Original article

Relationship between tumor imaging enhancement by measuring attenuation and clinicopathological characteristics in intrahepatic cholangiocarcinoma

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Running title: Tumor enhancing in cholangiocarcinoma

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ABSTRACT

Purpose: Arterial enhancement of intrahepatic cholangiocarcinoma (ICC) has been noted. To precisely identify the characteristics of tumor enhancement patterns, we examined the relationship between CT attenuation in the tumor and clinicopathological parameters or prognosis.

Methods: Subjects were 42 ICC patients who had undergone hepatectomy. Microvessel density (MVD) determined by CD34 staining was compared with imaging. Attenuation was calculated in images from multi-detector CT of tumor and non-tumorous regions. Enhancement patterns were divided into two groups: arterial enhancement with higher attenuation (>16 HU; Hyper group, n=12); and arterial enhancement with lower attenuation (Hypo group, n=30).

Results: Univariate analysis identified high tumor marker level, increased size, less-differentiation, incomplete resection, increased bleeding and lower MVD as significantly associated with poor survival ($p<0.05$). Increased attenuation throughout the whole ICC correlated significantly with radiological findings and MVD. Concomitant hepatitis, well-differentiation, and smaller tumor were more significantly frequent in the Hyper group than in the Hypo group ($p<0.05$). Postoperative early recurrence was significantly less frequent in the Hyper group, and overall survival was significantly better in the Hyper group ($p<0.05$).

Conclusions: Increased CT attenuation correlated with ICC tumor vascularity. Increased tumor enhancement in the arterial phase was associated with chronic hepatitis, lower malignancy and better survival.

Key words: intrahepatic cholangiocarcinoma-tumor enhancement-multidetector computed tomography-prognosis-hepatectomy

Intrahepatic cholangiocarcinoma (ICC) is a well-characterized liver cancer for which various prognostic risk factors have been clarified [1-3]. ICC is composed of cells that mostly resemble those of the peripheral bile ducts, but recent reports have suggested that ICC may not always display uniform characteristics [4-8]. ICC can be classified into subgroups according to macroscopic findings, with clinical and pathological characteristics and prognosis differing significantly among subgroups [9, 10]. While diagnosis of the ICC subtype would be useful for predicting prognosis and determining the need for adjuvant treatments with hepatic resection, clearly distinguishing subgroups is often difficult, particularly in advanced tumors. Although a variety of prognostic parameters have been identified in recent research, the definitive factors associated with poor prognosis have not been well clarified [11, 12]. Recent histological and molecular studies have revealed that tumor vascularity of ICC is closely associated with malignant behavior and patient prognosis [13, 14]. Higher tumor vascularity might be related to the presence of chronic viral hepatitis, which is also related to carcinogenesis for hepatocellular carcinoma (HCC) [15]. We have previously examined histological parameters in ICC and clarified that higher tumor vascularity as detected by CD34 staining was significantly associated with patient prognosis [16-18]. Tumor vascularity could thus represent an important marker for evaluating malignant characteristics of ICC. However, this marker is only observable in resected specimens, so a preoperative marker of tumor vascularity is needed. At this stage, no useful markers of tumor vascularity have been confirmed.

Contrast-enhanced computed tomography (CT) and other imaging modalities are useful to diagnose liver tumors, including ICC, and to determine the extent of tumor involvement [19]. The most common pattern of tumor enhancement in ICC is reportedly as a lower-density mass (hypovascular) compared with non-cancerous liver parenchyma, showing delayed enhancement in the peripheral part of the tumor [20, 21]. In contrast, some ICCs show tumor enhancement in the arterial phase, similar to classical HCC [22, 23]. Differences in these enhancement patterns in ICC may also reflect differences in cellular characteristics or clinical features. Recent reports have shown that early enhancement of ICC is associated with presence of chronic viral hepatitis and tumor characteristics resembling those of HCC [24-26]. Our previous study and others have already reported relationships between the enhancement pattern of ICC and malignant behavior or patient prognosis [16, 17]. Enhancement of ICC in the arterial

phase is associated with a similar clinical background to HCC and better survival compared to hypovascular ICC. To evaluate tumor enhancement, parameters that can be objectively determined are needed. Recent improvements in high-resolution CT allow precise determination of subtle changes in X-ray density, suggesting a potential method for objective evaluation [27].

To the best of our knowledge, the relationship between numerical evaluation of the tumor enhancement pattern in ICC and clinicopathological characteristics has yet to be clarified. The present retrospective study was designed to clarify the relationship between the enhancement pattern of ICC as indicated by attenuation in Hounsfield units (HFU) on computed tomography (CT) and the various clinicopathological features, biological factors, and patient survival after hepatic resection in 42 ICC patients. The overall aim of this study was to more precisely identify specific characteristics of the different patterns of tumor enhancement by calculating CT attenuation, and to allow preoperative definition of malignant behaviors and predict the risk of tumor recurrence and/or patient outcomes more clearly in comparison with the previous study [16].

Patients and methods

Patients

A total of 42 consecutive patients with ICC (29 men, 13 women) who were admitted to the Division of Surgical Oncology, Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences (NUGSBS) between 2006 and 2010 were analyzed retrospectively in this study. Mean age of patients in the present study was 70.2 ± 8.5 years (range, 42-81 years). All 42 patients underwent hepatic resection; surgical procedures included segmentectomy (n = 11), sectoriectomy (n = 17), right or left hepatectomy (n = 12), and extended hepatectomy (n = 2). All study protocols were approved by the ethics review board at our institution. Data were retrieved from both anesthetic and patient charts, plus the NUGSBS database, to cover the period of hospitalization following hepatectomy.

Indocyanine green retention rate at 15 min (ICGR15) was routinely examined to define the preoperative functional liver reserve. A dose of 0.5 mg ICG/kg body weight was injected intravenously and the retention rate was measured at 15 min without blood sampling using a photopiece applied to the fingertip (Sumitomo Electric, Tokyo, Japan) [28]. The volume of the liver to be resected was determined preoperatively based on both the results of ICGR15 and the estimated resected liver volume, excluding tumor volume, as measured by CT volumetry [28]. In cases where the permitted resected volume was greater than the estimated resected liver volume, hepatectomy was performed as planned.

Each enhancement pattern on CT was compared with regard to patient demographics, tumor markers, liver function, histological findings, immunohistochemical staining for CD34 (as a measure of microvessel count) or proliferative cell nucleolar antigen (PCNA), and patient survival after surgery.

Immunohistochemical Staining

The resected specimen was fixed in 10% formalin and embedded in paraffin. Thin sections (4mm) were deparaffinized twice by xylene and rehydrated in a series of ethanol solutions (100%, 90% and 80%). Briefly, sections were placed in 0.01 M trisodium citrate dehydrate buffer (pH 6.0) and treated in a microwave oven for 10 min at 500 Watts. For CD34 staining, tissue sections were digested with 0.2% trypsin in 0.01 M phosphate-buffered saline for 20 min at 37°C. In the next step, tissues were immersed in 3% H_2O_2 with distilled water for 10 min to inactivate endogenous peroxidase. After blocking

non-specific binding by normal goat serum, sections were then treated overnight at 4°C with 1:25 diluted mouse anti-monoclonal CD34 antibody (QB-END/10; Novocastra Laboratories, Newcastle, UK), or for 1 h at room temperature with 1:100 diluted monoclonal mouse anti-PCNA antibody (PC-10; Dako, Carpinteria, CA) as the primary antibody, respectively. This was followed by reaction with biotinylated anti-immunoglobulin and reagent using a labeled streptavidin-biotin peroxidase kit (Dako). The peroxidase reaction was visualized with 0.01% H_2O_2 and 3,3'-diaminobenzidine under light microscopy (×200). For microvessel count based on CD34 staining, we determined the average count in the five most-vascular areas examined at ×200 magnification. CD34 expression was evaluated in the areas surrounding the tumor. The average percentage of cells showing positive PCNA nuclear expression among 1,000 nuclei in five areas of the tumor was estimated. Pathological and morphological parameters and Japanese tumor-node-metastasis stage were defined using the criteria of the Liver Cancer Study Group of Japan [29].

Image analysis

CT studies were performed using a 64 detector-row CT (Definition; Siemens, Erlangan, Germany, or Aquilion TSX-101A; Toshiba, Tokyo, Japan). Nonionic iodinated contrast medium (iomeprol; Iomeron® 350/135 mL; Eizai, Tokyo, Japan) was rapidly injected using a calibrated power injector (100 mL/30 s) at a rate of 600 mg iodine/kg body weight. Images were obtained at 35 s (arterial phase), 70 s (portal phase) and 150 s (equilibrium phase) after injection. Tumor enhancement patterns were classified as hypervascular and hypovascular ICC based on the consensus diagnosis of two radiologists. Attenuation in the region of interest (ROI) was calculated using Aquarius NetStation version 1.5 software (TeraRecon, Foster City, CA). We examined 3 patterns of attenuation in the arterial phase of enhancement, as follows: 1) increased attenuation over the entire area of the tomographic ICC image (Fig. 1B) in comparison with that in non-enhanced CT (Fig. 1A); 2) increased attenuation in the most enhanced area of the tomographic ICC image (Fig. 1C) in comparison with that in non-enhanced CT (Fig. 1A); and 3) difference of attenuation in the ICC (Fig. 1B) and non-tumorous lesion (Fig. 1D) at the same arterial phase. The calculated median attenuation was set as the cut-off value.

Statistical analysis

All continuous data are expressed as mean ± standard deviation. Data from different groups were compared using one-way analysis of variance and examined using Student's *t*-test or Dunnet's multiple comparisons test. Disease-free and overall survival rates after surgery were calculated according to the Kaplan-Meier method, and differences between groups were tested for significance using the log-rank test. Two-tailed P values <0.05 were considered significant. Statistical Package for the Social Science (SPSS) version 18.0 software (SPSS, Chicago, IL) was used for all statistical analyses.

Results

Patient demographics

Background liver diseases included normal liver function (n=24) and chronic viral liver disease (n=18; hepatitis B virus [n=3] and hepatitis C virus [n= 15]). Concomitant cholecysto- or hepatico-lithiasis was observed in 11 patients (26%). Mass-forming type was observed in 18 patients and mass-forming type with ductal invasion type was seen in 24. Lymph node metastasis was observed in 20 patients (48%). R0 resection was achieved in 37 patients (88%) and R1 in 5 (12%). Postoperative tumor recurrence was observed in 12 patients (29%), involving the liver in 6 patients, local stump in 2, peritoneum in 3, lymph node in 1, and bone in 1. Three- and 5-year tumor-free survival rates were 30 and 22%, respectively, and the median tumor-free survival period was 31 months. Three- and 5-year overall survival rates were 46% and 36%, and the median tumor-free survival period was 62 months. Univariate analysis identified preoperative serum carbohydrate antigen (CA)19-9 level >37 U/mL (normal upper range), increased tumor size >5 cm, moderately or poorly differentiated adenocarcinoma, R1 resection, intraoperative blood loss >1000 ml and low vessel density as detected by CD34 (<140/mm^2) were significantly associated with poor overall survivals ($p<0.05$).

Analysis of changes of attenuation of CT images in the tumor

Increased attenuation over the entire tumor ranged between -78 and 78 HU and the median value was 16 HU. Increased attenuation in the most enhanced area of tumor ranged between 1 and 97 HU, with a median of 37 HU. Differences in attenuation between tumor and non-tumor regions ranged between -37 and 106 HU, with a median difference of 11 HU. Each counted HU was measured between hypovascular and hypervascular ICC diagnosed by radiologists. Increased attenuation in the whole ICC was significantly higher for hypervascular ICC (n=12; 35.2±22.9 HU) in comparison with hypovascular ICC (n=30; 8.2±6.9 HU as above; p=0.0018). Increased attenuation in the most enhanced region of ICC was significantly higher in hypervascular ICC (n=12; 48.9±27.6 HU) than in hypovascular ICC (n=30; 26.2±28.2 HU; p=0.084). Differences in attenuation between ICC and non-tumorous regions were significantly higher in hypervascular ICC (n=12; 31.1±27.7 HU) than in hypovascular ICC (n=30; 7.9±32.9 HU; p=0.104). Fig. 2 shows the correlation between HU and histological microvessel density

as detected by CD34. Changes in attenuation for the entire ICC or in the most enhanced region of ICC showed a significant positive correlation with MVD ($p<0.05$). Hence, differences in attenuation between ICC and non-tumorous regions did not correlate significantly with MVD. As HFU mostly reflected tumor vascularity, HFU for the entire ICC in the representative section was applied for subsequent analyses.

Table 1 shows the relationship between changes in attenuation on contrast-enhanced CT and various clinicopathological parameters or tumor relapse. A median of 16 HFU was set as the cut-off value. Tumors showing increased attenuation >16 HFU were grouped as the Hyper group, while those showing ≤16 HU was grouped as the Hypo group. Age and sex were unrelated to tumor enhancement. In the Hyper group, a background of chronic viral hepatitis was significantly more frequent compared to the Hypo group ($p<0.05$). Co-existence of gallstones or hepaticolithiasis was not associated with tumor enhancement. Serum levels of tumor marker were likewise not associated with tumor enhancement. Tumors in the Hyper group were significantly larger than those in the Hypo group ($p<0.05$). Macroscopic findings of ICC were not associated with tumor enhancement. Histological components of well-differentiated adenocarcinoma were significantly more frequent in the Hyper group than in the Hypo group ($p<0.05$). Prevalence of intrahepatic metastasis or node metastasis did not differ between groups. Prevalence of postoperative tumor recurrence within 2 years after surgery was significantly better in the Hyper group than in the Hypo group ($p<0.05$).

Fig. 3 shows the relationship between enhancement pattern and overall survival after hepatectomy. Overall survival was significantly better in the Hyper group than in the Hypo group ($p<0.05$); the 1-, 3-, and 5-year survival rates were 78%, 69%, and 69% in the Hyper group, and 49%, 17%, and 17% in the Hypo group, respectively.

Discussion

ICC is a devastating malignancy that is showing increasing incidence worldwide [8]. Current imaging modalities such as CT and magnetic resonance imaging provide useful diagnostic information on malignant liver tumors [19], and imaging analysis is important for defining the indications for surgery [30]. The predominant radiological characteristic of ICC is that of hypovascular or marginally delayed enhancement of the mass in the portal phase [8, 20, 21, 31, 32], but tumor morphology and vascularity show different characteristics in some ICCs, particularly peripheral lesions [20, 21, 33-36]. Differences in tumor vascularity may be caused by differences in the background liver injury or cancer cell origin. Nakanuma and Xu et al. recently clarified a close relationship between specific histological characteristics of ICC and tumor vascularity on image analysis [24-26]. Our previous study clarified the relationship between tumor vascularity and pathogenesis or prognosis [16]. However, in this preliminary report, limitations to the definition of enhancement pattern were encountered, because this represented a subjective finding by the radiologist. We therefore attempted to calculate enhancement as an objective parameter using attenuation from high-resolution CT and analytical software to achieve more reliable evaluation.

In the present study, subjects were also divided into two types according to the pattern of ICC enhancement by applying a cut-off value for increased attenuation of 16 HU. In primary liver cancer, tumor enhancement in the early arterial phase is a basic characteristic of HCC [34, 35]. This characteristic is sometimes observed in some ICCs, excluding combined-type HCC with an ICC component . Our series identified a hypervascular tumor pattern in 43% of cases. In comparison with subjective findings, evaluations using attenuation consistently showed clear correlations between vascularity and tumor characteristics. To define tumor vascularity, a set of criteria for measuring the area of the ICC was necessary, because ICC usually shows heterogeneity of tumor components. We examined three patterns of measuring vascularity, as shown above, and measuring HU for the entire area of the ICC on tomography mostly reflected tumor vascularity as determined from radiologist findings and microvessel counts using CD34 in the present study. In cases where only highly enhanced lesions were measured, this level of attenuation may reflect the highest malignant potential, but setting a clear ROI in such an area was difficult. Differences in HU between tumor and non-tumor areas may

also reflect tumor vascularity, but this evaluation seemed to be reflected by the inflammatory or fibrotic status of the background liver parenchyma. As a result, at this stage, measuring HU in the whole tumor would be better for evaluating tumor vascularity, which was applied to examine the clinical significance of various factors in the present study.

Analysis of patient demographics revealed that the presence of chronic viral hepatitis was associated with hypervascular ICC, as seen in our pilot study [16]. The relationship between ICC and hepatitis viral infection closely related such a type of ICC, in which cancer cell origin or stem cells would be caused by chronic hepatitis [37-40]. Hepatocyte-originated tumors might be expected to show early enhancement, as seen in HCC [33, 35]. Carcinoembryonic antigen (CEA) and CA19-9 are useful diagnostic markers for both enhancement-rich tumors and hypovascular ICC [41]. However, significant differences in levels of these markers, including alpha-feto protein (AFP), a specific marker of HCC, was not observed between hyper- and hypovascular ICC in the present study. Yamamoto et al. reported that peripheral mass-forming ICC mimics the clinical characteristics of HCC, including imaging findings [42]. Mass-forming-type ICC with high vascularity may show HCC-like characteristics, but with significant differences in enhancement pattern and tumor morphology [43]. Larger tumor size or lower degree of histological differentiation were related to the classical hypovascular ICC. Such results may imply that hypervascular ICC represents an earlier stage before obtaining advanced malignant characteristics. At this stage, however, evidence confirming relationships between enhancement patterns and the degree of ICC development is lacking. On the other hand, in the present study, tumor vascularity pattern was not associated with other novel parameters of ICC malignancy, such as node metastasis. If this parameter of attenuation is associated with poor prognosis as described below, this might offer a promising prognostic marker.

Analysis of post-operative patient survival revealed that hypervascular ICC was closely associated with better overall survival, similar to our previous results [16, 17]. In other reports, hypovascularity or delayed enhancement have been associated with poor survival [34, 43], agreeing with the present results. ICC showing CT enhancement might thus indicate cancer aggressiveness or patient prognosis. As described above, hypervascular ICCs showed fewer malignant behaviors such as histological differentiation and may display cellular characteristics similar to HCC [24-26]. As the recurrence rate in

the early phase after hepatectomy was lower for patients with hypervascular ICC, tumor enhancement was markedly associated with malignant characteristics of ICC. As no clear reasons explaining such survival differences are apparent at this stage, further study with molecular analysis for circulating tumor cells or stem cells may help to clarify the cellular characteristics of ICC in detail [44, 45].

Conclusions

In conclusion, we examined the objective parameter of tumor enhancement using CT attenuation in patients with ICC who underwent hepatectomy. Hypervascular ICC showing attenuation >16 HFU in the arterial phase was associated with the presence of chronic viral hepatitis, well-differentiated adenocarcinoma, smaller tumor, lower frequency of early recurrence, and better survival after hepatectomy. Early enhancement in the arterial phase is useful for estimating lower malignant potential and better postoperative survival in ICC patients.

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Figure legends

Fig. 1. Measurement of HU by enhancement of ICC in the arterial phase on computed tomography. a) non-enhanced (plain) CT, b) region of interest (ROI) in the whole enhanced tumor at the arterial phase, c) ROI in the most enhanced area of the tumor at the arterial phase, d) ROI in the enhanced area of the non-tumorous liver

Fig. 2. Correlation between HU and histological microvessel density as detected by CD34.

Fig. 3. Relationship between patterns of tumor enhancement defined by increased HFU in the whole ICC on contrast-enhanced CT and overall survival in patients who underwent hepatic resection. Median survival was calculated using the Kaplan-Meier method, with differences between groups tested for significance using log-rank test.

Fig. 1.

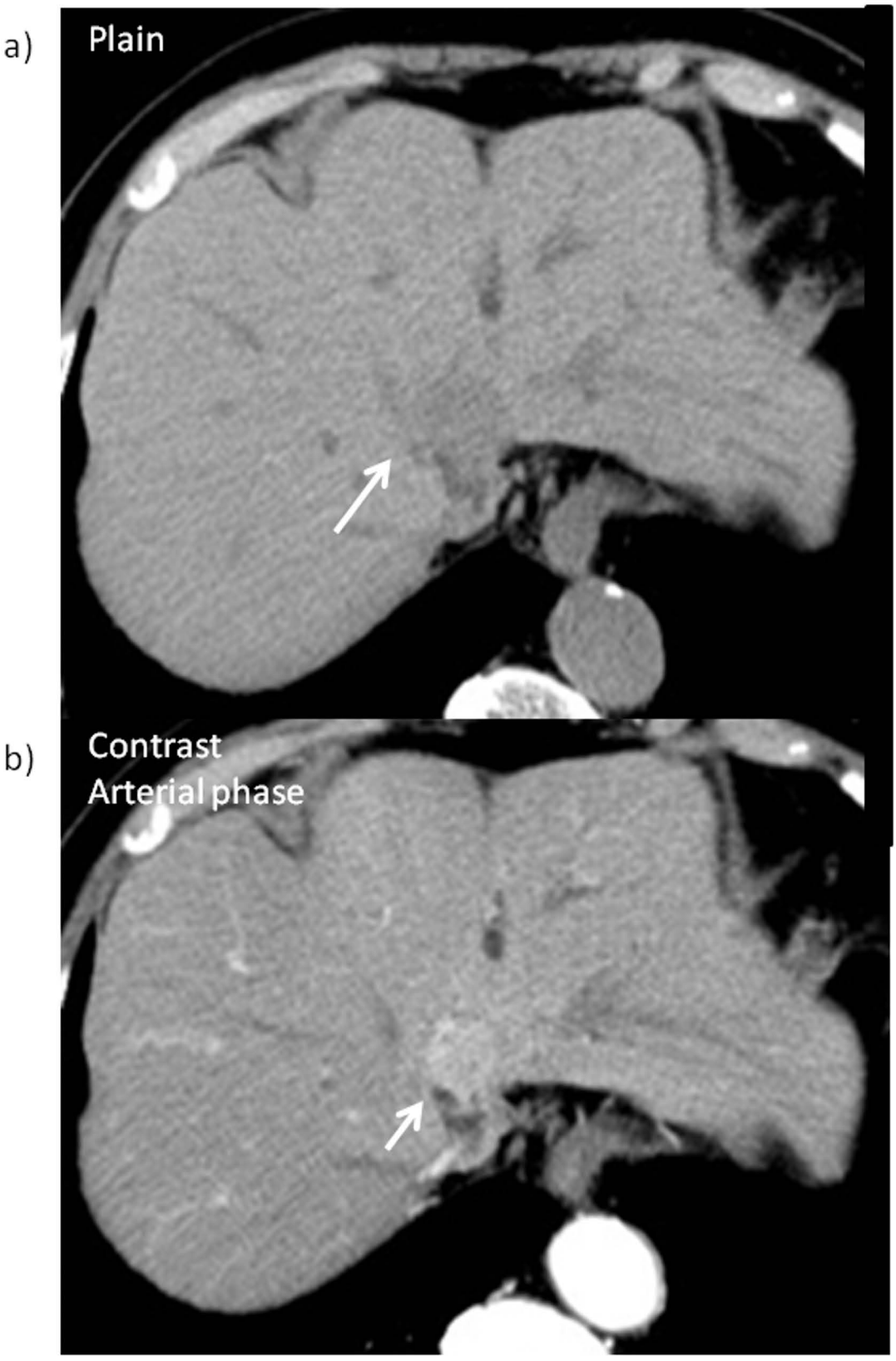

Fig. 1.

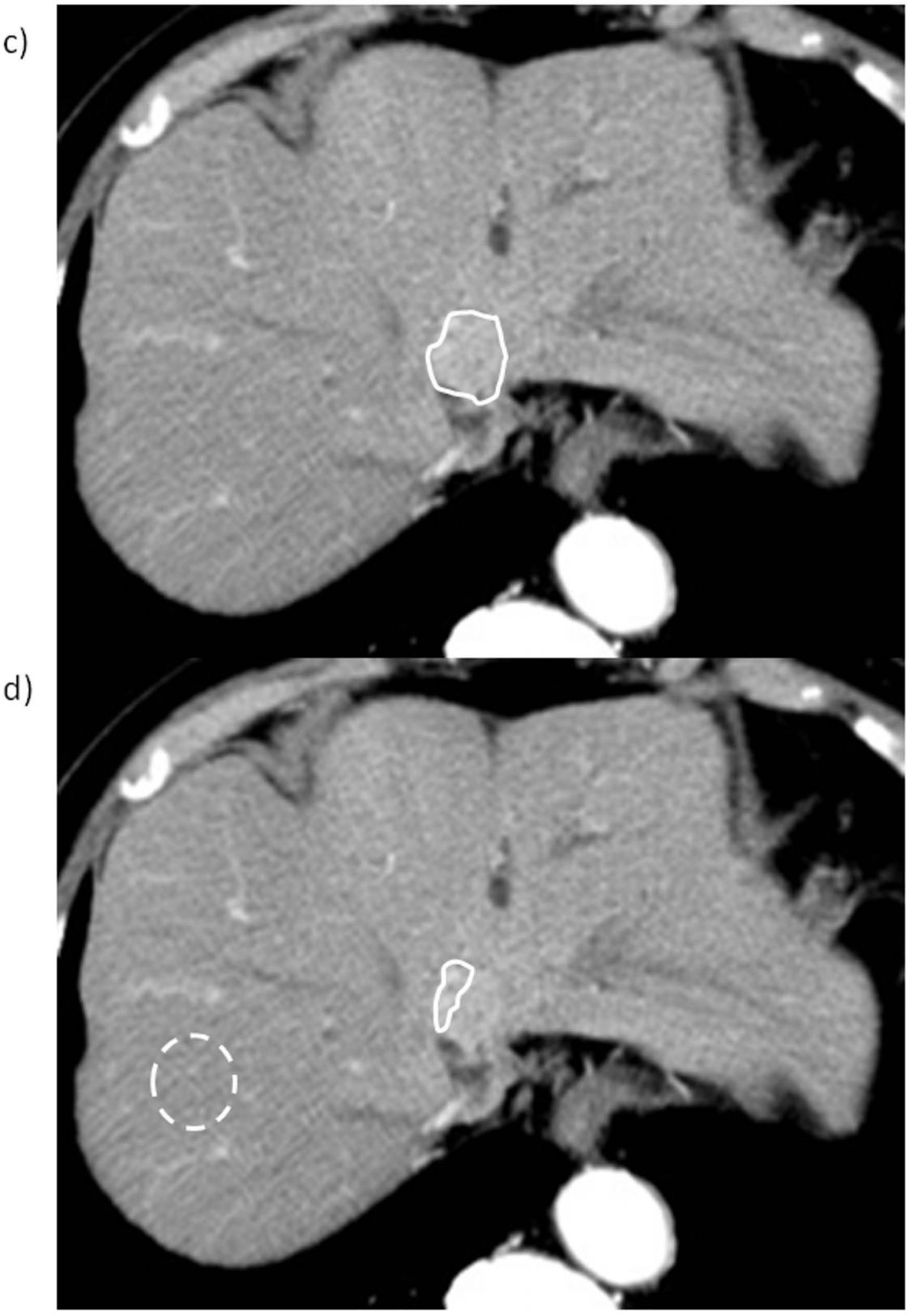

Fig. 2.

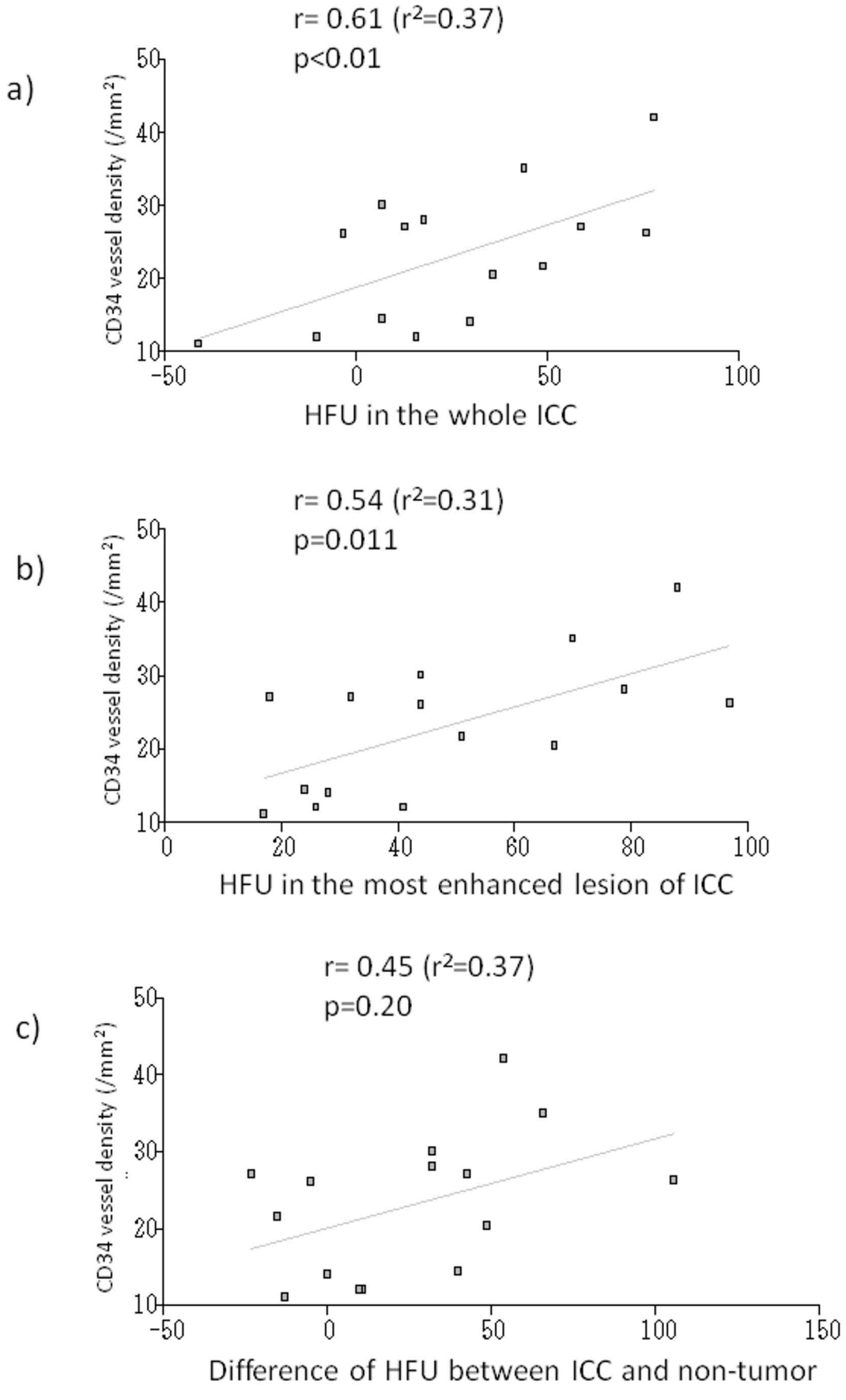
a)
r= 0.61 (r²=0.37)
p<0.01
CD34 vessel density (/mm²)
HFU in the whole ICC
b)
r= 0.54 (r²=0.31)
p=0.011
CD34 vessel density (/mm²)
HFU in the most enhanced lesion of ICC
c)
r= 0.45 (r²=0.37)
p=0.20
CD34 vessel density (/mm²)
Difference of HFU between ICC and non-tumor

Fig. 3.

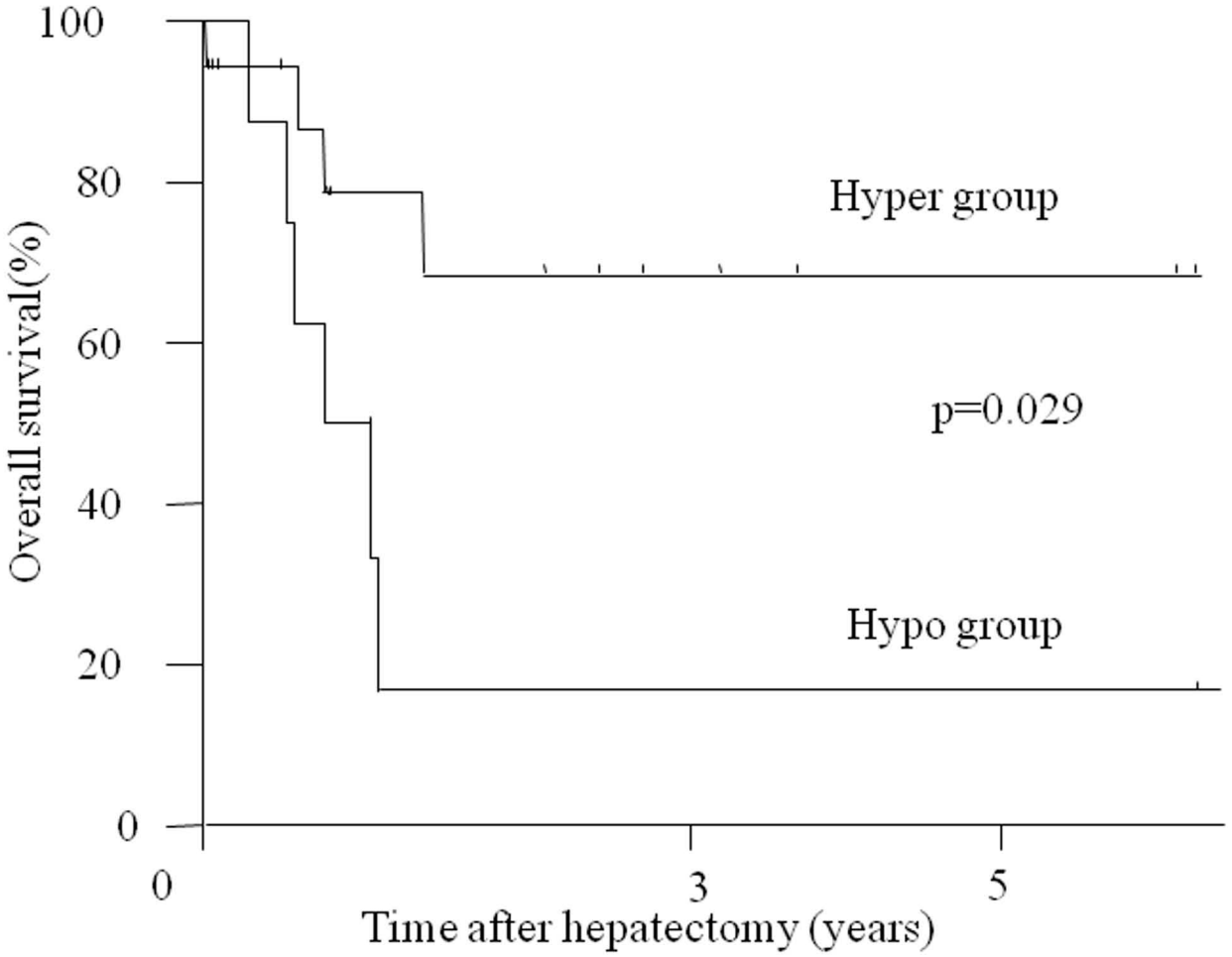
100
80
60
40
20
0
Overall survival(%)
Hyper group
p=0.029
Hypo group
0
3
5
Time after hepatectomy (years)

Table 1. Relationship between changes in attenuation on contrast-enhanced CT and various clinicopathological parameters or tumor relapse

	Tumor enhancement		P value
	Hypo group (n=24)	Hyper group (n=18)	
Gender			
Male/ female	17/7	12/6	0.87
Age (years)	70±9	71±8	0.92
Chronic viral hepatitis, no/ yes	20/ 4	7/ 11	0.045
Cholecysto- or hepaticolithiasis, no/yes	19/ 5	13/ 5	0.88
Serum tumor marker			
CEA (≤5 / >5 ng/ml)	12/ 12	13/ 5	0.26
CA19-9 (≤37 / >37 U/ml)	10/ 14	5/ 13	0.69
AFP (≤20 / >20 ng/ml)	22/ 2	16/ 2	0.99
Tumor size, ≤5 / >5cm	7/ 17	14/ 4	0.021
Macroscopic classification*			
Mass-forming/mass-forming with ductal invasion	11/ 13	7/ 11	0.86
Histological differentiation, well/ moderately/ poorly	2/15/ 7	8/ 6/ 4	0.023
PCNA labeling index(/1000 nuclei/5 fields)	176±143	157±111	0.80
Intrahepatic metastasis, no/ yes	18/ 6	11/ 0	0.15
Lymph node metastasis, no/ yes	16/ 8	8/ 10	0.23
Curability, R0/ R1	3/ 21	4/ 14	0.44
Postoperative tumor relapse within 2 years, no/ yes	10/14	14/ 4	0.035