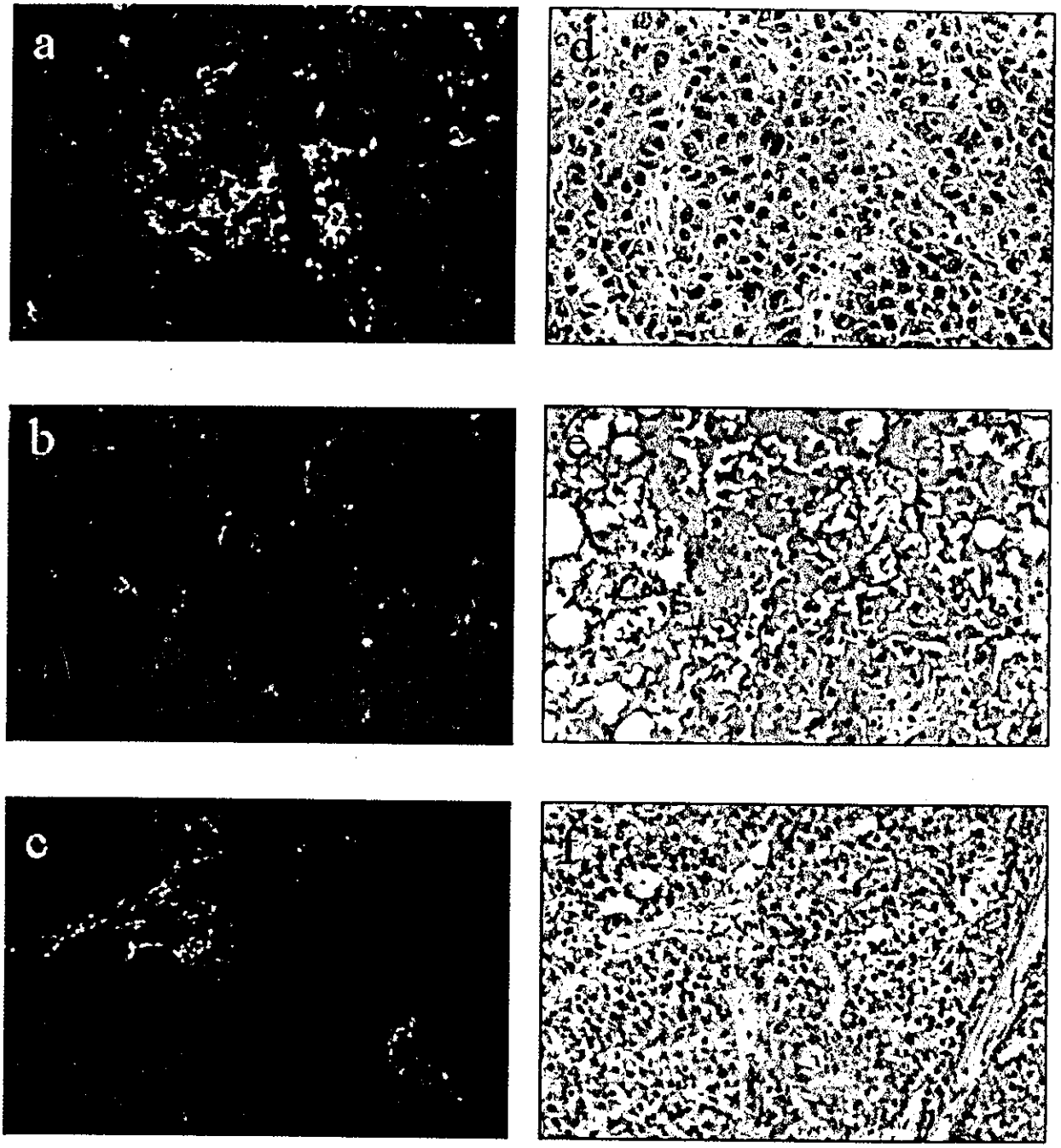


FIGURE 4 – Immunofluorescence staining for HLA-DR protein in representative cases. (*a*) A case (HCV10T) with score 2 in which more than 30% of tumor cells are stained. (*b*) A case (HCV45T) with score 1 in which about 10% of tumor cells are stained. (*c*) A case (HCV20T) with score 0. Note that some populations of stromal cells are stained, but none of the tumor cells are stained. All 3 tumors were judged as moderately differentiated HCC. (*d–f*) H&E staining of tumors corresponding to HCV10T, HCV45T and HCV20T, respectively (object lens: ×20).

FIGURE 5 – Levels of HLA-DR protein and *HLA-DRA* mRNA in HCCs. (*a*) Levels of HLA-DR protein and *HLA-DRA* mRNA in individual tumors. Note that there was an association between DNA microarray data and quantitative RT-PCR data in 30 samples ($r = 0.625$ and $p = 0.0001$). (*b*) DNA microarray data showing association between levels of HLA-DR protein and *HLA-DRA* mRNA in tumors ($p < 0.0001$ by ANOVA). (*c*) Quantitative RT-PCR showing association between levels of HLA-DR protein and *HLA-DRA* mRNA in tumors ($p = 0.0001$ by ANOVA). Score 0, less than 5% of tumor cells were stained; Score 1, 5–30% of tumor cells were stained; Score 2, more than 30% of tumor cells were stained. The RT-PCR result was based on mean of duplicated experiments in selected 30 samples.

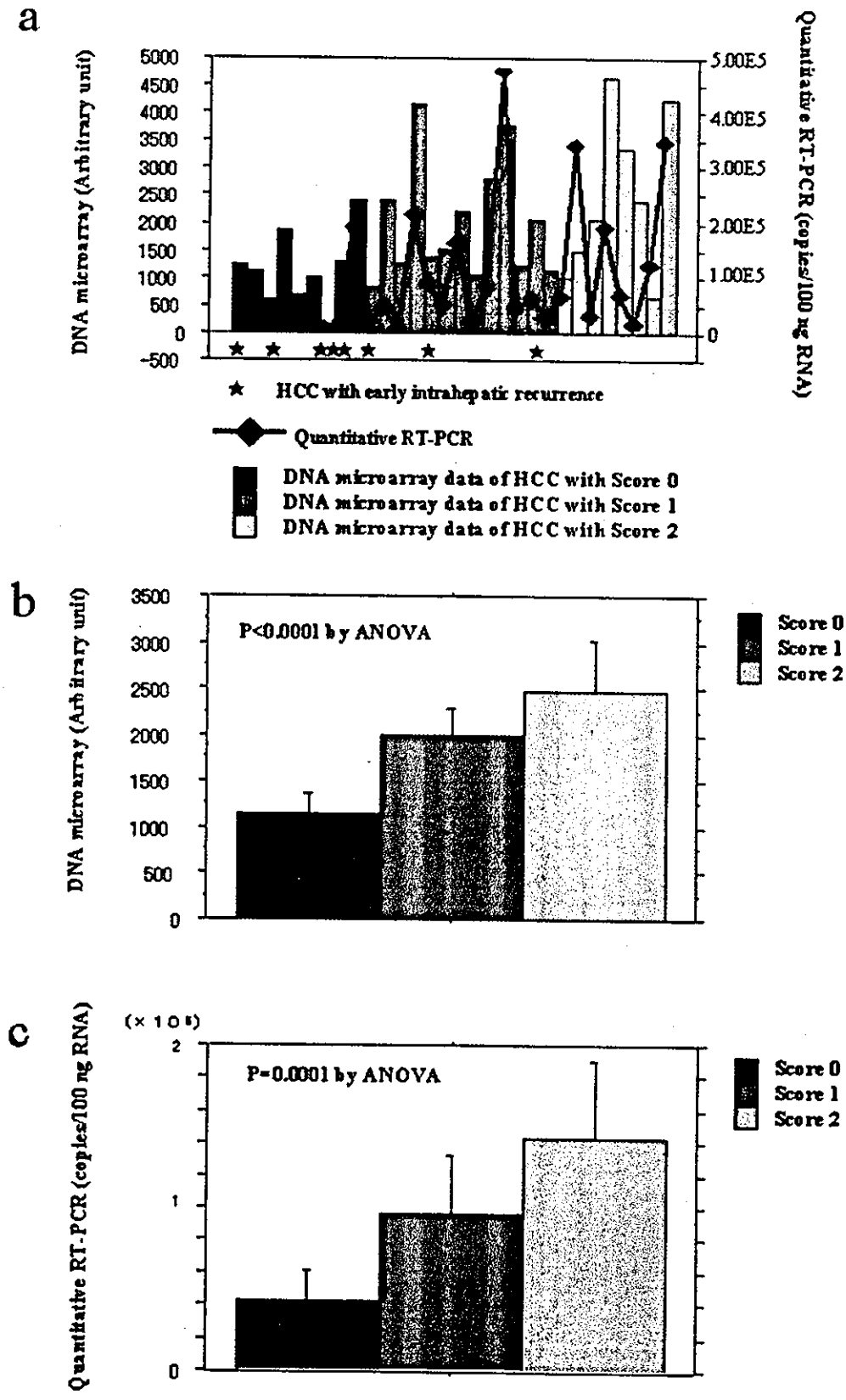
a
DNA microarray (Arbitrary unit)
Quantitative RT-PCR (copies/100 ng RNA)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
-500
5.00E5
4.00E5
3.00E5
2.00E5
1.00E5
0
HCC with early intrahepatic recurrence
Quantitative RT-PCR
DNA microarray data of HCC with Score 0
DNA microarray data of HCC with Score 1
DNA microarray data of HCC with Score 2
b
DNA microarray (Arbitrary unit)
3500
3000
2500
2000
1500
1000
500
0
P<0.0001 by ANOVA
Score 0
Score 1
Score 2
c
Quantitative RT-PCR (copies/100 ng RNA)
(× 10⁵)
2
1
0
P=0.0001 by ANOVA
Score 0
Score 1
Score 2

FIGURE 5.

TABLE II – RELATIONS BETWEEN TUMOR HLA-DR PROTEIN LEVELS AND CLINICOPATHOLOGIC FACTORS

| Clinicopathologic factors | Score of HLA-DR protein staining | | | *p* value |
|---|---|---|---|---|
| | 0 | 1 | 2 | |
| Sex | | | | N.S. |
| Male | 13 | 16 | 8 | |
| Female | 6 | 3 | 4 | |
| Age (year) | 61.9+/−2.4 | 63.5+/−1.2 | 64.0+/−2.3 | N.S. |
| Tumor size (cm) | 5.5+/−0.7* | 3.2+/−0.4* | 4.6+/−1.1 | *$p = 0.015$ |
| Tumor differentiation | | | | N.S. |
| Well | 0 | 2 | 0 | |
| Moderately | 17 | 12 | 11 | |
| Poorly | 2 | 5 | 1 | |
| Venous invasion | | | | N.S. |
| (−) | 13 | 15 | 10 | |
| (+) | 6 | 4 | 2 | |
| pTNM stage of UICC | | | | **$p = 0.098$ |
| I | 5 | 11 | 8 | |
| II | 10 | 7 | 2 | |
| IIIA | 4 | 1 | 2 | |

*ANOVA with Fisher's PLSD test.–**Fisher's exact test, N.S., not significant.

TABLE III – FACTORS RELATED TO EARLY INTRAHEPATIC RECURRENCE

| Factor | Early intrahepatic recurrence | | *p* value |
|---|---|---|---|
| | (+) (n = 17) | (−) (n = 33) | |
| Sex | | | *$p = 0.334$ |
| Male | 14 | 23 | |
| Female | 3 | 10 | |
| Age (year) | 60.2+/−2.5 | 64.5+/−1.1 | **$p = 0.120$ |
| Tumor size (cm) | 5.4+/−0.9 | 3.9+/−0.4 | **$p = 0.167$ |
| Tumor differentiation | | | *$p = 0.485$ |
| Well | 0 | 2 | |
| Moderate | 13 | 27 | |
| Poor | 4 | 4 | |
| Venous invasion | | | *$p = 0.012$ |
| (−) | 9 | 29 | |
| (+) | 8 | 4 | |
| pTNM stage of UICC | | | *$p = 0.038$ |
| I | 4 | 20 | |
| II | 9 | 10 | |
| IIIA | 4 | 3 | |
| HLA-DR protein staining | | | *$p = 0.013$ |
| score 0 | 11 | 8 | |
| score 1 | 5 | 14 | |
| score 2 | 1 | 11 | |

*Fisher's exact test.–**Mann-Whitney *U* test.

($p = 0.015$ by ANOVA with Fisher's PLSD test) (Table II). Low levels of tumor HLA-DR protein tended to be associated with advanced tumor stage ($p = 0.098$ by Fisher's exact test) (Table II). There were no associations between tumor HLA-DR protein levels and other clinicopathologic factors.

Univariate analysis showed that tumor HLA-DR protein levels, pTNM stage and venous invasion were associated with early IHR ($p = 0.013$, $p = 0038$ and $p = 0.012$) (Table III). There were no associations between early IHR and other clinicopathologic factors. Multivariate analysis showed that tumor HLA-DR protein levels and venous invasion were independent risk factors for early IHR (Table IV).

## Discussion

We herein present the molecular signature linked to early IHR of HCC by applying a supervised learning method to DNA microarray technology. HCC patients have various backgrounds and divergent clinical courses, resulting in much heterogeneity among tumor samples examined.[8,17] To address this heterogeneity among patients, we applied the Fisher ratio and the random permutation test to DNA microarray data.[7–10] Thus, the 46 genes selected as described above represent the molecular signature specific to IHR in a larger number of HCC cases. In particular, when we compared the present data with our previous microarray data,[7,8,10] we found that there was no overlap between the 46 genes and virus- and liver cirrhosis-related genes. This means that the 46 genes are potential biomarkers and/or molecular targets for detection or treatment of early IHR in HCC and that the potential is independent of viral and nontumorous factors.

The 46 genes included 10 immune system-related genes, all of which were downregulated in HCCs with early IHR. This is one of the most striking findings of our present study. Several investigators including ourselves have proposed the clinical efficacy of immune therapy against HCC.[18–20] Therefore, our present result suggests that downregulation of host immune response plays a central role in early IHR of HCC. Four genes (*HLA-DRA*, *HLA-DRB1*, *HLA-DG* and *HLA-DQA*) of the same family of HLA class II antigens were coordinately downregulated in HCCs with early IHR in comparison to those with nonrecurrence. Their downregulation in HCC with early IHR was observed not only in training samples but also in blinded samples, indicating their predictive value for early IHR.

We focused our investigation on *HLA-DRA* of the 4 MHC-class II genes and showed for the first time that HLA-DR protein plays an important role in early IHR of HCCs. Several studies have

TABLE IV – INDEPENDENT RISK FACTORS FOR EARLY INTRAHEPATIC RECURRENCE

| Variable | Regression coefficient | Standard error | Risk ratio (95%CI) | $p$ value |
|---|---|---|---|---|
| Venous invasion | | | | |
| (+) | 2.981 | 1.169 | 19.699 (1.922–194.803) | $p = 0.011$ |
| HLA-DR protein expression | | | | |
| Score 1 | −1.704 | 0.811 | 0.182 (0.037–0.893) | $p = 0.036$ |
| Score 2 | −3.689 | 1.558 | 0.025 (0.001–0.530) | $p = 0.018$ |
| Sex | | | | |
| Male | 2.459 | 1.277 | 11.694 (0.957–142.848) | $p = 0.054$ |

shown that HLA-DR protein can be expressed by HCC cells; however, its relation to metastatic potential has not been discussed.[21,22] HLA-DR protein is involved in the antigen presenting function of macrophages including dendritic cells.[22] Activated lymphocytes also express the HLA-DR antigen.[23] Previous studies revealed the infiltration of HLA-DR-positive immune cells into HCC tissues following adoptive immune therapy.[24] Improved prognosis of HCC following infiltration by $CD8^+$ and $CD4^+$ T lymphocytes was found by Wada *et al.*[25] Because we did not perform laser capture microdissection, it is possible that the cancer samples tested contained stromal cells. In our previous microarray study,[5] we found that *vimentin* was preferentially produced by stromal cells in cancer tissues, and its downregulation was associated with early IHR of HCC. Taken together, these reported findings suggest that HLA-DR protein can be expressed in both tumor cells and stromal cells in HCC tissues. To identify the major site of HLA-DR protein biosynthesis, we carried out immunofluorescence staining of fresh tumor samples. Our present results showed that the HLA-DR protein was preferentially located in the cytoplasm of tumor cells, but not in that of stromal cells, and HLA-DR protein levels in cancer cells were consistent with *HLA-DRA* mRNA levels determined by quantitative RT-PCR and DNA microarray analysis. These results indicate that major sources of *HLA-DRA* mRNA and HLA-DR protein in tumor tissues are HCC cells themselves.

Multivariate analysis showed that low expression of HLA-DR protein in tumor is an independent risk factor for early IHR, suggesting its potential as a predictive marker for HCC with high metastatic potential. However, it remains unclear how tumor HLA-DR protein is related to intrahepatic metastasis of HCC. It is reasonable to assume the immunologic role considering that many immune-response related genes were downregulated in HCCs with early IHR as well as *HLA-DRA* and that no recurrence was found in 25 of 31 HCCs with HLA-DR overexpression (score 1 or 2). Many studies have showed that MHC-class II antigens play a role in progression of malignant tumors *via* immunologic modification.[26–31] Our current results were consistent with their results. In this regard, our finding is not a new one. However, in our study, it is noteworthy that the MHC-class II genes were selected without any bias from thousands of genes for association with early IHR in HCC. Interestingly, a microarray study by Ramaswamy *et al.*[32] showed similar finding that the decreased levels of *HLA-DP beta1* encoding 1 MHC class II antigen at the metastatic sites were commonly observed in a variety of adenocarcinomas. Recently, Herkel *et al.*[33] reported that MHC Class II-expressing hepatocytes can function as antigen-presenting cells and activate specific CD4 T lymphocytes. Epigenetic inactivation of the transcriptional activator for MHC class II genes is associated with the loss of HLA-DR expression in tumor cells.[34] Sartoris *et al.*[35] showed increased expression of HLA-DR in HCC cell lines by transfection of the transcriptional activator for MHC class II genes and found that the transfectant can serve to activate HLA-DR-restricted T cell line. In conjunction with these reports, our present results suggest immunologic roles of HLA-DR protein in early IHR of HCC. However, it should be noted that the HLA-DR protein was exclusively localized in the cytoplasm rather than on the cell membrane, raising the possibility that tumor HLA-DR has functions other than antigen-presenting function.[36] Further studies are needed to elucidate the biological function in early IHR of HCC.

In conclusion, our study shows that HLA-DR protein produced by tumor cells can be useful as a predictive marker for early IHR of HCC. Because the prognosis of HCC is extremely poor even when curative surgery is performed,[4,5] HLA-DR protein may also function as a molecular target to improve the poor prognosis.

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*Proteomics* 2005, *5*, 0000–0000 DOI 10.1002/pmic.200401022



REGULAR ARTICLE

# Overexpression of alpha enolase in hepatitis C virus-related hepatocellular carcinoma: Association with tumor progression as determined by proteomic analysis

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To identify proteins that could be molecular targets for diagnosis and treatment of hepatitis C virus-related hepatocellular carcinoma (HCV-related HCC), we used a proteomic approach to analyze protein expression in samples of human liver. Twenty-six pairs of tumorous and corresponding nontumorous liver samples from patients with HCV-related HCC and six normal liver samples were analyzed by two-dimensional gel electrophoresis and liquid chromatography-tandem mass spectrometry. One of the numerous spots that showed stronger intensity in tumorous than in nontumorous samples was identified as alpha enolase, a key enzyme in the glycolytic pathway. Expression of this protein increased with tumor dedifferentiation and was significantly higher in poorly differentiated HCC than in well-differentiated HCC. This pattern was reproduced by immunoblot analysis and immunohistochemistry. Expression of alpha enolase also correlated positively with tumor size and venous invasion. These results suggest that alpha enolase is one of the candidates for biomarkers for tumor progression that deserves further investigation in HCV-related HCC.

Received: July 5, 2004
Revised: August 6, 2004
Accepted: September 10, 2004

**Keywords:**
Alpha enolase / Hepatitis C virus / Hepatocellular carcinoma

## 1 Introduction

Hepatocellular carcinoma (HCC) is one of the most fatal cancers worldwide, with a high incidence in many countries [1]. Infection by hepatitis virus, alcoholic liver dysfunction, autoimmune hepatitis, and exposure to aflatoxin B1 are some recognized causes of HCC. Hepatitis B virus (HBV) and hepatitis C virus (HCV) are the most clearly established risk factors for HCC. Although the properties of these two viruses differ, damage to hepatocytes by chronic viral infection is believed to induce HCC [2, 3]. Persistent infection with hepatitis virus leads to chronic hepatitis (CH) and then to liver cirrhosis (LC). Development of HCC is a multistep process based on histologic changes. HCC tumors are initially well-differentiated (G1 tumor), but with time, they dedifferentiate to moderately (G2 tumor) or poorly (G3 tumor) differentiated types with high proliferation rates. The pathogenesis of HCC is not entirely clear. Molecular biological studies have been helpful in the effort to elucidate this process.

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**Abbreviations: AFP**, alpha-fetoprotein; **HCC**, hepatocellular carcinoma; **HCV**, hepatitis C virus; **LC**, liver cirrhosis; **NL**, normal liver

The combination of 2-DE and MS is a proteomic method of high-throughput analysis of protein expression. Several proteomic studies have identified diverse proteins that may be involved in the pathogenic mechanism of HCC [3–12]. In the present study, we analyzed protein expression in 26 pairs of HCV-related HCC samples and corresponding nontumorous liver samples and in six samples normal liver (NL) to find proteins that might be involved in tumor differentiation and progression.

## 2 Materials and methods

### 2.1 Tumor samples

Twenty-six pairs of HCC samples and corresponding nontumorous liver samples were obtained from patients diagnosed with HCC who underwent surgical liver resection at the Department of Surgery II, Yamaguchi University Hospital. Following resection, samples were frozen immediately and stored at −80°C until use. Written informed consent was obtained from all patients before surgery. None of the patients received any preoperative therapy such as chemotherapy, percutaneous ethanol injection, or transcatheter arterial embolization. The study protocol was approved by the Institutional Review Board for Human Use of the Yamaguchi University School of Medicine. Histologic diagnosis of HCC was made on formalin-fixed, paraffin-embedded sections according to World Health Organization criteria. The HCC samples consisted of six G1, 15 G2, and five G3 tumors. Nontumorous samples consisted of nine CH and 17 LC. Serologically, all patients were positive for HCV antibody and negative for HBs antigen. Clinical characteristics of the patients are shown in Table 1.

### 2.2 Control liver samples

Normal liver samples were obtained from six patients who underwent hepatic resection for benign liver tumors or metastatic liver tumors due to gastrointestinal cancer. All were negative for both hepatitis B surface antigen and HCV antibody, and the tissues were histopathologically normal.

### 2.3 Sample preparation

Sample preparation was performed essentially as described previously [9, 12]. Liver samples were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, 165 mM sodium chloride, 10 mM sodium fluoride, 1 mM sodium vanadate, 1 mM PMSF, 10 mM EDTA, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1% NP-40). Suspensions were incubated for 2 h at 4°C, centrifuged at 15 000 × *g* for 30 min at 4°C, and the supernatants were stored at −80°C until use.

**Table 1.** Clinical characteristics of 26 HCC samples

| Differentiation of tumor[a)] | G1 | G2 | G3 |
|---|---|---|---|
| Number | 6 | 15 | 5 |
| Gender male/female | 3/3 | 10/5 | 3/2 |
| Age (mean) | 58–74 (69.3) | 50–76 (66.1) | 39–78 (64.2) |
| Histology of nontumorous lesion | | | |
| CH | 2 | 7 | 0 |
| LC | 4 | 8 | 5 |
| Tumor size (cm) (mean) | 1.2–3.2 (2.15) | 1.5–13.0 (5.47) | 3.4–12.0 (7.28) |
| Incidence of Serum AFP >20 ng/mL | 3/6 | 10/15 | 3/5 |
| Incidence of serum PIVKA-II > 40 U/mL | 2/6 | 12/15 | 4/5 |
| Venous invasion no/yes | 6/0 | 8/7 | 1/4 |

a) Assessment based on classification of malignant tumors by the International Union against cancer. G1, G2, and G3 indicate well-, moderately-, and poorly-differentiated HCC, respectively.

AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonist-II.

### 2.4 2-DE and SDS-PAGE

Three hundred micrograms of protein was used for each 2-DE assay. The first-dimensional IEF was performed on 7 cm, immobilized, pH 3–10 linear gradient strips (Amersham Biosciences, Uppsala, Sweden) at 20°C and 50 μA/strip. The strips were rehydrated with 125 μL of sample solution (8 M urea, 2% CHAPS, 0.01% bromophenol blue, 0.56% 2-mercaptoethanol, and 0.5% IPG buffer) for 14 h. IEF was run in three steps: 500 V for 1 h, 1000 V for 1 h, and 8000 V for 3 h. Voltage increases were carried out in a gradient. The second-dimensional run was performed on precast polyacrylamide gels (2-D homogeneous 12.5; Amersham Biosciences) in two steps: 600 V, 20 mA for 30 min and 600 V, 50 mA for 70 min. After electrophoresis, the gels were stained with CBB R-250 (Nacalai Tesque, Kyoto, Japan) for 24 h. Gels were destained with 10% acetic acid in water containing 30% methanol for 30 min and then destained with 7% acetic acid and used for in-gel digestion. Fifty micrograms of protein was used for each SDS-PAGE sample. Sample lysates were mixed with 4 μL of 5 × SDS sample buffer. Samples were denatured for 5 min at 95°C and separated on 10% SDS-PAGE gels. Twenty microliters of lysate was applied to each lane. Electrophoresis was carried out at 15 mA/gel.

### 2.5 In-gel digestion

CBB dye was removed by rinsing three times in 60% methanol, 50 mM ammonium bicarbonate, and 5 mM DTT for 15 min and twice in 50% ACN, 50 mM ammonium bicarbo-

nate, and 5 mM DTT for 10 min. The gel piece was dehydrated three times in 100% ACN for 30 min and then rehydrated with an in-gel digestion reagent containing 10 μg/mL sequencing-grade modified trypsin (Promega, Madison, WI, USA) in 30% ACN, 50 mM ammonium bicarbonate, and 5 mM DTT. The in-gel digestion was performed overnight at 30°C. The samples were rinsed in 30% ACN, 50 mM ammonium bicarbonate, and 5 mM DTT for 2 h and lyophilized overnight at −30°C.

### 2.6 Amino acid sequencing by LC-MS/MS

Lyophilized samples were dissolved in 20 μL of 0.1% formic acid and centrifuged at 15 000 × *g* for 5 min. Peptide sequencing of identified protein spots was performed by LC-MS/MS with a Spectrum Mill MS Proteomics Workbench (Agilent Technologies, Santa Clara, CA, USA).

### 2.7 Immunoblot analysis

After SDS-PAGE, fractionated proteins were transferred electrophoretically onto PVDF membranes (Immobilon-P; Millipore, Bedford, MA, USA), and the membranes were blocked overnight at 4°C in TBS containing 5% skim milk. The primary antibody was an anti-enolase polyclonal antibody (1:100, #sc-7455; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were incubated for 1 h at 4°C, washed four times with TBS containing 0.05% Tween 20, and incubated for 1 h at 4°C with horseradish peroxidase-conjugated secondary antibody (1:2000, #55360; ICN Pharmaceuticals, Aurora, OH, USA). The reaction was visualized with a chemiluminescence reagent (ECL; Amersham Biosciences).

### 2.8 Immunohistochemistry

Formalin-fixed, paraffin-embedded samples were sectioned at 4 μm thickness. Sections were treated with protease for antigen retrieval and then stained with anti-enolase polyclonal antibody (1:500, #sc-15343; Santa Cruz Biotechnology). Avidin-biotin/3,3'-diaminobenzidine (Ventana Medical Systems, Tucson, AZ, USA) was used for detection, and hematoxylin was used for counterstaining. All procedures were performed automatically with BenchMark IHC/ISH Staining Module (Ventana Medical Systems).

### 2.9 Statistical analysis

Expression levels of proteins on 2-DE gels were quantified by analyzing the intensity of each spot with ImageMaster 2D Platinum (v5.0 software; Amersham Biosciences). Differences in expression levels between tumorous samples and corresponding non-tumorous samples, and between samples with and without venous invasion were analyzed by Student's *t*-test. Analysis of alpha enolase level in all the histologic groups was performed by ANOVA with Bonferroni-Dunn test. Correlation of alpha enolase expression with tumor size, serum alpha-fetoprotein (AFP) levels, and serum protein induced by vitamin K absence or antagonist-II (PIVKA-II) levels was determined by Pearson correlation coefficients. $p < 0.05$ was considered significant in Student's *t*-test and Pearson correlation coefficients, and $p < 0.0033$ in ANOVA with Bonferroni-Dunn test.

## 3 Results

### 3.1 Identification of proteins on 2-DE gels

Protein expression was assessed in all tumorous, nontumorous, and NL samples. 2-DE was executed three times for each sample and almost same results were obtained. Hundreds of protein spots were visualized on 2-DE gels, and differences in spot intensities were compared visually and with a computerized approach for each gel. A spot of approximately 48 kDa in mass and a p*I* of 7.0 showed stronger intensity in G3 tumors than in nontumorous samples, G1 tumors, or G2 tumors (Figs. 1, 2). We thought that this protein might be involved in tumor differentiation. The spot was digested and used for MS analysis. This sample provided a good spectrum of amino acid sequences by LC-MS/MS and the protein was identified as alpha enolase, an isoenzyme of enolase, which is a key protein in the glycolytic pathway. The result of LC-MS/MS for the spot is shown in Table 2.

### 3.2 Alpha enolase expression in HCC

Immunoblot analysis for alpha enolase was performed on each differentiated HCC sample, corresponding nontumorous sample, and NL samples. Repeated analyses showed almost the same results. A slightly increased intensity of the band was identified in G2 tumors, and even more increased intensity was identified in G3 tumors. The intensity was equivalently weak in G1 tumor, nontumorous, and NL samples (Fig. 3). Immunohistochemical examination showed rare staining of cells in NL, in the presence of CH or LC, and in G1 tumors (Figs. 4A–D). Moderate, heterogeneous staining was identified in G2 tumors (Fig. 4E), and strong staining was identified in G3 tumors (Fig. 4F).

### 3.3 Alpha enolase expression and tumor differentiation

Spot intensity in 2-DE gels was quantified, and the expression level of alpha enolase was analyzed statistically. Spots showing differences in expression levels greater than two-fold between tumorous and nontumorous samples were considered upregulated. Upregulation of alpha enolase in each differentiated HCC (G1, G2, and G3 tumors) occurred in 1/6 (17%), 4/15 (27%), and 4/5 (80%) samples, respectively. The numerical values of expression levels were shown in Table 3. G3 tumor samples showed significant upregula-

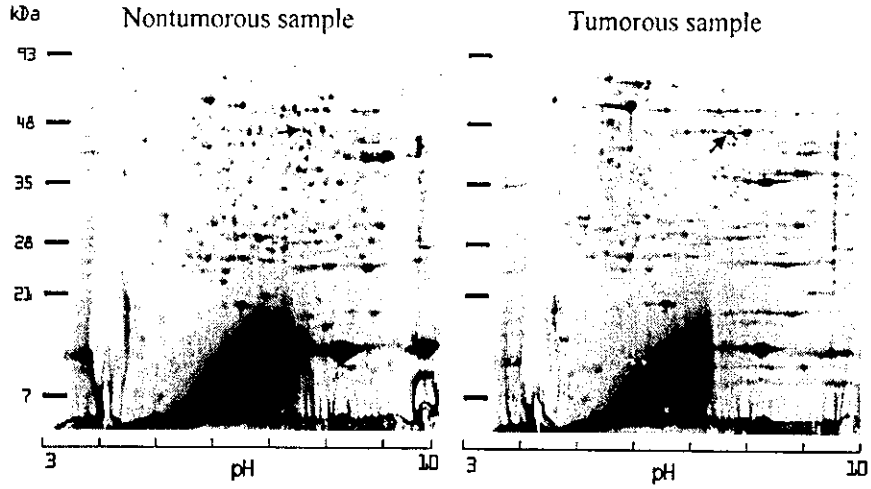


**Figure 1.** 2-DE pattern of HCV-related HCC sample and corresponding nontumorous sample. Proteins were prepared as described in the Section 2 and were separated on pH 3–10 linear IPG strips and then by 12.5% SDS-PAGE. Gels were stained with CBB R-250. A spot of approximately 48 kDa in mass and a p*I* of 7.0 showed strong intensity in tumorous samples compared to nontumorous samples (arrow).

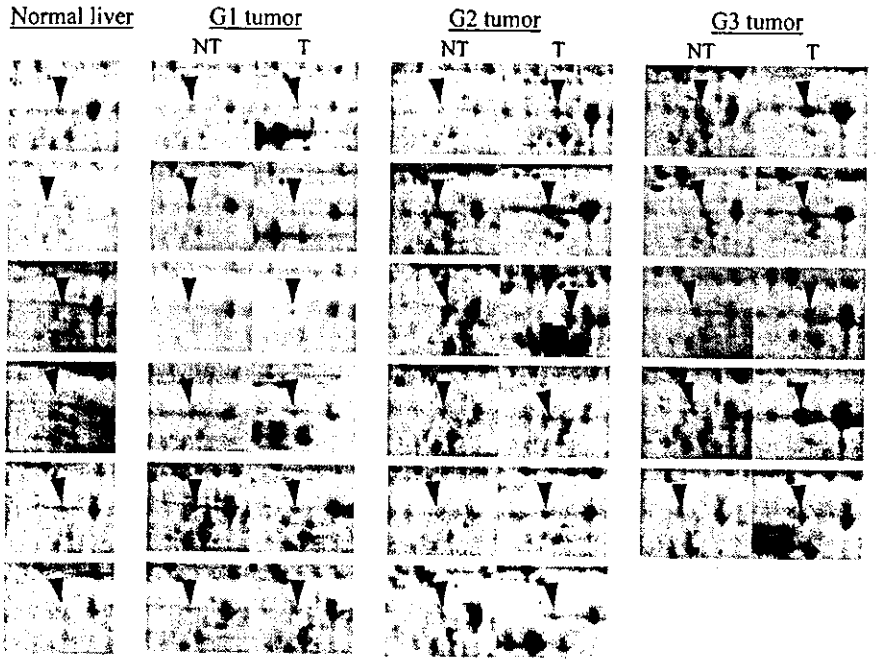


**Figure 2.** Differences in expression levels of the spot indicated in Fig. 1. Expression levels were greater in tumorous samples of G3 tumors than in other groups. In NL samples, this spot showed low expression, approximately equivalent to that in nontumorous samples. NT, corresponding non-tumorous sample; T, tumorous sample; G1, G2, and G3 tumor indicate well-, moderately-, and poorly differentiated HCC, respectively.

tion when compared with that in corresponding nontumorous samples ($p < 0.05$). In all histologic groups, expression levels of alpha enolase in G3 tumors were significantly upregulated compared to the level in G1 tumors. Alpha enolase in G2 tumors showed a tendency toward upregulation compared to that in G1 tumors ($p = 0.0289$). Alpha enolase in G3 tumors showed a tendency toward upregulation compared to that in G2 tumors ($p = 0.0057$), but the differences were not statistically significant. There was no significant difference in expression of alpha enolase between NL, CH, LC, and G1 tumor samples (Fig. 5).

### 3.4 Correlation with clinical characteristics

Correlation of alpha enolase expression levels with tumor size, serum AFP levels, serum PIVKA-II levels, and venous invasion was analyzed. Upregulation of alpha enolase was significantly correlated with tumor size ($p < 0.05$, $R^2 = 0.290$) (Fig. 6A). To confirm the significance of the result, two proteins, heat shock 70 kDa protein 1 known to be upregulated in HCC and β-actin known to express constitutively were also assessed. However, none of the two showed significant correlation with tumor size ($R^2 = 0.13$ and 0.002). Patients with venous invasion expressed higher

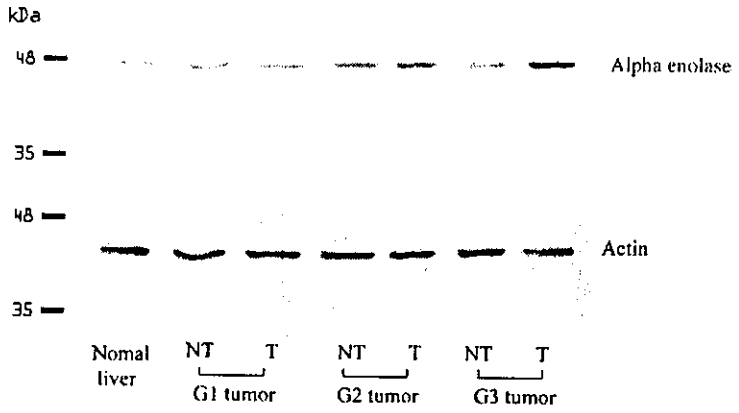


**Figure 3.** Immunoblot analysis of alpha enolase in NL and in each differentiated HCC. Intensity of the bands was slightly increased in G2 tumor and was further increased in tumorous sample of G3 tumor. The bands of actin are shown as a control. NT, corresponding nontumorous sample; T, tumorous sample; G1, G2, and G3 tumor indicate well-, moderately-, and poorly differentiated HCC, respectively.

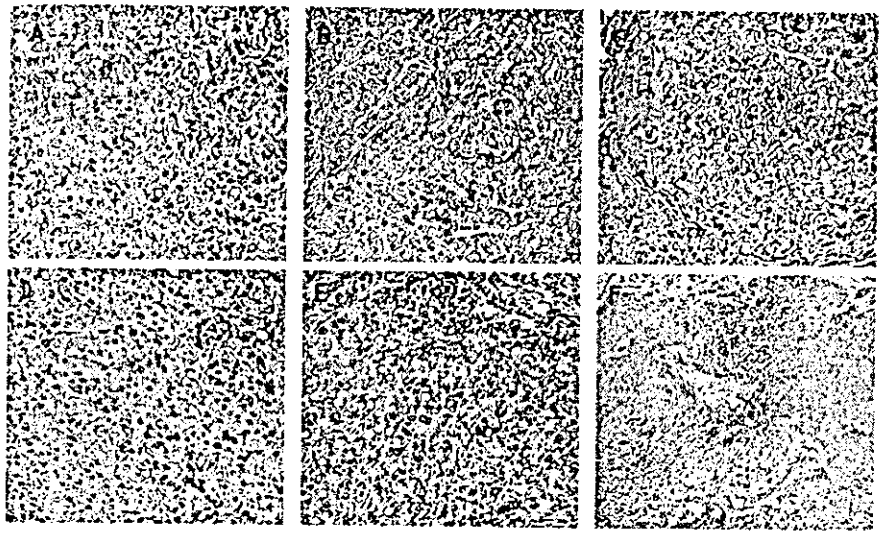


**Figure 4.** Immunohistochemical evaluation of alpha enolase. NL (A), CH (B), LC (C), and G1 tumor (D) samples showed rare positively stained cells. Moderate, heterogeneous staining was identified in G2 tumor (E) and strong staining was identified in G3 tumor (F). Original magnification, ×40.

levels of alpha enolase than patients without venous invasion (Fig. 6B). No significant correlation was observed between alpha enolase expression and serum AFP or serum PIVKA-II expression.

## 4 Discussion

The aim of this study was to identify proteins that could be molecular targets for diagnosis and treatment of HCV-related HCC. Our 2-DE and LC-MS/MS proteomic analyses of 26 HCC samples revealed that alpha enolase expression was upregulated in G3 tumors compared with that in G1 and G2 tumors, and expression levels were positively correlated with tumor size and venous invasion.

Alpha enolase is an isoenzyme of enolase, a key protein that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway. Three enolase isoenzymes have been identified. The alpha form is present in most tissues and embryonically, the beta form is expressed in muscle tissues, and the gamma form, also known as neuron-specific enolase (NSE), is found only in neuronal tissues. Alpha enolase has been detected not only in the cytoplasm but also at the membrane surface. Although the mechanism of surface expression and the orientation on the membrane are not well understood, it is known that the C-terminal amino acid, lysine, is exposed at the cell surface and is involved in binding to plasminogen, which is then activated and converted to plasmin [13, 14]. Plasmin, stabilized at the cell surface, induces fibrinolysis [15, 16].

In cancer cells, the rate-determining enzyme for glycolysis is converted to an isoenzyme different from that in normal cells and the capability of glycolysis is increased because of increased cell proliferation. Overexpression of alpha enolase, the embryonal form, could be part of this mechanism. In response to upregulated alpha enolase expression, the fibrinolytic system is inordinately accelerated. Consequently, increased local fibrinolysis may contribute to cancer cell invasion and metastasis. This is consistent with our results showing upregulated alpha enolase expression in

**Table 2.** The result of LC-MS/MS for the spot

| Start-end residues | $MH^+$ calculated (Da) | *m/z* measured (Da) | *z* | Sequence |
|---|---|---|---|---|
| 9–27 | 2154.072 | 719.09 | 3 | EIFDSRG NPTVEVDL FTSK |
| 64–79 | 1690.986 | 846.27 | 2 | AVEHINKT IAPALVSK |
| 64–79 | 1690.986 | 564.68 | 3 | AVEHINKT IAPALVSK |
| 120–131 | 1259.711 | 630.89 | 2 | AGAVEK GVPLYR |
| 132–161 | 3011.570 | 1004.98 | 3 | HIADLA GNSEVILPV PAFNVING GSHAGNK |
| 183–196 | 1597.907 | 533.62 | 3 | IGAEVYH NLKNVIK |
| 406–419 | 1691.897 | 846.93 | 2 | YNQLLRIE EELGSK |
| 406–419 | 1691.897 | 565.02 | 3 | YNQLLRIE EELGSK |
| 412–421 | 1103.595 | 552.49 | 2 | IEEELGSKAK |
| 426–433 | 959.543 | 480.44 | 2 | NFRNPLAK |

The matched peptides cover 26% of the protein.

**Table 3.** Expression of alpha enolase in each differentiated HCC

| | NT | | T | | *p*-value | Occurrence of upregulation |
|---|---|---|---|---|---|---|
| G1[a] | 0.053 | 0.014 | 0.073 | 0.021 | N.S. | 1/6 (17%) |
| G2[b] | 0.108 | 0.020 | 0.169 | 0.030 | N.S. | 4/15 (27%) |
| G3[c] | 0.118 | 0.028 | 0.302 | 0.066 | $p < 0.05$ | 4/5 (80%) |

Values are shown as mean ± SD (arbitrary unit).
a), b), c) G1, G2, and G3 indicate well-, moderately-, and poorly-differentiated HCC, respectively.
NT, Nontumorous samples; T, tumorous samples; N.S., Not significant.
Expression levels of proteins on 2-DE gels were quantified by analyzing the intensity of each spot with ImageMaster 2-D Platinum. Spots showing differences in expression levels greater than two-fold between tumorous and nontumorous samples were considered upregulated. Differences in expression levels between tumorous samples and corresponding nontumorous samples were analyzed by Student's *t*-test.

patients with large tumors. Alpha enolase has also been described as a stress protein induced by hypoxia [17]. In large tumors, oxygen is relatively decreased in the central region. Thus, it is reasonable that alpha enolase is upregulated in large tumors.

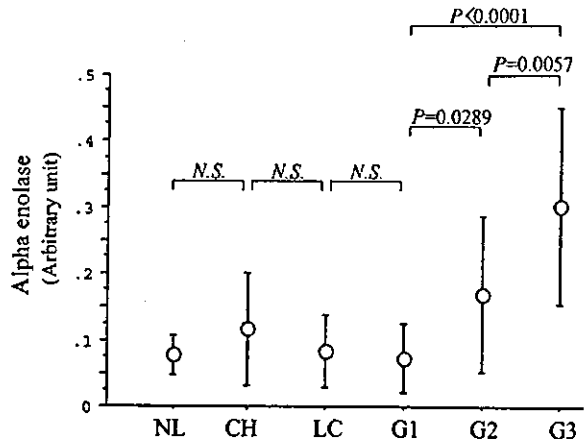


**Figure 5.** Quantitative analysis performed by ANOVA with Bonferroni-Dunn test. $p < 0.0033$ was considered significant. Comparing all histologic groups, expression of alpha enolase in G3 tumors was significantly upregulated compared to that in G1 tumors. Alpha enolase in G2 tumors showed a tendency towards upregulation, compared to that in G1 tumors ($p = 0.0289$), and alpha enolase in G3 tumors showed a tendency towards upregulation compared to that in G2 tumors ($p = 0.0057$). However, these differences were not statistically significant. There was no significant difference among NL, CH, LC, and G1 tumor samples. NL, normal liver; CH, chronic hepatitis; LC, liver cirrhosis; G1, G2, and G3 indicate well-, moderately-, and poorly differentiated HCC, respectively.

Overexpression of alpha enolase has been reported in a cervical cancer cell line [18, 19] and a colon cancer cell line [20], in endometrial hyperplasia and adenocarcinoma [21], in breast cancer [22, 23], and in Alzheimer's disease [24]. It has also been reported in an HCC cell line [25]. However, there have been reports of downregulation of alpha enolase expression in non-small cell lung cancer [26] and of downregulation of alpha enolase expression in HBV-HCC and non-B, non-C HCC, albeit at a low frequency [10]. The investigators used samples of differentiation grades I and II of the Edmondson-Steiner grading system, which are comparable to G1 and G2 tumors; this might explain these differences. Additional studies are necessary to confirm expression differences in HBV-HCC.

Transcriptome analysis of HCV-related HCC samples showed significant upregulation of alpha enolase in G3 tumors ($p < 0.05$) (unpublished data). The expression level of the mRNA and that of the encoded protein do not necessarily correlate because of the effects of mRNA lability, post-translational modification, and degradation of proteins. In the case of alpha enolase, however, mRNA levels do correlate with protein levels in HCV-related HCC.

Molecular biological techniques have developed rapidly in recent years and will play essential roles in clarifying the pathogenesis of HCV-related HCC. The overexpression of alpha enolase in G3 tumors and the positive correlation with tumor size and venous invasion suggest that alpha enolase is

 

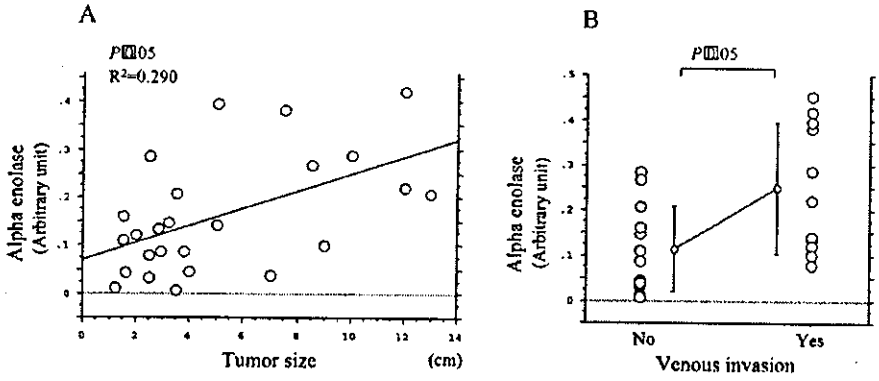


**Figure 6.** Relation between alpha enolase expression, tumor size, and venous invasion. (A) Upregulation of alpha enolase was significantly correlated with tumor size ($P < 0.05$, $R^2 = 0.290$). (B) Patients with venous invasion expressed high levels of alpha enolase compared to patients without venous invasion.

one of candidates for biomarkers for tumor progression that deserve further investigation and may constitute a target for gene and protein therapy in HCV-related HCC.

*This work was supported by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan (No. 15659315) and by the Venture Business Laboratory, Yamaguchi University.*

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# Tumor secreting high levels of IL-15 induces specific immunity to low immunogenic colon adenocarcinoma via CD8+ T cells

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Received March 31, 2004; Accepted May 24, 2004

**Abstract.** Although interleukin (IL)-15 augments innate and acquired immunities, IL-15 expression is controlled at the levels of transcription, translation and intracellular trafficking. We constructed plasmid vectors encoding the murine mature-IL-15 cDNA linked to an Igκ leader sequence and full-length murine IL-15 cDNA to evaluate the efficacy of the mature-IL-15 vector. Weakly immunogenic colon 26 cells were transfected with the above-mentioned vectors or with empty vector (mock). Transfectants with mature-IL-15 produced significantly higher levels of IL-15 than did transfectants with full-length IL-15. When injected into syngeneic BALB/c mice, transfectants secreting high levels of IL-15 were rejected completely. Depletion of natural killer cells or CD4+ T cells did not affect the growth of transfectants. In contrast, transfectants treated with anti-CD8 antibody re-grew 1 month later after implantation. These findings indicate that CD8+ T cells are required for complete rejection of the tumor. Gene therapy with transfectants expressing mature-IL-15 containing the Igκ leader sequence may be useful as a tumor vaccine.

## Introduction

Interleukin (IL)-15 is a 15-kDa cytokine (1,2) that uses the ß and γ chains of the IL-2 receptor (R) for signal transduction and shares biologic activities with IL-2. In particular, IL-15 promotes proliferation and activities of T, B and natural killer (NK) cells (3,4) and is a potent inducer of lymphokine-activated cytotoxic activity against tumor cells (5-7).

However, IL-15R contains a unique α chain that has a higher affinity and broader tissue distribution than that of the IL-2Rα chain (8,9). Indeed, there are several significant differences in the molecular and cellular features of IL-2 and IL-15 (10). IL-15 shows stronger mediation of NK and NKT cell differentiation and survival *in vivo* in comparison with IL-2 (11,12). Moreover, IL-15 can selectively promote proliferation and long-lasting survival of memory CD8+ T cells, in contrast to IL-2, which inhibits proliferation of CD8+ memory T cells (13-15). In addition, doses of IL-15 required to induced severe hypotension and pulmonary vascular leak syndrome were six times higher that those of IL-2, resulting in a higher therapeutic index for IL-15 (16). Such findings have led to a renewed clinical interest in IL-15.

Though IL-15 mRNA is expressed in a broad normal organization and tumor cells (1), it has been difficult to detect IL-15 protein in supernatants of many cells that express IL-15 mRNA (17). IL-15 expression is controlled at the levels of transcription, translation, and intracellular trafficking (18-20). Although there was a 4- to 5-fold increase in translation of IL-15 mRNA with the alternative short signal peptide in comparison with that of the wild-type 48-aa signal peptide (17,21,22), only IL-15 containing the 48-aa signal peptide is secreted (21). We previously reported that highly immunogenic tumor (Meth-A) cells transfected with IL-15 containing the 48-aa signal peptide can elicit an anti-tumor immune response (23). However, in a preliminary study we found that these anti-tumor effects were not present in the weakly immunogenic colon 26 cells.

In the present study, we constructed plasmid vectors encoding the murine mature-IL-15 cDNA linked to a high efficiency Igκ leader sequence and evaluated the efficacy of high IL-15-producing tumor. We report that high IL-15 production caused complete rejection of weakly immunogenic colon 26 cells and induced long-lasting CD8+ T cell-mediated specific anti-tumor immunity.

## Materials and methods

*Reverse transcription-PCR and primers.* RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) were carried out as described previously with some modifications (24). Briefly, cells ($5x10^6$) were lysed in 1 ml of TRIzol reagent (Life Technologies, Grand Island, NY), and total cellular RNA was isolated according to the manufacturer's instructions. One microliter of total RNA (1 μg) was added to 19 μl of RT-mixture (Takara, Ohtsu, Japan). After mixing, the samples were incubated at 30°C for 10 min, 55°C for 30 min, 95°C

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*Key words:* IL-15, CD8, signal peptide, gene therapy, cancer

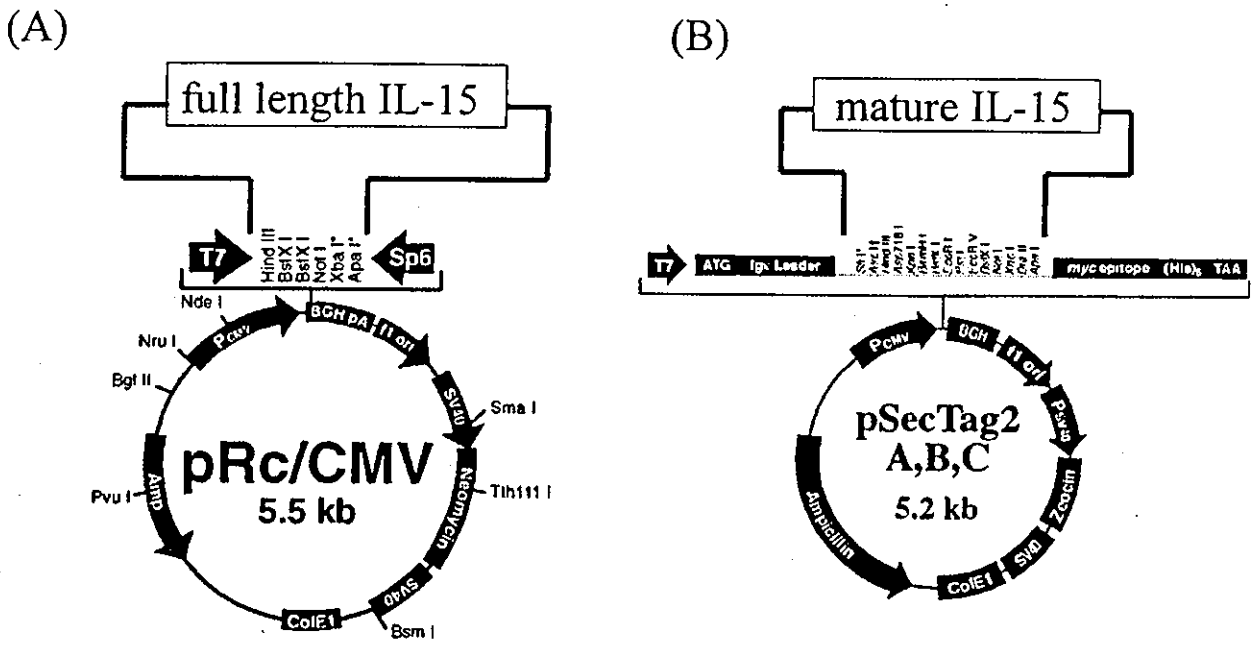


Figure 1. (A), Illustration of full-length IL-15 expression vector. (B), Illustration of mature-IL-15 expression vector.

for 5 min, and 4°C for at least 5 min. PCR-mixture (80 µl) (Takara) containing 100 nM primers was added to each RT reaction. PCR amplification consisted of 34 cycles of denaturation at 94°C for 1 min, annealing at 58 or 68°C for 1 min, and extension at 72°C for 2 min. The annealing temparature was 68°C for vector construction and 58°C for mRNA detection.

The primer sequences for murine full-length IL-15 vector construction were sense, 5'-AGCTGCGGCCGCATGAAAA TTTTGAAACCATAT-3' and antisense, 3' primer 5'-AGCT AGTCTAGATCAGGACGTGTTGATGAACAT-3'. Those for murine mature-IL-15 vector construction were sense, 5'-CCTCGTGAATTCGCCAACTGGATAGATGTAAGA-3' and antisense, 5'-TACACACTCGAGTCAGGACGTGTTGA TGAACAT-3'. Primers for detection of murine IL-15 mRNA were sense, 5'-TAGATATAAGATATGACCTGGA-3' and antisense, 5'-TGTTGAACATTTGGACAAT-3', and those for detection of murine ß-actin mRNA were sense, 5'-TCGA CAACGGCTCCGGCATGT-3', and antisense, 5'-GCTGAT CCACATCTGCTGGAA-3'. The expected sizes of the PCR products were 513, 372, 330, and 1046 bp, respectively. The PCR products were separated by electrophoresis on 1% agarose gels and visualized by staining with ethidium bromide and UV transillumination.

*Murine full-length-IL-15 expression vector (Fig. 1A).* A murine IL-15 cDNA was isolated by RT-PCR from total RNA of lipopolysaccharide-stimulated murine spleen cells. The PCR fragment was cloned into the *NotI/XbaI* site of the pRC/CMV eukaryotic expression vector (Invitrogen, San Diego, CA). The nucleotide sequence was confirmed by sequence analysis with the T7 promoter.

*Murine mature-IL-15 expression vector (Fig. 1B).* A mature-IL-15 cDNA was isolated by PCR with the full-length IL-15 expression vector as a template. The PCR fragment was cloned into the *EcoRI/XhoI* site of the pSecTag2B eukaryotic expression vector (Invitrogen), which contains the Igκ leader sequence. The nucleotide sequence was confirmed by sequence analysis from the T7 promoter.

*Tumor cells and transfection.* Colon 26 cells (25), a murine colon adenocarcinoma cell line derived from BALB/c mice, were maintained in RPMI 1640 (Life Technologies) medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin G, and 100 µg/ml streptomycin. Sub-confluent cultures in 100-mm petri dishes were transfected with 5 µg of murine full-length or mature-IL-15 expression plasmid or vector alone with Lipofectamine reagent (Life Technologies) according to the manufacturer's instructions. G418 (100 µg/ml) (Life Technologies), which selects for the pRC/CMV vector, and Zeocin (100 µg/ml) (Invitrogen), which selects for the pSecTag2B vector, were added to the cells 48 h later. G418- or Zeocin-resistant clones were isolated and expanded in culture medium containing 100 µg/ml of G418 or Zeocin as appropriate.

*Bioassay for IL-15 production by transfectants.* Because an ELISA kit for murine IL-15 is not available, the activity of IL-15 in culture supernatants was quantified with the CTLL-2 bioassay as descrived previously (23). Briefly, each transfectant line ($1x10^6$ cells/ml) was cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum without G418 or Zeosin for 48 h. Culture supernatants were collected by centrifugation for 5 min at 400 g and filtered (0.22 µm) prior to bioassay. IL-15 and IL-2 dependent CTLL-2 cells (4000 cells/well) in 96-well flat-bottom microtiter plates were incubated with culture medium supplemented with $5x10^{-5}$ M 2-mercaptoethanol in the presence of culture supernatant with or without anti-murine IL-15 monoclonal anti-

body (G277-3588, PharMingen, San Diego, CA). After 20-h incubation, 50 µg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) (Chemicon International, Temecula, CA) was added to each well, and the reaction was allowed to incubate for an additional 4 h at 37˚C. Isopropanol with 0.04 N HCl (100 µl) was then added to each well. Color development at a wavelength of 540 nm was monitored with an ELISA reader (SLT Labinstruments, Austria). Serial dilutions of murine recombinant IL-15 (BioSource, Camarillo, CA) were used as a standard.

*Animal studies.* Seven-week-old female BALB/c mice were purchased from Japan SCL (Hamamatsu, Japan). Mice were inoculated with freshly prepared suspensions of tumor cells at a concentration of $2x10^6$ cells/ml. Animals were inoculated with a total of $5x10^5$ cells; all were inoculated subcutaneously in the right lower abdominal quadrant with a 27-gauge needle. Tumor volumes were measured in $mm^3$ with a venire caliper and calculated according to the following formula: a x $b^2/2$, where a is the larger and b is the smaller of the two dimensions. All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Yamaguchi University School of Medicine.

*Re-challenge with parental cells (colon 26) and Meth-A.* Sixty days after disappearance of the initial implant of mature-IL-15/colon 26 cells, 10 mice were injected with $5x10^5$ parental colon 26 cells in the previously uninjected side, left lower abdominal quadrant. Meth-A cells, which were derived from a methylcholanthrene-induced fibrosarcoma in BALB/c mice (26), were also injected into both nonimmunized (n=10) and immunized (n=10) mice.

*In vivo depletion of NK, CD4⁺ and CD8⁺ T cells.* Depletion of NK, CD4⁺ and CD8⁺ T cells was carried out as described previously (27). Briefly, to deplete NK cells, 200 µl of a 1:15 dilution of anti-asialo GM1 antibody (Wako Fine Chemicals, Osaka, Japan) in phosphate-buffered saline (PBS) or control rabbit serum (diluted 1:15 in PBS) was injected intraperitoneally into mice 2 days prior to tumor challenge and 5, 7 and 11 days after tumor challenge. Monoclonal antibodies against CD4⁺ cells (GK1.5) and CD8⁺ cells (2.43) (both purchased from American Type Culture Collection, Rockville, MD) or HBSS (Gibco-BRL) (control) were injected intraperitoneally (1.0 mg) into mice (n=6) to deplete subsets of immune cells 3 days before and once each week after the inoculation of tumor cells. Flow cytometric analysis was performed with an EPICS XL (Beckman Coulter, Fullerton, CA) to verify 95% depletion of specific cell subsets in the spleen after the administration of depleting antibodies. Tumor volume was recorded twice a week.

*Histologic evaluation for immune cells infiltrating into tumor tissues.* On days 7 and 14 after inoculation, tumors were dissected, fixed in 10% neutral buffered-formalin, and embedded in paraffin. Sections (4 µm) were stained with hematoxylin and eosin. For immunohistochemical staining, tissues were embedded in OCT compound (Ames Division, Miles Laboratories, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at -80˚C. Acetone-fixed 6-µm cryostat sections were blocked with goat serum and then immunostained with optimal dilutions of the following rat mAbs: L3/T4 (CD4, Becton Dickinson, Franklin Lakes, NJ) and KT15 (CD8, Serotec, Sapporo, Japan). Slides were then sequentially incubated with biotinylated goat anti-mouse IgG (Zymed laboratories, South San Francisco, CA) and ABComplex (Dako, Tokyo, Japan). Each incubation step lasted at least 30 min and was followed by a 10-min wash with PBS. Sections were then incubated with 0.03% $H_2O_2$ and 0.06% 3,3-diaminobenzidine for 2-5 min, rinsed with tap water, and counterstained with hematoxylin.

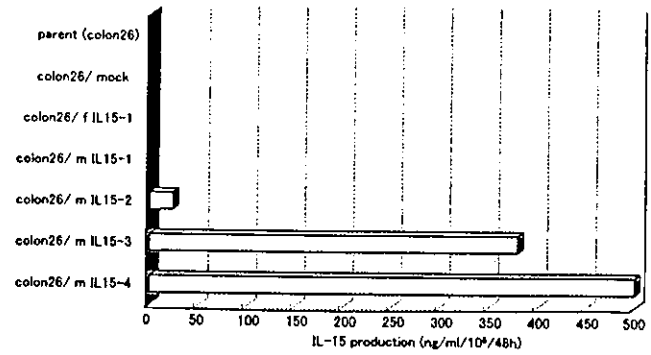


Figure 2. Production of IL-15 by transfectants was confirmed by CTLL-2 proliferation assay. Levels of IL-15 produced by mature-IL-15 transfectants (clones 1, 2, 3 and 4) were between 0 and 500 ng/$10^6$ cells/48 h, whereas full-length IL-15 transfectants and mock transfectants did not produce IL-15.

*Statistical analysis.* Statistically significant differences were evaluated with Student's t-test. A value of $P<0.05$ was considered statistically significant. Results are presented as mean ± SE.

## Results

*Expression of IL-15 mRNA by transfectants.* Four independent G418-resistant full-length IL-15 clones (named colon 26/fIL-15-1, 2, 3 and 4) and four independent Zeocin-resistant mature-IL-15 clones (named colon 26/mIL-15-1, 2, 3 and 4) were isolated and expanded. RNA was isolated, and RT-PCR was performed. Three of 4 (75%) clones (clones 1, 2 and 4) expressed full-length IL-15 mRNA, and 4 of 4 (100%) clones expressed mature-IL-15 mRNA. IL-15 mRNA was not detected in colon 26 cells transfected with empty vector or parental colon 26 cells (data not shown).

*Bioassay for IL-15 produced by transfectants (Fig. 2).* Production of IL-15 protein was confirmed by CTLL-2 proliferation assay. IL-15 levels in the supernatants of mature-IL-15 transfectants ($10^6$ cells/48 h) were 0 ng, 24 ng, 380 ng and 500 ng for clones 1, 2, 3 and 4, respectively. The bioactivity of each culture supernatant was neutralized completely by 10 µg/ml anti-IL-15 antibody. Colon 26 cells transfected with vector expressing full-length IL-15, mock transfected cells and parental colon 26 cells did not produce bioactive IL-15.

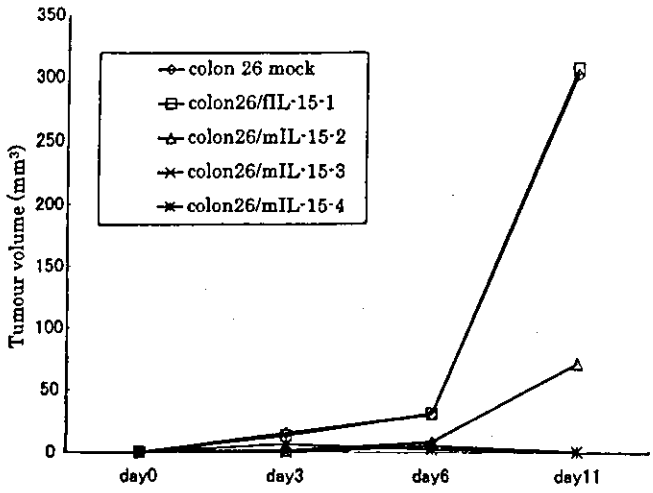


Figure 3. Tumorigenicity of full-length IL-15, mature-IL-15 (clones 1, 2, 3 and 4) and mock transfectants were examined by subcutaneous injection into BALB/c mice. Mean tumor volumes of clones 3 and 4 were significantly smaller on day 11 ($P<0.01$), whereas mock transfectants and clone 1 grew progressively. Each SEM was <10%.

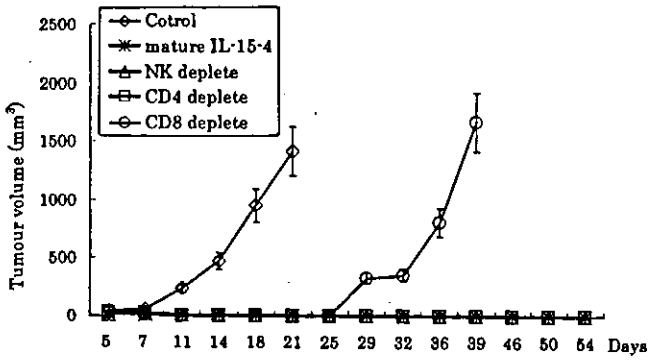


Figure 4. Depleted mice and control mice (HBSS only) were implanted with colon 26/mIL-15-4 cells. There was no difference in tumor growth between the NK-depleted mice, CD4⁺-depleted mice, and control mice. Tumors in mice treated with anti-CD8⁺ antibodies was initially rejected but grew rapidly approximately 1 month after transplantation.

*Inhibition of tumor growth in vivo (Fig. 3).* Transfection of colon 26 cells with full-length or mature-IL-15 expression vector did not alter the growth properties of the cells *in vitro* as assessed by doubling time or morphology (data not shown) in comparison with parental or mock transfected cells. The tumorigenicity of full-length IL-15, mature-IL-15, and mock transfected cultures were examined by subcutaneous injection into BALB/c mice. The mean tumor volumes of the mature-IL-15, -3, -4 were significantly reduced (n=7), whereas mock transfectants grew progressively (n=7). Full-length IL-15 transfectant showed growth similar to that of mock transfected cells.

*Re-challenge with parental colon 26 cells and Meth-A.* We next examined whether primary rejection of IL-15 transfectants led to protective immunity. Sixty days after the disappearance of the initial mature-IL-15/colon 26 implants, immunized mice (n=7) were injected with $5x10^5$ parental colon 26 cells in the lower left abdomen. Seven non-immunized mice were injected in the same manner as controls. Tumor rejection was observed in all immunized mice, whereas colon 26 cells grew progressively in nonimmunized mice (data not shown). To confirm the specificity of the protective immunity, $5x10^5$ Meth-A cells were injected into both nonimmunized and immunized mice. There was no significant difference in growth of implanted Meth-A cells between nonimmunized and immunized mice (data not shown).

*Effects of anti-asialo GM1, anti-CD4 and anti-CD8 antibodies on growth of mature-IL-15 clones (Fig. 4).* Depleted mice and control mice were inoculated with mature-IL-15-expressing cells. There was no difference in tumor growth among the NK-depleted group (n=6), CD4⁺-depleted group (n=6) and the control group (n=7). Tumors in mice treated with anti-CD8 antibody were initially rejected completely but grew rapidly approximately 1 month after transplantation in comparison with those in mice treated with HBSS (control).

*Histology at the site of tumor cell injection (Fig. 5).* To characterize the host cellular responses augmented by IL-15 production, histological analysis of the injection site was performed 14 days following the injection of tumor cells. Immunohistochemical analysis of the site of injection of mature-IL-15 transfectants (clone 4) revealed infiltration of CD4⁺ and CD8⁺ lymphocytes and Mac-1-positive monocytes. This was not observed with mock transfectants.

## Discussion

The anti-tumor effects of IL-15-transfected tumor cells have been described. Two areas have been the focus of recent studies, the low efficiency signal peptide of IL-15 and the induction of anti-tumor effector cells by IL-15.

Both murine and human IL-15 contain an unusually long 48-aa signal peptide, and an alternative short 21-aa signal peptide is also found in human (17,21) and an alternative 26-aa form is present in mice (22). We previously reported that highly immunogenic tumor (Meth-A) cells transfected with the human IL-15 containing the 48-aa signal peptide can elicit local and systemic T cell-dependent immunity (23). However, secretion of bioactive IL-15 was low, and these anti-tumor effects were not evident with weakly immunogenic colon 26 cells (Figs. 2 and 3). Although Kimura *et al* (28) reported the efficacy of Meth-A cells transfected with the alternative form of murine IL-15, which produces relatively large amounts of intracellular IL-15, they did not examine the efficacy with weakly immunogenic tumor cells.

We previously reported that replacement of the endogenous IL-18 leader sequence with the Igκ signal peptide caused efficient secretion of bioactive IL-18 protein (27). We constructed plasmid vectors containing the murine mature-IL-15 linked to the Igκ leader sequence and evaluated the efficacy of high IL-15-producing tumor. Tumorigenicity of mature-IL-15 transfectants of colon 26 cells was decreased in proportion to the level of IL-15 secretion. The clone with the highest production of IL-15 was rejected completely when injected subcutaneously (Figs. 2 and 3).

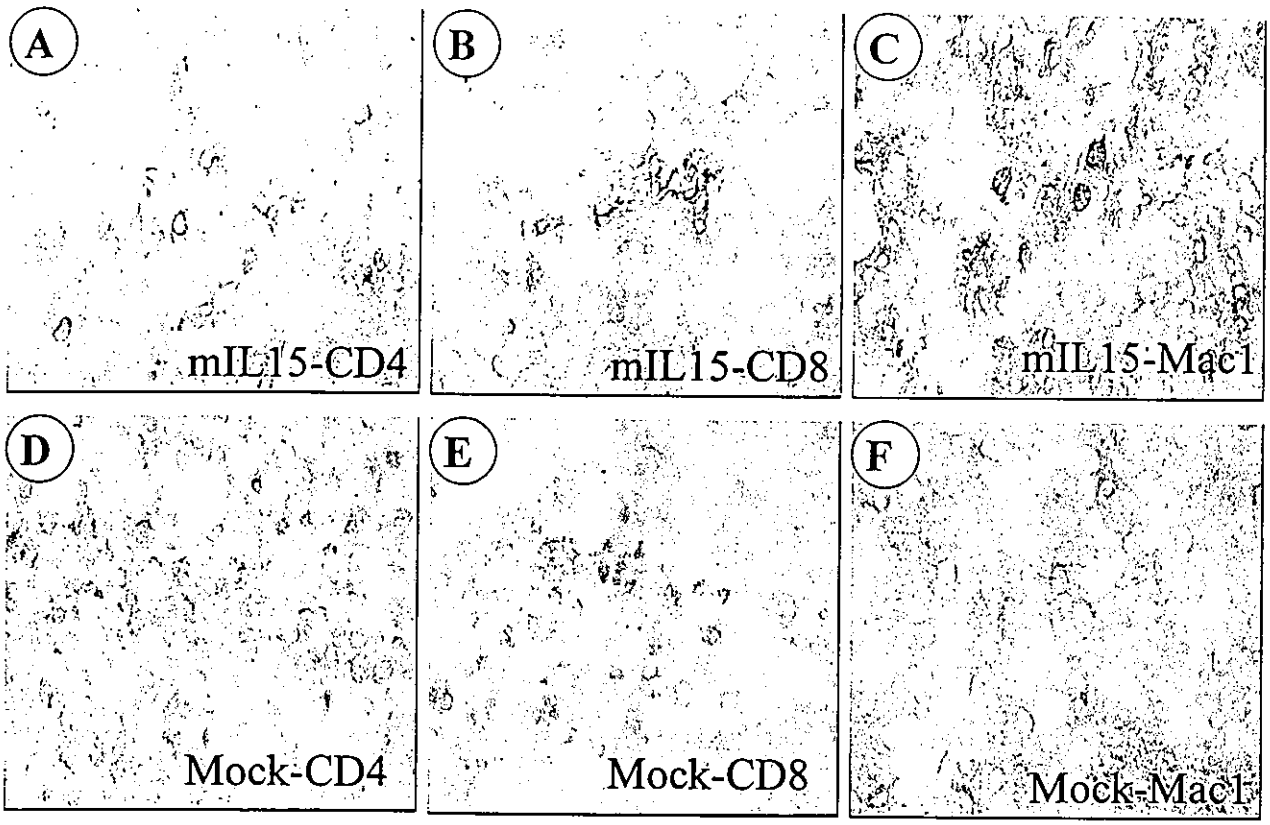


Figure 5. Immunohistochemical analysis of tumor implantation sites in BALB/c mice 14 days after subcutaneous injection of colon 26/mIL-15-4 cells (A-C) and mock transfectants (D-F). Staining with L3/T4 (CD4) (A), KT15 (CD8) (B) and Mac-1 (CD11b) (C) revealed infiltration of $CD4^+$ and $CD8^+$ lymphocytes and Mac-1-positive monocytes, respectively. Such infiltration was not observed in mice treated with mock transfectants (D-F).

Fourteen days after subcutaneous injection of colon 26/mIL-15-4 cells infiltration of $CD4^+$ and $CD8^+$ lymphocytes and Mac-1-positive monocytes was observed at the site of injection (Fig. 5). These findings were supported by previous reports that described the function of IL-15 in proliferation and functional activation of T, B and NK cells (3,4) and monocytes (29). To further clarity of anti-tumor mechanisms, we performed *in vivo* depletion of NK cells, $CD4^+$ and $CD8^+$ T cells. The anti-tumor effects of colon 26/mIL-15-4 cells were partially abrogated by treatment with anti-$CD8^+$ antibodies but not by depletion of NK cells or $CD4^+$ T cells (Fig. 4). In $CD8^+$ T cell-depleted mice, colon 26/mIL-15-4 cells disappeared temporarily, but re-grew 1 month after implantation. These results indicate that inoculation of IL-15-secreting tumor cells may mediate initial anti-tumor effects through $CD8^+$ T cells, $CD4^+$ T cells, NK cells and a variety of immunocompetent cells and that long-lasting specific immunity is mediated only through $CD8^+$ T cells. However, Meazza *et al* (30) reported that TS/A tumor cells, which secrete high levels of IL-15, reduced tumorigenicity, and that depletion of $CD8^+$ T cells or NK cells abrogated the efficacy of IL-15. This inconsistency may be due to differences in IL-15 between murine and human. Although murine and human IL-15 cross-react, there is only 73% amino acid identity between murine and human IL-15 (1). Therefore, it may be more appropriate to use murine IL-15 in murine studies. Indeed, Yajima *et al* (31) reported that murine IL-15 transgenic mice have anti-tumor activity against MHC class I-negative and -positive malignant melanoma through augmented NK activity and cytotoxic T-cell response, respectively. The present study is the first to show that murine tumor cells secreting high levels of murine IL-15 can mediate complete rejection of weakly immunogenic tumor cells and induce long-lasting specific anti-tumor immunity.

These results suggest that IL-15 is important in tumor immunity and that IL-15 may be an excellent candidate for a tumor-vaccine adjuvant for boosting $CD8^+$ memory T cells as therapy for weakly immunogenic human cancers.

## Acknowledgements

This study was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (project No. 09470268).

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総　説

東女医大誌　第74巻　第6・7号
頁　291～299　平成16年7月

シリーズ「画期的に進歩した最新の治療法」(3)

# 消化器癌における免疫療法

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（受理　平成16年2月27日）

**Series "Epoch Making Progress in Treatment for Malignant Diseases" (3)**

## New Perspectives on Immunotherapy of Digestive Cancer

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In the living body, two immune systems, "natural immunity" and "acquired immunity", are working, and two types of immunological response exist by "effector phase" in which a direct effect cell stands face to face against a neoplasm or by "priming phase" in which the specific immunity to an antigen is guided. A cellular immunotherapy is a cancer treatment by using T lymphocytes, natural killer cells and dendritic cells which are activated ex vivo and again transferred in vivo. We performed (1) adoptive immunotherapy with tumor-specific CTLs in the patients with multiple liver cancer and (2) adoptive immunotherapy with CD 3-activated T (CAT) in the patients with multiple lung metastases. Each rate of a successful treatment (CR + PR) is 40%, or 50% and the disease control (stable disease: SD) during a treatment period became possible at 91% or 92%. At present, we are trying such new clinical studies as (3) cancer vaccine therapy with tumor or peptide-pulsed dendritic cells, (4) direct injection of dendritic cells into advanced tumors. All treatments with any effector cells transferred, toxicity and side effect were tolerable and not critical. We have now started a new trial of cellular immunotherapy after the curative operation to prevent recurrence or metastasis. Although there are several problems which should cope with them from now on, the cellular immunotherapy is safe, and useful tool for cancer treatment.

**Key words:** cancer immunotherapy, cell therapy, CTL, dendritic cell, cancer vaccine

## 緒　言

人の体では2つの免疫系が働いている．1つは自然免疫といわれ，生体が生まれつき保有している抵抗力であり，怪我をして傷口に細菌が付着しても自然に傷口を治したり，ウイルス感染に対してそれらを排除する免疫力を指す．これに対して2つ目は獲得免疫と呼ばれ，成長の過程で体が繰り返し教え込まれることにより獲得する特異的な免疫力である．日本脳炎，破傷風，天然痘などの病気では自然免疫のみで十分な対処ができずに重篤な病態に陥る危険があるため，あらかじめ予防接種を行い体に抗原を教え込ませ特異的免疫を獲得させることにより，実際の感染の時にウイルスや細菌を効率よく排除することが可能となる．

生体内に癌細胞が発生した時も同様にまず生体の自然免疫が働いて癌細胞を排除し，さらにその破片を捕獲した抗原提示細胞がリンパ節や脾臓に移動し，癌抗原を提示して獲得免疫を誘導することができれば，強力に癌細胞を排除することが可能となるであろう．しかし，癌細胞は本来自己の細胞であるために非自己に対するような強力な免疫が励起され難く，またMHC分子を欠損したり，免疫担当細胞を

表1　癌に対する免疫細胞療法の分類

| |
|---|
| (1) 利用する免疫担当細胞（白血球）の種類から分類する |
| ・αβT細胞，γδT細胞，NK細胞，NKT細胞などのリンパ球 |
| ・樹状細胞などの抗原提示細胞 |
| (2) 免疫応答の機序から分類する |
| ・自然免疫を利用した「抗原非特異的」な細胞療法 |
| ・獲得免疫を利用した「抗原特異的」な細胞療法 |
| (3) 免疫サイクルの過程から分類する |
| ・Priming phaseを強化する「樹状細胞療法」 |
| ・Effector phaseを強化する「活性化リンパ球療法」 |

抑制する因子を産生して生体の免疫応答から回避していると考えられている．生体側の要因として自然免疫や獲得免疫の働きが弱い場合にも癌は増殖し進行していく．

癌に対する免疫細胞療法とは生体の自然免疫，獲得免疫を担う免疫担当細胞を利用する治療法であり，主に白血球の中のNK（natural killer）細胞，Tリンパ球，抗原提示細胞（樹状細胞）を利用している．実際の治療手段は，生体内における免疫サイクルのどの作用機序を強化するかにより“effector phase”での免疫効果を強化する方法と，“priming phase”の免疫獲得を強化する方法が考案されている（表1）．

**1．癌に対する生体の免疫サイクル**（図1）

正常細胞に変異が生じ，癌が発生するとまず生体の自然免疫が働き，マクロファージなどの貪食細胞，NK細胞，好中球などが癌細胞を攻撃し排除する．自然免疫の攻撃を受けて破壊された癌細胞の一部を抗原提示細胞（樹状細胞）が取り込み，リンパ節や脾臓に移動して，そこで癌細胞の特徴（癌抗原）をTリンパ球やBリンパ球に教え込む教官の役割を果たす．特異的な癌抗原の情報を教え込まれたTリンパ球は細胞性免疫，Bリンパ球は抗体を産生して液性免疫の主体となり，誘導された癌特異的細胞傷害性Tリンパ球（cytotoxic T lymphocyte: CTL）や癌特異抗体が癌細胞を集中攻撃する．

このような癌特異的免疫の獲得とその免疫応答が不十分であると，癌は免疫系の攻撃を受けずに増殖していくことになる．癌はIL-10，TGF-βなどの免疫抑制性サイトカインを産生したり，癌表面にFasなどの分子を表出してリンパ球を細胞死に陥らせて免疫系の攻撃を回避する．これらの生体内での免疫サイクルを考慮して，①癌に対する自然免疫応答を強化する，②癌抗原感作を強化する，③癌特異的攻撃細胞や癌特異抗体を強化する目的で免疫細胞療法が研究されており，利用される免疫担当細胞が効率よく効果を発揮するためには細胞投与経路の工夫が必要とされる．

**2．免疫サイクルと免疫細胞療法**（図2）

1）自然免疫を強化する免疫細胞療法

自然免疫を強化する目的で非特異的に癌細胞を傷害するキラー細胞（NK細胞など）を体外で増殖，活性化して生体内に投与する治療法がある．代表的な方法は1980年代に米国NCIを中心に実施されたLAK（lymphokine-activated killer）療法と呼ばれる方法で，ヒト末梢血リンパ球をIL-2（インターロイキン2）と呼ばれるサイトカインで刺激して誘導した細胞を投与する治療法である．LAK細胞はCD3陽性Tリンパ球，NK細胞など種々の細胞が含まれた集団であるが，抗腫瘍活性はその中のCD16陽性CD56陽性細胞（NK細胞群）が主体と考えられる．この細胞群は非特異的に腫瘍細胞を傷害し，特にMHC抗原陰性腫瘍を強力に傷害するといわれている．

最近，我々はNK細胞のみを選択的に活性化する方法を確立し，活性化NK細胞（LANK）を用いた臨床治療を検討している．NK細胞は抗原特異性がなく，標的腫瘍への集積能に乏しいため，細胞を直接腫瘍に注入するか腫瘍栄養動脈から移入するなどのcell deliveryの工夫が必要である．

2）癌抗原感作を強化する免疫細胞療法（図3）

樹状細胞は生体内での強力な抗原提示細胞であり，近年末梢血からの分離培養法が確立された．樹状細胞はその名のごとく樹枝状の形を呈し，貪食した抗原を処理してMHCと結合して細胞表面に提示することにより体内で癌抗原特異的Tリンパ球（CTL）を誘導する．樹状細胞はIL-12などのリンパ球活性化サイトカインを産生し，細胞表面上にB7（CD80，CD86）分子を表出して効率良くCTLを誘導できる．この樹状細胞のMHCに癌抗原を提示させた「樹状細胞がんワクチン」を皮下または皮内に投与して，癌抗原に対する特異的免疫応答を励起させる試みが1990年代後半より開始された．樹状細胞に表出している空のMHC分子に人工的に合成した9基程のアミノ酸基を直接結合させてTリンパ球のTCRにシグナルを送り，生体内で抗原特異的CTLを誘導することができる．

1990年代より腫瘍関連抗原の同定が精力的に進