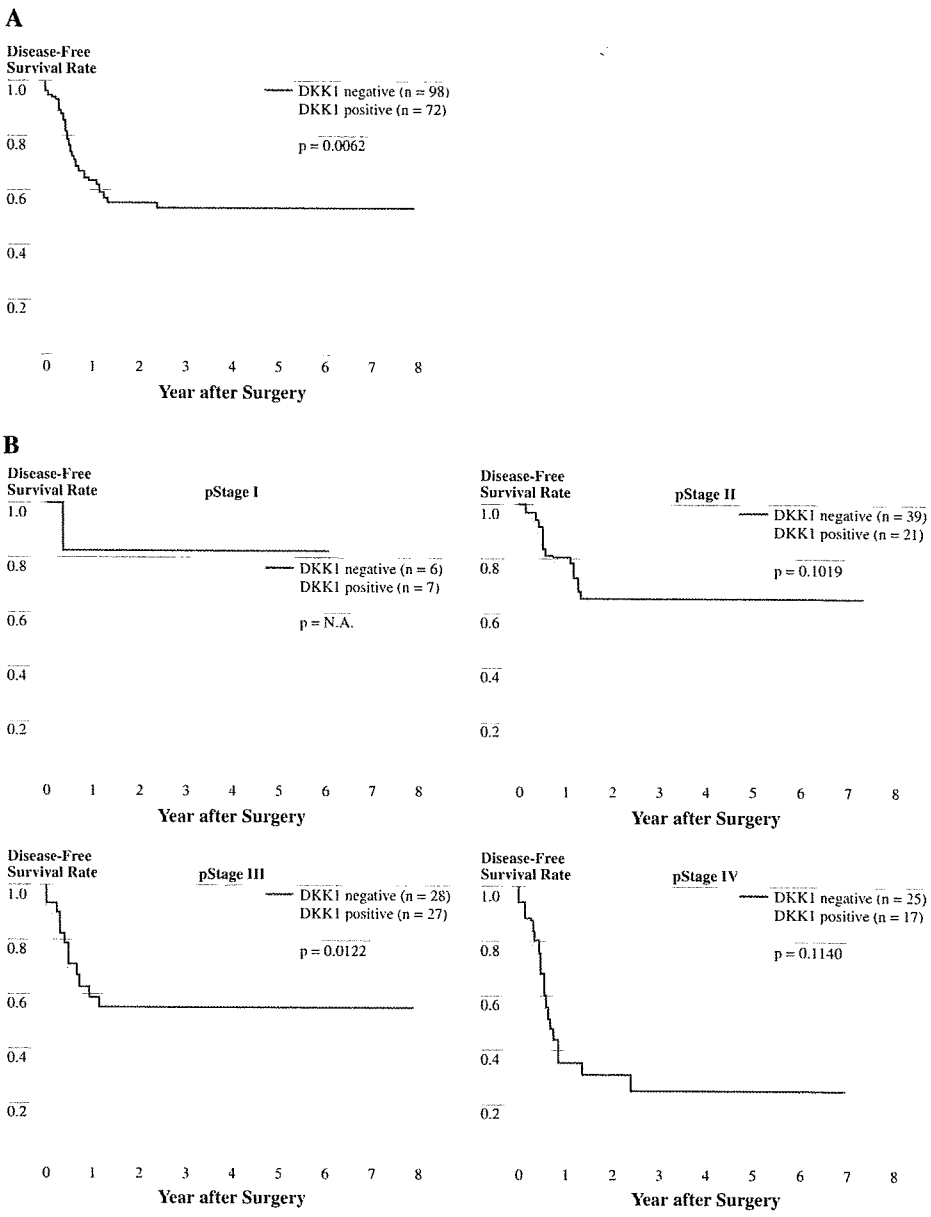

FIG. 2 Survival curves according to DKK1 expression. **a** Disease-free survival curve classified according to DKK1 expression for all patients plotted by Kaplan–Meier methods. **b** Disease-free survival curves according to DKK1 expression at each pathological stage. Differences between two groups were evaluated by log-rank test. Ordinate: disease-free survival rate, abscissa: time after surgery (years)

for DKK1 could be potentially considered a predictor of unfavorable prognosis of patients who undergo ESCC resection.

The human DKK-related gene family is composed of DKK-1, DKK-2, DKK-3, and DKK-4, together with a unique DKK-3-related protein termed Soggy (Sgy). hDKKs 1–4 contain two distinct cysteine-rich domains in which the positions of ten cysteine residues are highly conserved between family members.[25] Members of the human DKK-related family differ not only in their structures and expression patterns but also in their abilities to inhibit Wnt signaling, which is thought to regulate the proliferation and renewal of stem cells, whereas dysregulated activation of Wnt/β-catenin signaling has been implicated in carcinogenesis.[26] DKK1 is a 35-kDa protein that contains signal peptide sequence and functions as a negative regulator of the Wnt signaling through disruption of the complex formation of Wnt and its receptors, LRP5/6 and Fz receptor.[27] DKK1 is reported to play a crucial role in head formation in vertebrate development, although there is a big question mark on its role in cancer and whether it is similar to its known function in normal cells or embryogenesis.[4,8,25,28,29]

In human cancers, DKK1 is overexpressed in breast cancer, lung cancer, esophageal carcinomas, ovarian endometrioid adenocarcinomas, human hepatoblastomas, Wilms' tumors, HCC, and multiple myeloma.[9–14] However,

TABLE 3 Univariate survival analysis of disease-free survival by Cox's proportional hazard model

Parameters	Number of cases	hazard ratio	95% CI	*P*
Age (<65 years/≥65 years)	95/75	1.153	0.759–1.752	0.5056
Gender (male/female)	152/18	1.016	0.510–2.022	0.9646
Histology (mod-poor/well)[a]	127/43	1.677	0.974–2.889	0.0623
Location (lower/upper-middle)[b]	73/97	1.018	0.673–1.540	0.9333
Neoadjuvant chemotherapy (yes/no)	95/75	1.521	0.996–2.324	0.0521
pT (T3,4/T1,2)[c]	117/53	2.944	1.713–5.059	<0.0001
Number of pN (≥4/<4)	56/114	2.836	1.866–4.309	<0.0001
Lymphatic invasion (present/absent)	145/25	2.892	1.336–6.262	0.0070
Venous invasion (present/absent)	88/82	1.251	0.827–1.892	0.2882
DKK1 expression (positive/negative)	72/98	1.763	1.167–2.663	0.0071

CI confidence interval

[a] Well-, moderately, and poorly differentiated squamous cell carcinoma

[b] Middle, lower, and upper thoracic esophagus

[c] pT and pN based on TNM classification

TABLE 4 Multivariate analysis of disease-free survival by Cox's proportional hazard model

Parameters	Number of cases	HR	95% CI	*P*
pT (T3,4/T1,2)	117/53	2.053	1.157–3.645	0.0140
Number of pN (≥4/<4)	56/114	2.107	1.362–3.260	0.0008
Lymphatic invasion (present/absent)	145/25	2.055	0.925–4.565	0.0770
DKK1 expression (positive/negative)	72/98	1.813	1.195–2.751	0.0052

For abbreviations, see Table 3

Gonzalez-Sancho et al. reported the loss of DKK1 expression in colon cancer, suggesting that colon carcinogenesis involves the removal of the inhibitory effect of DKK1 on the Wnt/β-catenin pathway.[16] Only a few studies reported the clinical or prognostic significance of DKK1. Forget et al. found preferential expression of DKK1 in hormone-resistant breast tumors, which was associated with poor prognosis.[9] Yamabuki et al. examined the possible role of DKK1 as a serologic and prognostic biomarker for lung and esophageal carcinomas.[10] They evaluated DKK1 expression by IHC using tumor tissue microarrays and suggested its association with poor prognosis for ESCC, although they failed to identify it as an independent prognostic factor. They also showed experimentally that exogenous expression of DKK1 increased the migration/invasion activity of mammalian cells, suggesting a significant role for DKK1 in progression of human cancer. Considered together, the above results and our findings suggest that DKK1 expression seems to play an important role in the development and/or progression of certain types of human tumors including ESCC, although the link between DKK1 and Wnt signaling pathway in the context of cancer progression or carcinogenesis remains under investigation at present.

With respect to the results of survival analysis, our survival data by pathological stage based on TNM classification were comparable to those of previous reports on ESCC in Japan.[3] The 5-year DFS of pStage III DKK1-negative tumors determined in the present study was 55.1%, which was equivalent to 56.8% of DFS for pStage II tumors in the above study. On the other hand, the 5-year DFS of pStage III DKK1-positive tumors in our study was 17.6%, which was similar or rather worse than that of 20.4% of pStage IV tumors. Therefore, for prediction of prognosis, especially for patients with pStage III tumors, it might be useful to integrate DKK1 expression level with the pathological TNM classification although other stages, especially stage IV, had too small samples to detect a prognostic significance of DKK1 expression.[3] Furthermore DKK1 expression could be a valuable guide for treatment strategy of ESCC. Patients with pStage III, pT3/4 or pN1 DKK1-positive tumors, who are highly likely to show disease recurrence, should receive chemotherapy and be followed up closely; a second line taxan-based chemotherapy for nonresponders to NACT (or the same protocol as NACT for responders) could be an effective postoperative chemotherapy. It should be noted, however, that one cannot apply our results in ESCC to adenocarcinomas of the esophagus, which is the commonest histopathological type in Western countries, because this study involved only analysis of squamous cell carcinoma, which is common in East Asian countries.

In conclusion, DKK1 expression as determined immunohistochemically was significantly associated with poor prognosis of patients with ESCC. However, because Wnt signaling pathway alone could not provide an explanation for the prognostic significance of DKK1, further studies should evaluate the potential mechanisms of increased DKK1 expression and poor prognosis. Nevertheless, we hope the findings of this study open the door for exploration of efficacious treatment strategies and development of new therapeutic approaches, such as antibody therapy or functional inhibition of expression, for ESCC.

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The feasibility of using biopsy samples from esophageal cancer for comprehensive gene expression profiling

MASAAKI MOTOORI[1,2], ICHIRO TAKEMASA[1], MAKOTO YAMASAKI[1], TAKAMICHI KOMORI[1], ATSUSHI TAKENO[1], HIROSHI MIYATA[1], SHUJI TAKIGUCHI[1], YOSHIYUKI FUJIWARA[1], TAKUSHI YASUDA[1], MASAHIKO YANO[2], NARIAKI MATSUURA[3], KENICHI MATSUBARA[4], MORITO MONDEN[1], MASAKI MORI[1] and YUICHIRO DOKI[1]

[1]Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka Suita, Osaka 565-0871; [2]Department of Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases, 1-3-3 Nakamichi, Higashinari-ku, Osaka 537-8511; [3]Department of Functional Diagnostic Science, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871; [4]DNA Chip Research Inc., 1-1-43 Suehiro, Tsurumi, Yokohama, Kanagawa 230-0045, Japan

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Abstract. Advanced esophageal cancer has been recently treated by multimodal therapy including preoperative chemotherapy or chemoradiotherapy and surgery. A biopsy sample provides a valuable specimen for understanding the biological characteristics of individual esophageal cancer. Pretreatment prediction of the response to chemotherapy or radiotherapy based on biological characteristics using biopsy samples is a desirable goal. In using biopsy samples for molecular analysis, there are two problems; the proportion of cancer cells and the intratumor heterogeneity. This study was conducted to investigate the feasibility of using endoscopic biopsy samples of esophageal squamous cell cancer (ESCC) for comprehensive gene expression profiling (GEP). Comprehensive GEP was performed in 40 bulky ESCC specimens and 10 normal esophageal epithelial specimens from patients who underwent esophageal resection and 52 endoscopic ESCC biopsy samples from 26 patients (two samples per one patient). Unsupervised hierarchical cluster analysis showed distinct profiles between the bulky ESCC specimens and normal epithelial specimens. Also, unsupervised hierarchical cluster analysis revealed distinct profiles between the biopsy ESCC samples and normal epithelial specimens. Moreover, a couple of biopsy samples taken from different locations of the same tumor were closely clustered together. That is, biopsy ESCC samples were distinguished from normal esophageal epithelial specimens and the intratumor heterogeneity of GEP was smaller than intertumor heterogeneity. GEP using biopsy ESCC samples is feasible and has the potential to represent the biological properties.

Correspondence to: Dr Ichiro Takemasa, Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka Suita, Osaka 565-0871, Japan
E-mail: alfa-t@sf6.so-net.ne.jp

Key words: esophageal cancer, endoscopic biopsy sample, gene expression profiling, intratumor heterogeneity

Introduction

Advanced esophageal cancer, which has a poor prognosis, has been previously treated by multimodal therapy including preoperative chemotherapy or chemoradio therapy and surgery (1,2). Neoadjuvant therapy has been shown to improve the prognosis of responders. On the other hand, non-responders not only suffer from side effects but also lose precious time to take advantage of other possible treatments (3,4). Therefore, pretreatment prediction of the response to chemo- or radiotherapy is one of the most desirable goals in clinical practice, but pretreatment clinocopathological factors are unable to predict the response and there is no reliable method. Biological characteristics of a tumor are important factors affecting the malignant potential and sensitivity to chemotherapy or radiotherapy.

A pretreatment biopsy sample provides a valuable specimen for understanding the biological characteristics of individual esophageal cancer. Molecular analyses, such as RT-PCR and immunohistochemistry, of pretreatment endoscopic biopsy samples of esophageal cancer have been performed to understand the biological characteristics of esophageal cancer (5-7). However, only one gene or a few genes have been addressed in these studies. Multiple genetic alterations are involved in the development and progression of esophageal cancer and these aberrations may affect the expression of a large number of genes (8,9) and numerous molecular pathways may contribute to the sensitivity of chemotherapy or radiotherapy. Gene expression profiling (GEP) allows assessment of expression of thousands of genes simultaneously and is one of the powerful tools for understanding the biological characteristics of each tumor. In fact, this approach has already been used to identify genes that could serve as molecular markers of cancer classification and

outcome prediction (10-14). In esophageal cancer, GEP using surgical resection samples has been performed (15-17). However, these results can be used only in the selection of post operative adjuvant therapy or follow-up schedules. To apply the results of GEP to therapeutic planning of esophageal cancer in clinical practice, pretreatment endoscopic biopsy samples should be analyzed. Recently, GEP using not only surgically resection samples but also biopsy samples has been successfully performed (18-21).

Endoscopic biopsy samples are usually small and morphologically esophageal cancer often displays intratumor macroscopic and microscopic heterogeneity. If the biopsy samples used for molecular analysis contain no or few cancer cells, it would not represent the biological characteristics of a tumor. If the gene expression of samples taken from different locations in the same tumor is drastically different, biological classification based on molecular analysis of biopsy samples may not be suitable. That is, in using biopsy samples for molecular analysis, there are two problems; the proportion of cancer cells and the intratumor heterogeneity of gene expression. However, there have been few studies addressing these problems (7).

This study investigated whether biopsy ESCC samples can be distinguished from normal esophageal epithelial specimens by GEP and assessed the intratumor heterogeneity of GEP by analyzing a couple of biopsy samples taken from different locations of the same tumor.

Materials and methods

Patients and clinical samples. Esophageal squamous cell cancer (ESCC) samples were obtained from 40 patients and normal esophageal epithelial specimens from 10 patients who underwent a surgical resection. The clinicopathological characteristics of the resected ESCC specimens are listed in Table I. Normal esophageal epithelial specimens were collected from the area normally stained by the Lugol dye. In addition, a couple of endoscopic biopsy samples of ESCC were obtained from 26 patients and assayed separately (Fig. 1). The clinicopathological characteristics of the biopsy ESCC specimens are listed in Table II. None of the patients received either chemotherapy or radiotherapy before the surgery or endoscopy. Tissue specimens were disrupted in RNAlater (Ambion, Austin, TX) and stored at 4˚C for 1-2 h, then at -80˚C until use. For each biopsy specimen, an adjacent cancer tissue biopsy was given to a pathologist for assessing the presence of cancer and its histology. Routine hematoxylin and eosin- (H&E) stained slides were used. All aspects of this study protocol were performed according to the ethical guidelines set by the committee of the three Ministries of the Japanese Government and a signed consent form was obtained from each subject.

Cellular composition of the biopsy specimens. The cellular composition of biopsy specimens was determined by an evaluation of the cell squares in the H&E-stained slides using light microscopy. These results were recorded as percentages. A total 110 biopsy samples from 45 ESCC patients, partly including patients enrolled in the microarray analysis, were analyzed.

Table I. The clinicopathological characteristics of the resection ESCC specimens.

Characteristics	No. of patients
Gender	
Male/Female	030/10
Age	
Median	64
Tumor location[a]	
Cervical esophagus	1
Upper thoracic esophagus	7
Middle thoracic esophagus	17
Lower thoracic esophagus	15
Pathological T category[a]	
pT1	6
pT2	3
pT3	26
pT4	5
Pathological N category[a]	
pN0	9
pN1	19
pM1(LYM)	12
Pathological disease stage[a]	
pStage I	2
pStage II	11
pStage III	13
pStage IV	14

[a]According to TNM classification.

Extraction and quality assessment of RNA. Total RNA was purified from clinical samples utilizing TRIzol reagent (Invitrogen, San Diego, CA) as described in the accompanying protocol. The integrity of RNA was assessed by Agilent 2100 Bioanalyzer and RNA 6000 LabChip kits (Yokokawa Analytical Systems, Tokyo, Japan). Only high-quality RNA with intact 18S and 28S ribosomal RNA was used for the subsequent analysis. For control reference, 15 RNA samples from normal esophageal epithelial specimens were mixed.

Preparation of fluorescent-labeled aRNA targets and hybridization. The extracted RNA samples were amplified with T7 RNA polymerase using Amino Allyl MessageAmp™ aRNA kit (Ambion) according to the manufacturer's protocol. The quality of each Amino Allyl-aRNA sample was checked by Agilent 2100 Bioanalyzer. Five μg of control and experimental aRNA samples were labeled with Cy3 and Cy5, respectively, mixed and hybridized on an oligonucleotide microarray covering 30,000 human probes (AceGene Human 30K; DNA Chip Research Inc. and Hitachi Software Engineering Co., Ltd., Yokohama Japan). The experimental protocol is available at http://www.dna-chip.co.jp/thesis/AceGeneProtocol.pdf. Thereafter, the microarrays were scanned using the ScanArray 4000 (GSI Lumonics, Billerica, MA).

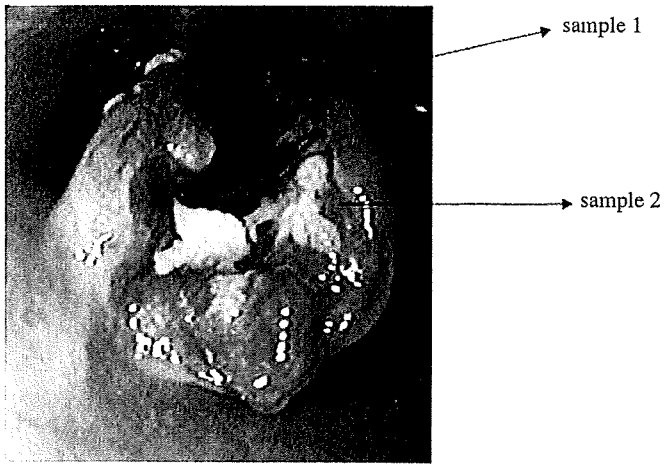

Figure 1. Sampling method for the biopsy ESCC specimens. A couple of biopsy samples were collected from each patient during a routine endoscopic examination.

Table II. The clinicopathological characteristics of the biopsy ESCC specimens.

Characteristics	No. of patients
Gender	
Male/Female	19/7
Age	
Median	67
Tumor location[a]	
Upper thoracic esophagus	11
Middle thoracic esophagus	11
Lower thoracic esophagus	4
Pretherapeutic clinical T category[a]	
cT1	1
cT2	5
cT3	14
cT4	6
Pretherapeutic clinical N category[a]	
cN0	0
cN1	13
cM1(LYM)	13
Pretherapeutic clinical stage[a]	
cStage I	0
cStage II	2
cStage III	9
cStage IV	15

[a]According to TNM classification.

Analysis of microarray data. Signal values were calculated by DNASISArray software (Hitachi Software Inc. Tokyo, Japan). Following background subtraction, data with low signal intensities were excluded from additional investigation. In each sample, the Cy5/Cy3 ratio values were log-transformed and global equalization to remove a deviation of the signal intensity between whole Cy3- and Cy5-fluorescence was performed by subtracting a median of all log(Cy5/Cy3) values from each log(Cy5/Cy3) value. Genes with missing values in >10% of the samples were excluded from further analysis. Hierarchical cluster analysis (HCA) with Euclidean distance as a similarity coefficient and Ward as a clustering algorithm was performed using GeneMath 2.0 software (Applied Maths, Inc., Austin, TX).

Up- or down-regulated genes. Commonly up-regulated genes were defined when their expression levels were 2-fold or more against the control reference in at least 50% of the samples. In addition, commonly down-regulated genes were defined when their expression levels were half-fold or less against the control reference in at least 50% of the samples.

RT-PCR. To verify our microarray data, RT-PCR was performed for two of the commonly up-regulated genes (*MMP9* and *SPARC*). Total RNA (2 μg) from eight biopsy ESCC specimens and control reference (mixture of fifteen RNAs from normal esophageal epithelial specimens) was used for the reverse-transcription reaction with oligo-(dT) primer, using the Reverse Transcription System (Promega, Madison, WI). PCR was performed in a 25 μl reaction mixture containing 1 μl of cDNA template, 0.2 mmol/l of each primer and 1 unit of Taq DNA Polymerase (AmpliTaq Gold, Roche Molecular Systems, Pleasanton, CA), as follows; one cycle of 95°C for 12 min, followed by 25 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1.5 min, followed by 72°C for 10 min. The primers were designed by using Web-based Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). GAPDH was also amplified as a marker to confirm the amounts of cDNA generated from each sample. PCR products were visualized with ethidium bromide following separation by electrophoresis on 2% agarose gel.

Results

Cellular composition of biopsy specimens. The mean percentage of tumor cells and stromal cells were 46 and 26% with a standard deviation of 20 and 16, respectively.

Total RNA yield from biopsy specimens. The average and minimum volume of total RNA from one biopsy sample was 17.7 and 2.2 μg, respectively. The quality of all the extracted RNAs was sufficient for comprehensive GEP with intact 18S and 28S ribosomal RNA.

Gene expression profiling between resection ESCC specimens and normal esophageal epithelial specimens and between biopsy ESCC specimens and normal esophageal epithelial specimens. First, GEP was compared between resection ESCC specimens and normal esophageal epithelial specimens. After gene processing described previously, 18,718 genes were used for further analysis. Unsupervised HCA using all 18,718 genes showed distinct profiles between the two groups (Fig. 2). All resection ESCC specimens were distinguished from normal esophageal epithelial specimens by GEP.

Secondly, GEP was compared between biopsy ESCC specimens and normal esophageal epithelial specimens. After gene processing, 18,734 genes were used for further analysis.

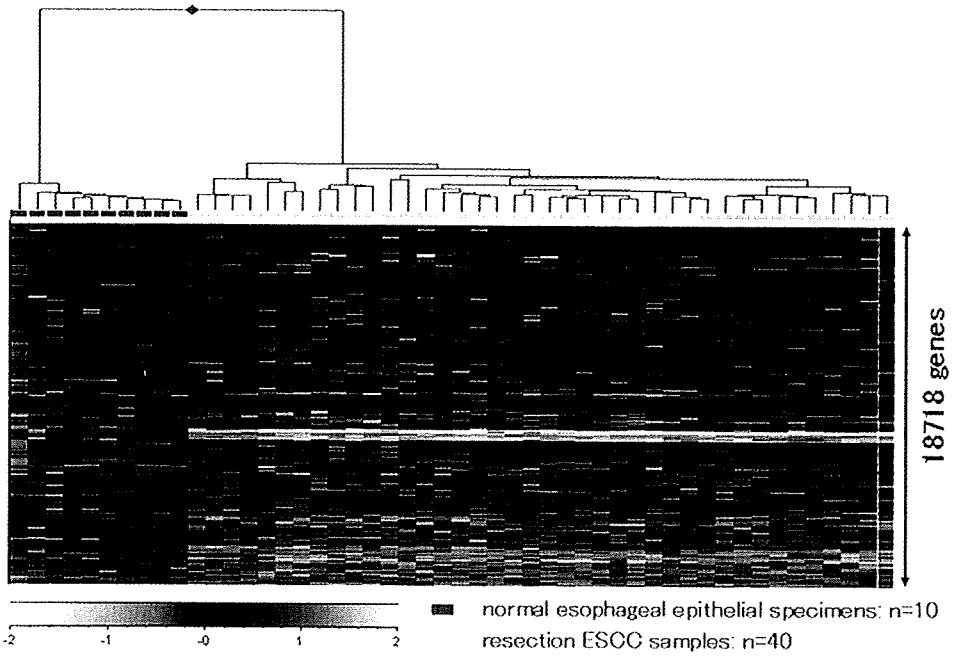

Figure 2. Hierarchical cluster analysis with 18,718 genes in 40 resection ESCC samples and 10 normal esophageal epithelial specimens. The rows and columns represent genes and samples, respectively. The color scale at the bottom indicates the relative expression levels in terms of standard deviations from the median.

Unsupervised HCA using all 18,734 genes showed distinct profiles between the two groups (Fig. 3). Almost all biopsy ESCC specimens except one specimen were distinguished from normal esophageal epithelial specimens by GEP. Moreover, a couple of biopsy samples taken from different locations of the same tumor were closely clustered together, except one case.

Commonly up- or down-regulated genes in resection and biopsy ESCC specimens. A total of 129 and 136 commonly up-regulated genes were identified in the resection and biopsy ESCC specimens, respectively and 85 genes (~65%) were overlapped in both groups. In addition, 518 and 506 commonly down-regulated genes were identified in resection and biopsy ESCC specimens, respectively and 444 genes (~85%) were overlapped in both groups. To confirm the microarray data, RT-PCR was performed for two of the commonly up-regulated genes (*MMP9* and *SPARC*) in eight biopsy samples. These genes have been reported to be associated with progression of esophageal cancer (22,23). The expression patterns of RT-PCR closely agreed with those of the microarray in both genes (Fig. 4).

Discussion

To understand the biological characteristics of individual esophageal cancer, molecular analysis of pretreatment endoscopic biopsy samples have been performed. Miyata *et al* performed immunohistochemical analysis of six molecules in pre radiation biopsy samples. The sensitivity of radiation therapy was significantly correlated with *p53* and *CDC25B* expression (6). Langer *et al* investigated expression of 12 molecules in pretreatment biopsy samples using a real-time RT-PCR analysis and compared the histological effect to cisplatin and 5-fluorouracil chemotherapy. *MTHFR*, *caldesmon* and *MRP1* were significantly associated with the response (7). However, it is clear that several genes will not define the biological characteristics of individual tumors. The properties of each tumor are likely to reflect the functions of all gene products. Therefore, multiple markers will be needed to adequately define the sensitivity of tumors to chemotherapy or radiotherapy and GEP, which can assess the expression of thousands of genes, will likely to be a suitable approach. This is a feasibility study of using biopsy samples in comprehensive GEP for future clinical application.

Cancer tissues consist of mixed populations of cancer cells and stromal cells, such as fibroblasts, infiltrating lymphocytes and endothelial cells. GEP of cancer is currently based on two main methods of RNA preparation; whole tissue RNA extraction and laser capture microdissection (LCM). LCM certainly can improve tissue sampling and achieve homogeneity of the tumor tissue. However, stromal elements play multiple roles in tumor growth and progression and also contribute to tumor response to chemotherapy or radiotherapy (24-26). The biological characteristics of a tumor are considered to be reflected by both the cancer cells and stromal cells, so whole tissues of the specimens were analyzed.

Endoscopic ESCC biopsy samples are small, so it is very difficult to assess the proportion of cancer cells by investigating a part of a sample. In the H&E-stained slides, the average ratio of cancer cells and stromal cells of biopsy specimens was 46 and 26%, respectively, although these samples were different from those actually used in the microarray analysis. If the biopsy sample using comprehensive GEP is composed of mostly normal cells, it would not represent the biological characteristics of a tumor. First, this study confirmed that resection ESCC specimens were distinguished from normal esophageal epithelial specimens by GEP. Then, it verified that biopsy ESCC specimens were distinguished from normal esophageal epithelial specimens by GEP. In this study, the

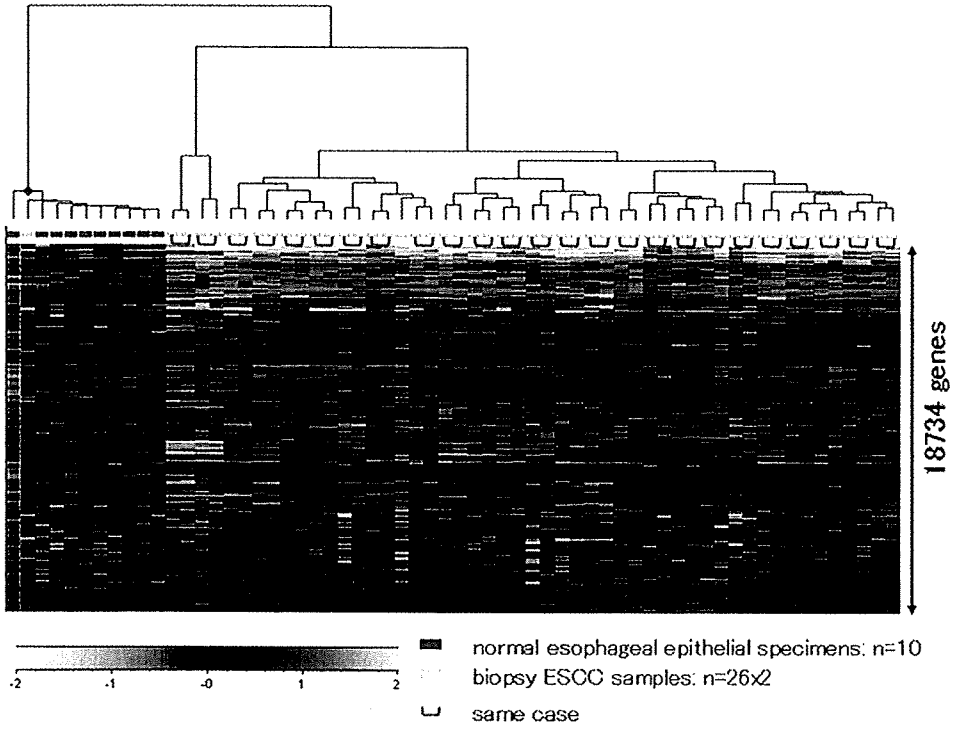

Figure 3. Hierarchical cluster analysis with 18,734 genes in 52 biopsy ESCC samples and 10 normal esophageal epithelial specimens. The rows and columns represent genes and samples, respectively. The color scale at the bottom indicates the relative expression levels in terms of standard deviations from the median.

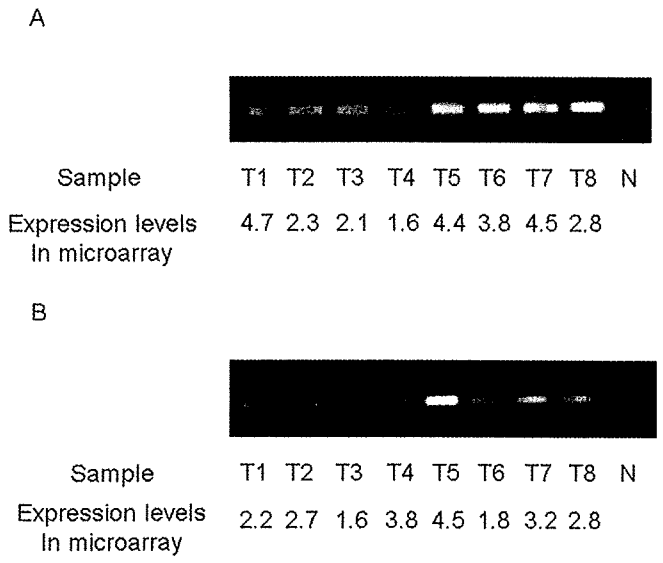

Figure 4. The expression patterns of RT-PCR. (A) *SPARC* and (B) *MMP9*. T1-T8, biopsy cancer sample. N, control reference.

reason that one biopsy specimen could not be distinguished from the normal esophageal epithelial specimens, perhaps, was that there was a low proportion of cancer cells due to a sampling error, though it was possibly adequate for the diagnosis of cancer by microscopy.

There is histopathological heterogeneity in esophageal cancer, as in other solid tumors. The influence of such morphological intratumor heterogeneity on GEP is not clear, though the heterogeneity is detected in individual genes (4,27,28). Therefore, the difference of GEP between biopsy samples taken from different locations in the same tumor should be elucidated. In the current study, a couple of endoscopic biopsy samples obtained from the same case were closely clustered together in almost all cases. This result means that the intratumor heterogeneity of GEP is smaller than intertumor heterogeneity in the superficial position of esophageal cancer and one biopsy specimen may represent GEP of the superficial position of esophageal cancer. Concerning the intratumor heterogeneity of GEP in other solid tumors, the degree of GEP variability within gastric cancer samples isolated from resection specimens of same patient was remarkably low (29). In surgically resected soft tissue sarcomas, the average intratumor distance was considerably shorter than the intertumor distance and intratumor heterogeneity seems to have only a small impact on the variability of GEP (30,31). In endoscopic biopsy samples of colorectal cancer, the intratumor heterogeneity of GEP is smaller than intertumor heterogeneity (21). The findings of these studies are consistent with the current results. It is not clear how many biopsy samples from one patient are sufficient to assess the intratumor heterogeneity. In normal rectal epithelial specimens, two biopsy samples per person are recommended for microarray analysis based on the variation in gene expression data within a person (32). In cervical cancer, although the majority of genes are expressed relatively uniformly, a subset of genes can be expressed quite variably within a single patient. Genes which have a wide variation within a single patient require several biopsies, sometimes >10 biopsies, based on a statistical analysis. However, the optimum number of biopsies cannot be chosen based on statistical reasoning alone, because in the clinical practice, the feasibility of taking many biopsies from one patient is a restrictive factor (33).

The question remains as to whether an endoscopic biopsy ESCC sample reflects the characteristics of the whole tumor. In breast cancer, by comparing GEP of tissue samples with the same cases of FNAB samples, the differences are looked closer (34). Komori *et al* reported that an endoscopic biopsy sample of colorectal cancer might give an accurate picture of the GEP in the whole tumor (21). In this study, although biopsy samples and resection samples were taken from different patients, ~65% of the commonly up-regulated genes and 85% of the commonly down-regulated genes were overlapped. This result indicates that the GEP of endoscopic biopsy samples of ESCC may potentially represent the GEP of whole tumors.

In summary, comprehensive GEP using biopsy ESCC specimens is feasible and has the potential to represent the biological properties of ESCC. Further studies with comprehensive GEP using biopsy samples would provide a novel prediction system of neoadjuvant chemotherapy or chemoradiotherapy for ESCC.

Acknowledgements

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LIVER TRANSPLANTATION 15:1738-1749, 2009

ORIGINAL ARTICLE

Differential Transcriptome Patterns for Acute Cellular Rejection in Recipients with Recurrent Hepatitis C After Liver Transplantation

Tadafumi Asaoka,[1] Tomoaki Kato,[2,5] Shigeru Marubashi,[1] Keizo Dono,[1] Naoki Hama,[1] Hidenori Takahashi,[3] Shogo Kobayashi,[1] Yutaka Takeda,[1] Ichiro Takemasa,[1] Hiroaki Nagano,[1] Hideo Yoshida,[2] Phillip Ruiz,[3] Andreas G. Tzakis,[2] Kenichi Matsubara,[4] Morito Monden,[1] Yuichiro Doki,[1] and Masaki Mori[1]

[1]Department of Surgery, Graduate School of Medicine, Osaka University, Osaka, Japan; [2]Division of Liver and Gastrointestinal Transplantation, Department of Surgery, and [3]Department of Pathology and Surgery, University of Miami School of Medicine, Miami, FL; [4]DNA Chip Research, Incorporated, Yokohama, Japan; and [5]Department of Surgery, Columbia University College of Physicians and Surgeons, New York, NY

Histopathological evaluation of the liver via biopsy remains the standard procedure for the diagnosis of both acute cellular rejection (ACR) and recurrent hepatitis C (RHC) after liver transplantation. Nevertheless, it is often difficult to diagnose ACR in hepatitis C virus–positive recipients because of changes in common and overlapping with RHC. The aim of this study was to identify potential target genes for ACR in recipients with RHC. We analyzed 22 liver biopsy samples obtained from 21 hepatitis C virus–positive recipients. The clinicopathological diagnosis based on biopsy examination was ACR-predominant with superimposed RHC in 9 samples (ACR group) and RHC without ACR (non-ACR group) in 13. Using oligonucleotide microarrays, we compared the transcriptional changes in the 2 groups and selected 2206 genes that were significantly modulated in ACR. We analyzed the regulatory networks in ACR with Ingenuity Pathway Analysis software, and we confirmed with quantitative real-time polymerase chain reaction the reproducibility of caspase 8, apoptosis-related cysteine peptidase and bone morphogenetic protein 2 up-regulation in another group of validation samples, representing 2 genes from the core network as the target genes for ACR. Our results demonstrated novel transcriptome patterns for ACR with concurrent RHC that were distinct from those of recipients with only RHC, suggesting that gene expression profiling may be useful in the diagnosis of ACR in recipients with hepatitis C. *Liver Transpl 15:1738-1749, 2009.* © 2009 AASLD.

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Although the rapid development of potent immunosuppressants in the last decades has enabled liver transplantation (LT) to be a well-established treatment for various end-stage liver diseases and acute liver failure, immune tolerance is not yet attainable, and acute cellular rejection (ACR) remains a common problem after LT. Despite continuous improvements in immunosuppressive therapy, ACR still occurs in 25% to 40% of

Additional Supporting Information may be found in the online version of this article.

Abbreviations: ACR, acute cellular rejection; AST, aspartate aminotransferase; BCL2, B cell lymphoma 2; BMP2, bone morphogenetic protein 2; CASP8, caspase 8, apoptosis-related cysteine peptidase; CCC, cholangiocellular carcinoma; CFLAR, caspase 8 and Fas-associated protein with death domain–like apoptosis regulator; CyA, cyclosporine A; FK, tacrolimus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFNAR1, interferon (alpha, beta, and omega) receptor 1; IGFBP3, insulin-like growth factor binding protein 3; IL12RB, interleukin 12 receptor beta; IPA, Ingenuity Pathway Analysis; IRAK2, interleukin 1 receptor-associated kinase 2; LC, liver cirrhosis; LT, liver transplantation; LTA, lymphotoxin α; MAP3K11, mitogen-activated protein kinase kinase kinase 11; MAPK10, mitogen-activated protein kinase 10; MMF, mycophenolate mofetil; NFAT, nuclear factor of activated T cells; NFATC3, nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 3; NS, not significant; qRT-PCR, quantitative real-time polymerase chain reaction; RAD9A, RAD9 homolog A; RHC, recurrent hepatitis C; RT-PCR, real-time polymerase chain reaction; STAT, signal transducer and activator of transcription; STK4, serine/threonine kinase 4; T-Bil, total bilirubin.

Address reprint requests to Shigeru Marubashi, M.D., Ph.D., Department of Surgery, Graduate School of Medicine, Osaka University, 2-2, Yamadaoka E-2, Suita 565-0871, Osaka, Japan. Telephone: +81-6-6879-3251; FAX: +81-6-6879-3259; E-mail: maru@surg2.med.osaka-u.ac.jp

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