Effect of Transarterial Immunoembolization as Preoperative Treatment for Hepatocellular Carcinoma

Takuro Saito¹, Takao Tsuchiya¹, Yoshihiro Sato¹, Akira Kenjo¹, Takashi Kimura¹, Takayuki Anazawa¹, Masanori Terashima¹, Atsushi Takahashi², Hiromasa Ohira³ and Mitsukazu Gotoh¹

¹Department of Surgery I, Fukushima Medical University, ²Department of Gastroenterology and Rheumatology, Fukushima Medical University

Abstract
Background: Hepatocellular carcinoma (HCC) frequently recurs after curative resection. In the present study, we assessed the survival of HCC patients with or without preoperative transarterial immunoembolization (TIE) in relation to changes in serum cytokine levels and histological characteristics of resected specimens.

Methods: After confirmation of the safety and feasibility of preoperative TIE in a preliminary study of 9 patients, 15 patients who planned to undergo curative resection of HCC were randomized to the TIE group (n=8) or the control group (n=7). TIE was performed with a mixture of OK-432, fibrinogen, thrombin, and lipiodol. Hepatic resection was planned 2 weeks after TIE.

Results: In the preliminary study, none of 9 patients with TIE developed complications that were severe enough to postpone the scheduled operation. Retrospectively, the 3-year disease-free and overall survival rate in the 9 patients who received preoperative TIE were both 100%; in contrast, the disease-free survival and overall survival were 27% and 64%, respectively, in 22 patients who received a hepatectomy around the same period without TIE. Furthermore, the prospective study revealed that the 3-year disease-free survival and overall survival in the TIE group were 88% and 100%, which were significantly higher than the corresponding rates in the control group (17% and 57%, respectively, both: p<0.05). Serum levels of interleukin-12 and interferon gamma were increased after TIE. Histologically, significant infiltrations of dendritic cells were observed in embolized areas, but the infiltration of FOXP3-positive cells was significantly suppressed after TIE.

Conclusion: Preoperative TIE suppresses recurrences of HCC after surgery. The suppressive effect might be caused by increased levels of helper T-cell 1 cytokines, the accumulation and maturation of dendritic cells, and the suppression of T-regulatory lymphocytes.

Key Words: Transarterial immunoembolization, hepatocellular carcinoma, immunotherapy, OK432.

(Received June 15, 2011; Accepted June 24, 2011)

Introduction
Hepatocellular carcinoma (HCC) is one of the most common causes of cancer-related deaths¹. A careful multidisciplinary assessment of tumor characteristics, liver function, and physical status is required for proper therapeutic management, even in patients with early stage tumors²-⁵. Treatments for HCC include hepatic resection, radiofrequency ablation, microwave ablation, cryoablation, percutaneous ethanol injection, and transcatheter arterial chemo embolization (TACE)⁶. Despite these treatments, HCC has a high rate of recurrence⁴,⁵. The most common site of recurrence is the remnant liver. Treatments to control recurrent HCC include repeat hepatectomy, ablation therapy, and TACE⁶. The establishment of effective adjuvant therapy to prevent HCC recurrence is necessary.

Tumor-infiltrating lymphocytes (TILs) in HCC have been characterized⁹,¹₀, and extensive infiltration is associated with reduced tumor recurrence following resection⁹,¹₀. Cytotoxic CD8+ lymphocytes (CTLs) and natural killer cells are potential effector cells in the control of tumor growth, although both require CD4+ T helper 1 immune responses for optimal function¹¹,¹²,¹³. T-regulatory lymphocytes (Treg cells) are a subgroup of CD4+ T cells with suppressor function characterized by the expression of the interleukin-2 receptor alpha chain (CD25) in the resting state¹⁴. The induction of Foxp3, a specific marker of Treg cells, is associated with suppressive activity¹⁵. An increased number of Treg cells is associated with tumor progression and poor prognosis in patients with HCC¹¹,¹²,¹³. Thus, the regulation of tumor immunity in the liver may play a role in the progression of HCC.
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Patients and methods

We conducted a preliminary study to confirm the safety and feasibility of preoperative TIE in 9 patients at our institute after obtaining written informed consent. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by our institutional review committee. Patients who planned to undergo curative resection of HCC were randomized to a TIE group (n=8) or a control group that did not receive TIE (n=7). Patients in the TIE group received TIE two weeks prior to surgical resection.

Transarterial immunoembolization

A vascular catheter was inserted into the tumor-feeding artery, such as the segmental artery or its branches. Transarterial immunoembolization was performed according to our protocol, as reported previously. OK-432 and thrombin were dissolved in saline at concentrations of 2.5 KE/ml and 2 U/ml, respectively (solution A). Solution B contained fibrinogen at 60 mg/ml in saline. One ml of solution A and an equivalent volume of solution B were mixed with 0.5 ml of lipiodol (iodized oil) for 30 seconds. The mixed solution was injected slowly over a period of 30 and 60 seconds via a catheter placed in the arterial branch feeding the tumor. Because of limited period of injection, some modification was introduced for easy-to-use approach in later cases. Five KE of OK-432 and 30 mg of fibrinogen were individually dissolved in 1.0 ml of saline and then mixed together with 2.0 ml of lipiodol. Four unit of thrombin was separately dissolved in 0.5 ml of saline and diluted with 0.5 ml of Omnipaque™ (iohexol) 350. First, the OK432 mixture was injected through the catheter, and then the thrombin-iohexol solution was injected to secure local trap of the drugs within the liver.

Evaluation of the effect of TIE

The direct effects of TIE were examined by assessing the cytokine profile and the histological characteristics of resected specimens. The final effect of TIE was evaluated by comparing the patient survival and disease-free survival between the TIE group and the control group.

Cytokine measurements

The serum levels of interleukin-6, interleukin-10, interleukin-12, TNF-alpha, and interferon gamma in both the preliminary and randomized cases were measured before and after TIE by enzyme immunoassay.

Histological changes after TIE

Resected specimens in both the preliminary and randomized cases were fixed with 10% buffered formalde-
samples were stained with hematoxylin and eosin or immunostained with various antibodies by using the avidin-biotin peroxidase complex method. Antibodies used in this study included: polyclonal anti-S-100 antibody (Nichirei, Tokyo, Japan), monoclonal anti-CD1a antibody (Novocastra Laboratories, Newcastle, UK), monoclonal anti-Fascin antibody (Dako Corporation, CA, USA), monoclonal anti-CD83 antibody (Santa Cruz Biotechnology, CA, USA), monoclonal anti-CD4 antibody (Novocastra Laboratories, Newcastle, UK), monoclonal anti-CD8 antibody (DakoCytomation, Glostrup, Denmark), monoclonal anti-CD25 antibody (Abcam, Tokyo, Japan), and monoclonal anti-Foxp3 antibody (Abcam, Tokyo, Japan). The infiltrating cells in embolized areas were scored according to the number of antigen-presenting cells per high-power field as follows: 0 to 10 cells (scored “0”), 11 to 50 cells (scored “1”), 51 to 100 cells (scored “2”), and more than 100 cells (scored “3”).

**Statistical analysis**

Clinical and histological findings and anticancer effects were compared between the groups by using the Student’s t test, Fisher’s exact test, or Chi-square analysis. Postoperative survival and disease-free survival rates were compared with the log-rank test. P-values less than 0.05 were considered to be statistically significant.

### Results

**Adverse events associated with TIE in the preliminary study**

The adverse events that occurred after TIE in the preliminary study are listed in Table 1. All patients who received TIE developed a fever with temperatures above 38°C for various lengths of time. Transiently elevated liver enzymes and an increased number of leukocytes in the peripheral blood were also observed. However, none of these adverse events were severe enough to postpone the scheduled hepatic resection. Accumulation of pleural effusion was observed in only one of 9 patients after the hepatic resection performed 2 weeks after TIE.

**Patient characteristics, operative procedures and prognostic factors in the randomized controlled study**

Patient characteristics including age, sex, viral infection, and liver damage were not significantly different between the TIE and control groups (Table 2). Prognostic factors, such as TNM stage, histological findings, and surgical procedure, were similar between the groups (Table 3).

**Overall survival and disease-free survival after hepatic resection**

In the preliminary study, the 3-year disease-free survival and overall survival rate in patients who received preoperative TIE were both 100%, which were higher than those in the control groups.

### Table 1. Adverse events after TIE in the preliminary study

| Adverse events after TIE | No. of patients (%) |
|-------------------------|---------------------|
| Fever                   | 9/100               |
| within 3 days           | 5/56                |
| 4 – 7 days              | 3/33                |
| over 7 days             | 1/11                |
| Laboratory data         |                     |
| GPT >100 IU/L           | 3/33                |
| WBC>10000/μl            | 2/22                |

### Table 2. Patient characteristics in the randomized controlled trial

| Variables          | control (n=7) | TIE (n=8) |
|--------------------|---------------|-----------|
| Age (years)        | 68 ± 4        | 60 ± 11   | N.S.     |
| Sex men/women      | 5:2           | 7:1       | N.S.     |
| Viral infection    |               |           |          |
| HBV                | 2             | 2         | N.S.     |
| HCV                | 5             | 5         |          |
| none               | 0             | 1         |          |
| Liver damage       |               |           |          |
| A                  | 7             | 8         |          |
| B                  | 0             | 0         |          |
| C                  | 0             | 0         |          |

### Table 3. Prognostic factors in the randomized controlled study

| Variables                     | control (n=7) | TIE (n=8) |
|-------------------------------|---------------|-----------|
| Operative procedure           | 3/4/0         | 4/3/1     | N.S.     |
| pTNM stage (I/ II/ III/ IV)   | 1/5/3/0       | 2/3/3/0   | N.S.     |
| Fibrosis score; F (0/ 1)      | 5/2           | 5/3       | N.S.     |
| Classification of differentiation | 2/5/0     | 1/6/1     | N.S.     |
than the corresponding rates in 22 patients who received a hepatectomy at the same period without TIE (27% and 64%, respectively, Fig. 1-A, 1-B).

The randomized controlled study revealed that 5 of the 7 patients in the control group developed tumor recurrence within 3 years after hepatic resection. In contrast, only 1 of 8 patients in the TIE group developed a tumor recurrence within 3 years (p<0.05). The 3-year disease-free survival rate was 88% in the TIE group and 15% in the control group (Fig. 2-A; p<0.05). Furthermore, the 3-year overall survival rate was 100% in the TIE group but only 66% in the control group (Fig. 2-B).

**Changes in cytokine levels after TIE**

Serum levels of IL-6, IL-10, and TNF-alpha were significantly elevated 1 to 3 hours after TIE as compared to before TIE (p<0.05, Fig. 3 A, B, C). Serum levels of IL-12 were elevated 3 to 24 hours after TIE with statistically significant differences at 6 and 24 hours after TIE as compared with before TIE (p<0.05, Fig. 3D). Serum levels of INF-gamma were elevated 6 to 24 hours after TIE with a statistically significant difference at 24 hours after TIE as compared with before TIE (p<0.05, Fig. 3E).

**Histological changes after TIE**

Hematoxylin-eosin staining of resected specimens obtained 2 weeks after TIE revealed coagulation necrosis and lytic necrosis of the main tumor along with the presence of infiltrating cells such as macrophages and lymphocytes (Fig. 4).

Immunohistochemical staining of these specimens by using antibodies specific for pan-DC (anti-S-100 antibody), immature DC (anti-CD1a antibody), and mature DC (anti-Fascin antibody and anti-CD83 antibody) revealed marked infiltration of these cells at the embolized area with a significantly greater number of cells in the TIE group as compared to the control group (Fig. 5-A, 5-B). Immunohistochemical staining with anti-CD8 antibody revealed marked infiltration of these cells around the embolized area with a significantly higher number of cells as compared with the control group (Fig. 6-A, 6-B). However, the number of infiltrating cells in the embolized area that were stained with anti-CD4 antibody and anti-CD25 antibody was not significantly different as compared with the control group. In contrast, the number of anti-FOXP3 antibody positive cells around the embolized area was significantly decreased in the TIE group as compared with the control group.

**Discussion**

The curative treatments for HCC, including surgical resection, do not efficiently prevent tumor recurrence\(^{1-5}\). Although a number of studies have examined adjuvant or neo adjuvant treatment in combination with surgical resection, no effective treatments have been established\(^{23}\). Patients with HCC have been found to have impairments in their immune system, including impaired cytotoxic T lymphocyte (CTL) inductivity and poorly functioning dendritic cells\(^{8,13,24}\). Whereas restoration of CTL by intratumoral dendritic cell infusion or the effect of postoperative adoptive immunotherapy with autologous lymphocytes activated with recombinant interleukin-2 and antibody to CD3 has been reported\(^{20,26}\). Thus, immunological alteration might be a promising option to improve the surgical outcome of patients with HCC. Preliminary studies of TIE with OK432 for unresectable HCC with intrahepatic metastases or TIE as a preoperative treatment have shown the effectiveness of TIE as compared to TAE\(^{19,20}\). In the present study, preoperative TIE with OK432 prevented the recurrence of HCC after surgical resection by suppression of tumor recurrence within 3 years. Both the disease-free survival and overall patient survival were improved by TIE without severe adverse events.

The intrahepatic recurrence of HCC occurs as a result of residual intrahepatic metastasis or metachronous multicentric liver carcinogenesis\(^{27,28}\). Sakon et al. reported that intrahepatic metastases of HCC occurred within 2 years after hepatic resection, whereas multicentric liver carcinogenesis occurred more than 2 years after resection\(^{29}\). Therefore, preoperative TIE might effectively control potential intrahepatic metastases, because tumor recurrence within 3 years was suppressed by TIE.

TIE with OK432 induced transient increases in the serum levels of IL-6, IL-10, and TNF-alpha and induced the systemic elevation of IL-12 and INF-gamma. OK432 induces IL-12 and polarizes the T-cell response to a helper T-cell 1 [Th1]-dominant state\(^{30}\). Furthermore, impaired production of INF-gamma in patients with HCC has been associated with impaired CTL inductivity\(^{24}\). Elevation of serum levels of IL-12 and INF-gamma by TIE with OK432 might induce a Th1-dominant state and restore CTL inductivity against HCC.

Resected specimens after TIE contained marked infiltration of mature and immature dendritic cells and CD8-positive T lymphocytes. Dendritic cells are the most potent type of antigen-presenting cells in the human body\(^{30-32}\). During dendritic cell development, immature dendritic cells process antigens, and mature dendritic cells present the antigens to resting lymphocytes and elicit T-cell responses\(^{33-36}\). We previously reported that OK432 induced Th1-type cytokines and mature DCs in vitro\(^{36}\). After TIE, locally injected OK432 with embolized materials induced lytic necrosis of the tumor. At the same time, induction of Th1-type cytokines and accumulation and maturation of DCs around the tumor occurred, and CTLs against tumors might be effectively induced.

In contrast, the number of FOXP3-positive lympho-
Serum levels of IL-6, IL-10, and TNF-alpha were significantly elevated 1 to 3 hours after TIE (Fig. 3 A, B, C). Serum levels of IL-12 were elevated 3 to 24 hours after TIE with statistically significant elevations at 6 and 24 hours after TIE (Fig. 3D). Serum levels of INF-gamma were elevated 6 to 24 hours after TIE with a statistically significant elevation at 24 hours after TIE (Fig. 3E) (*p<0.05).
cytes was significantly suppressed following TIE. An increased number of regulatory T cells correlates with CD8 T-cell impairment and poor survival in patients with HCC\textsuperscript{13}. The intratumoral balance of regulatory and cytotoxic T cells in resected specimens is also associated with prognosis, and a combination of depletion of Tregs and concomitant stimulation of effector T cells reduces recurrence and prolongs survival after surgery\textsuperscript{11,12}.

Alteration of the intratumoral balance by TIE might play an important role in the suppression of tumor recurrence.

In conclusion, preoperative TIE with OK432 suppresses recurrences of hepatocellular carcinoma after surgery. This suppression might be caused by elevated Th1 cytokines, the accumulation and maturation of dendritic cells, the induction of CTLs and the suppression of Treg lymphocytes. This strategy might be effective in combina-
tion with other local therapies including radiofrequency ablation. A prospective randomized trial of preoperative TIE is needed.

Conflict of interests
The authors declare that they have no conflict of interest.

Acknowledgement
This study was supported, in part, by a non-profit organization Epidemiological & Clinical Research Information Network (ECRIN).

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Fig. 6 Immunohistochemical findings of resected specimens stained with markers of lymphocytes. A: Immunohistochemical staining of resected specimens with specific antibodies for CD4, CD8, CD25, and Foxp3. B: Immunohistochemical staining with anti-CD8 antibody revealed marked infiltration of these cells around the embolized area, showing a significantly higher number of cells as compared to the control group (**p<0.01). The number of anti-FOXp3 antibody positive cells around the embolized area was significantly decreased in the TIE group as compared with the control group (**p<0.01).
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