Role of CD44 expression in non-tumor tissue on intrahepatic recurrence of hepatocellular carcinoma

Running head: CD44 expression in HCC

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Abstract

Background: CD44 is well known to be one of the cancer stem cell markers and is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion, and cell migration. We investigated the role of CD44 expression in both tumor and non-tumor tissues on recurrence of hepatocellular carcinoma (HCC).

Methods: Forty-eight patients with HCC who underwent hepatic resection at our institution were enrolled in this study. CD44 expressions in both tumor and non-tumor tissues were examined using real time reverse transcription-polymerase chain reaction. The patients were divided into two groups: high and low gene-expression group, based on the CD44 expression level. We compared the clinicopathological factors between the high expression and low expression groups in both tumor and non-tumor tissues.

Results: In the tumor tissues, the gene-expression levels of CD44 did not correlate with any clinicopathological parameters. The disease-free survival rate showed no significant difference between the two gourps. In non-tumor tissues, although there was no significant relationship between the CD44 expression levels and clinicopathological factors, disease-free survival rate in the CD44 low expression group was significantly better than that in the CD44 high expression group ($p<0.05$). In multivariate analysis, the risk factors in tumor recurrence were presence of microscopic portal invasion and high expression level of CD44.

Conclusion: The CD44 expressions in the non-tumor tissues may predict HCC recurrence.

Mini abstract

The CD44 expressions in the non-tumor tissues may be a significant prognostic factor in patients with hepatocellular carcinoma.

Key words: hepatocellular carcinoma, CD44, prognostic factor, non-tumor tissue

Abbreviations

HCC	hepatocellular carcinoma
RT-PCR	reverse transcription-polymerase chain reaction
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
AST	aspartate aminotransferase
ALT	alanine transaminase
WBC	white blood cell
PT	prothrombin time
$ICGR_{15}$	indocyanine green retention rate at 15 minutes
AFP	alpha-fetoprotein
DCP	des-gamma-carboxy prothrombin

Introduction

Hepatocellular carcinoma (HCC) is well known to be one of the most common malignancy and cause of cancer-related death. The HCC has poor prognosis, in advanced stages due to recurrence and distant metastasis [1, 2]. Some studies reported that the five-year survival rate of patients after curative resection of HCC were 30-50 percent [3]. Therefore, it is important to elucidate the prognostic factors and establish treatment strategy to overcome the poor prognosis of HCC.

CD44 is one of the cancer stem cell markers, highly glycosylated transmembrane protein, widely expressed cell surface hyaluronan receptor, with composes of a distal extracellular domain, a membrane-proximal region, a transmembrane spanning domain, and a cytoplasmic tail [4-6]. CD44 participates in a relatively diverse set of functions including lymphocyte homing, T-lymphocyte activation, signal transmission involved in cell proliferation, migration and apoptosis. CD44 also contribute to the activation of stem cell regulatory genes [5, 7, 8]. CD44 is essential to the physiological activities of normal cells, but it was also associated with pathologic activities of cancer cells [8].

Recently, a number of studies suggest that CD44 can involve for cancer stem cells. Jin L et al reported that CD44 is a key regulator function of leukemic stem cells in acute myeloid leukemia [9]. More recent studies have shown that stem cell-like properties are enriched in CD44-expressing subpopulations of some lung cancer cell lines and also reported that CD44 positive population may be the best to identify tumor initiating cells of human colon cancer [10, 11].

Compared with control counterparts, the enhanced expression of CD44 in malignant tissues can be detected in several cancers, such as breast cancer, colorectal cancer, renal cell carcinoma, HCC, gallbladder carcinoma, ovarian carcinoma [8-11]. Over expression of CD44 is also correlated with hallmarks of cancer biology including tumorigenesis, cell proliferation, and metastasis. CD44 is emerging as an important metastatic tumor marker and is also associated with an unfavorable prognosis for a variety of cancers, including HCC [6-8, 12-15].

However, the detailed pathological role of CD44 in HCC was unclear. The aim of this study

was, therefore, to investigate the expression of CD44 by real time reverse transcription-polymerase chain reaction (RT-PCR) in both tumor and non-tumor tissues, and to clarify its clinical significance.

Patients and methods

Patients

Forty-eight patients with HCC, who underwent hepatic resection at Tokushima University Hospital between 2005 and 2009, were included in this study.

Quantitative RT-PCR for CD44

The gene-expression levels of CD44 in the tumor and non-tumor liver tissues from the 48 HCC patients were evaluated by quantitative RT-PCR. Total RNA was extracted from using RNeasy Mini Kit (QIAGEN, Hilden, Germany). Quantitative RT-PCR was performed by the Applied Biosystems 7500 real-time PCR system, TaqMan Gene Expression Assays-on-demand, and TaqMan Universal Master Mix (Applied Biosystems). The following assays (assay identification number) were used: CD44 (Hs01075865_m1). TaqMan Human GAPDH Endogenous Control (4326317E) was used as control gene. Expression levels of the CD44 were calculated as a ratio to GAPDH. The thermal cycler conditions were as follows: 2 min at 50°C, 10 min at 95°C, then 40 cycles of 15 sec at 95°C and 1 min at 60°C. Amplification data were analyzed with an Applied Biosystems Prism 7500 Sequence Detection System version 1.3.1 (Applied Biosystems).

Patients follow-up

All patients were followed-up regularly in the outpatient clinic and monitored prospectively for recurrence by a standard protocol including serum AFP and des-gamma-carboxy prothrombin (DCP) level and ultrasound or contrast computed tomography (CT). Patients were followed up every 2 months during the first postoperative year and at least every 3–4 months afterward. AFP examination and liver ultrasonography were performed during each visit. CT scan of the abdomen was performed every 6 months. Bone scan or magnetic resonance imaging (MRI) was performed if localized bone pain was reported. A diagnosis of recurrence was based on typical imaging appearance in CT and/or MRI and an elevated AFP and/or DCP level.

Statistical analysis

All results were presented as a mean ± SD. For comparison of continuous variables, the Mann-Whitney U test was used, and the chi-squared test was applied for categorical data. Patient survival was calculated by the product limit method of Kaplan and Meier, and differences in survival rates between the groups were compared using the log rank test. Prognostic factors were examined using univariate and multivariate analyses (Cox proportional hazards regression model). The continuous variables were generally classified into two groups, according to the median value of each variable. All statistical analysis was performed using statistical software (JMP 8.0.1., SAS Campus Drive, Cary, NC). Statistical significance was defined as a P value less than 0.05.

Results

The expression level of CD44 mRNA in HCC

The patients were divided into two groups according to the median value of expression level in the tumor tissue: CD44 high expression group (n=25) and low expression group (n=23). There was no relationship between CD44 gene expression and clinicopathological factors. The expression level of CD44 mRNA did not correlate with any parameters, such as age, sex, HBsAg, HCVAb, tumor size, number and stage (Table 1). The disease-free survival rate showed no significant difference between the two gourps (Figure 1).

In the non-tumor tissue analysis, the 48 patients were also divided into two groups according to the median value of CD44 expression level: low expression group (n=24) and high expression group (n=24). There was no significant relationship between the expression level of CD44 mRNA and clinicopathological factors (Table 2). Liver function test such as serum albumin level, prothrombin time and $ICGR_{15}$ value didn't correlated with CD44 mRNA expression level. Similarly there was no significant correlation between tumor factors and CD44 mRNA expression. However, the disease-free survival rate in the CD44 low expression group was significantly higher than that in the CD44 high expression group (1-year: 70.1% vs. 50.1%, 3-year: 40.9% vs. 18.2%, $p<0.05$) (Figure 2).

Recurrence rate of CD44 high expression group was more frequent than that in the CD44 low expression group (70.8% vs. 41.7%), and most of the recurrent site was liver in both groups (Figure 3). The incidence of extrahepatic recurrence was higher in the CD44 high expression group (4/17) than that in the low expression group (1/10).

In the univariate analysis, Stage III or IV, microscopic portal invasion, intrahepatic metastasis, and CD44 high expression were determined as the significant risk factors of tumor recurrence. The multivariable analysis revealed presence of microscopic portal invasion and CD44 high expression were independent and significant risk factors of tumor recurrence (Table 3, 4).

Discussion

In this study, we investigated the expression of CD44 using quantitative RT-PCR and tried to clarify its clinical role. Our results showed that there was no significant relationship between the gene-expression levels of CD44 and clinicopathological factors in both the tumor tissues and the non-tumor tissues. The disease-free survival rate showed no significant difference between high and low CD44 expression group in the tumor tissues. In the non-tumor tissues, however, the disease-free survival rate in the CD44 low expression group was significantly higher than that in the CD44 high expression group. This is the first report suggesting that the gene-expression level of cancer stem cell markers in non-tumor tissues may be associated with a risk of tumor recurrence.

To clarify which cell types were responsible for the higher expression of CD44 in the non-tumor liver tissues, we investigated the CD44 expressions in the liver tissue specimens by immunohistochemical staining. However, we could not detect any CD44 expression in the non-tumor tissue (Figure 4). We speculated that there is no detectable CD44 expression at the protein level in each cell. We could detect CD44 expression in the non-tumor tissues by amplifying the expression at the mRNA level using RT-PCR.

There are at least two possible mechanisms to explain why the frequency of intrahehatic recurrence in the patients with high CD44 expression was significantly higher than that in those with low CD44 expression in the non-tumor tissues. The first is the possible explanation from the viewpoint of field cancerization, while the second is given from the viewpoint of micrometastasis.

Several studies have shown that the specific gene-expression patterns in cancerous tissues of HCC can accurately predict early recurrence, possibly due to the intrahepatic metastasis of HCC [16-18]. However, the outcomes of the patients with HCC even after a curative hepatectomy have been unsatisfied, at least partially, because of its multicentric origin [19, 20]. We have previously shown the importance to investigate the noncancerous portion of liver tissues to examine the molecular mechanisms during the process of liver carcinogenesis based on the idea of ‘‘field carcinogenesis’’ is necessary, because multicentric occurrence of HCC is mainly associated with underlying chronic liver damage rather than adverse tumor factors [21, 22]. The role of CD44 in

heopatocarcinogenesis remains unclear. However, Endo et al. have indicated that some isoforms of CD44 were positive in cirrhosis and chronic hepatitis, and these CD44 molecules might play roles in the early stage of HCC development [15]. Although a number of studies have shown that the tumor expression level of a cancer stem cell marker; CD44 is closely related to the tumor aggressiveness and poor prognosis, our findings suggest that the non-tumor expression level of CD44 may also be an important clinical indicator in terms of the hepatocarcinogenesis.

Another possible mechanism is a role of CD44 during the steps of tumor metastasis. Fundamentally, CD44 has a crucial role in regulating solid tumor cell adhesion to the surrounding normal tissues [23]. In the steps of tumor metastasis, cancer cells invade the basement membrane and underlying collagen matrix, migrate into the vascular system, adhere to endothelium of the target organ, and multiply to create a tumor. Invasion through the basement membrane represents one of the first step in this process, and CD44 was considered to be involved in this process of tumor metastasis [24, 25]. These findings explain the mechanisms by which high CD44 expression levels in tumor cells may be associated with a high potential of tumor metastasis. On the other hand, the contribution of CD44 to remodeling of microenvironment through the activation of cathepsin and metalloproteinase 9 has also been reported [24]. Therefore, it is conceivable that migrating HCC cells into the vascular system may be prone to adhere to non-tumor liver tissue, which is expressing high levels of CD44. Our data show that the pattern of recurrence was mainly intrahepatic recurrence. Although we did not determine the expression levels of CD44 in other organs, non-tumor liver tissues underlying chronic liver disease might express highest levels of CD44 compared to other organs.

In addition, a possible drawback of this study might be the relatively small number of patients examined. These findings and phenomenon should certainly be confirmed in larger number of patients with chronic liver disease and HCC.

In conclusion, the patients with high expression levels of CD44 in non-tumor liver tissues had a significantly poorer disease-free survival rate, mainly due to the intrahepatic recurrence. Therefore, the assessment of CD44 expressions in the non-tumor tissues may help us to identify a group of

patients who have a high risk for development of intrahepatic recurrence after hepatic resection for HCC.

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Figure legends

Figure 1

Disease-free survival in patients with low and high expression of CD44 in tumor tissue.

There was no significant difference between the two groups.

Figure 2

Disease-free survival in patients with low and high expression of CD44 in non-tumor tissue.

Disease free survival rate was significantly higher in CD44 low expression group than that in CD44 high expression group.

Figure 3

Recurrence pattern between CD44 high and low group.

Most of the recurrent site was liver in both groups.

MC: multicentric recurrence

Figure 4

CD44 expressions in the tumor and the non-tumor tissue by immunohistochemical staining.

CD44 expression in the non-tumor tissue was not observed.

T: tumor, NT: non-tumor

Table 1. Relationship between tumor tissue expressions of CD44 with clinicopathological variables

Factor	CD44 low (n=23)	CD44 high (n=25)	*P* value
Age (year)	66.7	68.1	0.60
Gender (M/F)	15/8	19/6	0.41
HBs-Ag (+/-)	6/17	9/16	0.46
HCV-Ab (+/-)	9/14	10/15	0.95
Albumin (g/dl)	3.6±0.9	3.6±0.5	0.90
T-Bil (mg/dl)	0.8±0.3	0.9±0.3	0.24
ALT (IU/L)	41±28	41±25	0.98
PT (sec)	11.2±2.6	12.0±0.8	0.13
$ICGR_{15}$ (%)	12.7±6.6	14.0±8.9	0.57
Stage (I, II / III, IV)	15/8	11/14	0.14
Maximum diameter (cm)	5.0±3.5	4.6±3.5	0.71
Number (single/multiple)	16/7	19/7	0.75
Background liver (LF, CH / LC)	15/8	17/8	0.92
fc (+/-)	16/7	16/9	0.75
vp (+/-)	6/17	8/17	0.55
Differentiation (well / mod, por)	3/20	4/21	0/75
AFP (>200ng/ml)	5/12	7/17	0.62
DCP (>400mAU/ml)	12/10	9/13	0.36

ALT: alanine transaminase, PT: prothrombin time, ICGR15: indocyanine green

retention rate at 15 minutes, LF: liver fibrosis, CH: chronic hepatitis, LC: liver cirrhosis, AFP: alpha-fetoprotein, DCP: des-gamma-carboxy prothrombin

Table 2. Relationship between non-tumor tissue expressions of CD44 with clinicopathological variables

Factor	CD44 low (n=24)	CD44 high (n=24)	*P* value
Age (year)	66.7	68.0	0.88
Gender (M/F)	20/4	14/10	0.06
HBs-Ag (+/-)	10/14	5/19	0.17
HCV-Ab (+/-)	8/16	11/13	0.38
Albumin (g/dl)	3.8±0.5	3.6±0.5	0.18
T-Bil (mg/dl)	0.9±0.3	1.0±0.3	0.55
ALT (IU/L)	42.7±28.2	43±27	0.76
PT (sec)	11.8±1.0	11.8±0.9	0.97
$ICGR_{15}$ (%)	13.9±7.6	14.4±8.6	0.91
Stage (I, II / III, IV)	16/8	10/14	0.08
Maximum diameter (cm)	4.4±4.0	4.1±2.8	0.23
Number (single/multiple)	19/5	16/8	0.26
Background liver (LF, CH / LC)	17/7	14/10	0.26
fc (+/-)	16/8	15/9	0.91
vp (+/-)	7/17	7/17	0.53
Differentiation (well / mod, por)	3/21	4/20	0.32
AFP (>200ng/ml)	6/18	6/16	0.86
DCP (>400mAU/ml)	8/15	13/8	0.07

ALT: alanine transaminase, PT: prothrombin time, ICGR15: indocyanine green

retention rate at 15 minutes, LF: liver fibrosis, CH: chronic hepatitis, LC: liver cirrhosis, AFP: alpha-fetoprotein, DCP: des-gamma-carboxy prothrombin

Table 3. Risk factors in tumor recurrence: univariate analysis

Variable		3 year survival (%)	p Value
Host factors at recurrence			
Gender			
male	(n=34)	34.9	0.5857
female	(n=14)	20.3	
Age			
≤70	(n=23)	41.6	0.7711
>70	(n=25)	19.5	
Diabetes			
absent	(n=30)	29.5	0.2901
present	(n=18)	36.0	
Hypertension			
absent	(n=24)	12.4	0.1606
present	(n=24)	44.3	
HBsAg			
negative	(n=33)	21.9	0.6620
positive	(n=15)	47.9	
HCV			
negative	(n=29)	49.7	0.1083
positive	(n=19)	9.9	
Child`s class			
A	(n=45)	33.0	0.4584
B	(n=3)	0	
PT			
≤12 sec	(n=29)	72.3	0.8053
>12 sec	(n=19)	35.5	
TBil			
≤1.0 mg/dL	(n=34)	37.4	0.0633
>1.0 mg/dL	(n=14)	13.4	
Albumin			
≥3.5 g/dL	(n=35)	33.0	0.5973
<3.5 g/dL	(n=13)	23.4	
ALT			
≤40 IU/dL	(n=31)	28.7	0.4434
>40 IU/dL	(n=17)	36.8	
ICG R_{15}			
≤10%	(n=18)	22.4	0.7765
>10%	(n=30)	37.9	
Background liver			
LF, CH	(n=32)	33.6	0.5349
LC	(n=16)	35.6	
Stage			
I, II	(n=26)	50.2	0.0275
III, IV	(n=22)	15.0	
AFP			
≤200 ng/ml	(n=34)	34.0	0.0723
>200 ng/ml	(n=12)	0	
DCP			
≤400 mAU/ml	(n=23)	32.9	0.5608
>400 mAU/ml	(n=21)	24.2	
Tumor factors			
Tumor number			
single nodule	(n=34)	40.4	0.4635
multiple nodules	(n=11)	20.0	
Tumor size			
≤3 cm	(n=23)	40.4	0.3709
>3 cm	(n=23)	26.7	

Histology			
Well	(n=6)	33.3	0.3966
Mod, por	(n=38)	40.0	
Fc			
absent	(n=15)	44.0	0.6814
present	(n=31)	32.0	
vp			
absent	(n=33)	44.0	0.0093
present	(n=12)	19.0	
Im			
absent	(n=37)	49.6	0.0012
present	(n=9)	0	
CD 44			
high	(n=24)	18.2	0.0337
low	(n=24)	50.1	

PT: prothrombin time, ALT: alanine transaminase, ICGR15: indocyanine green retention rate at 15 minutes, LF: liver fibrosis, CH: chronic hepatitis, LC: liver cirrhosis, AFP: alpha-fetoprotein, DCP: des-gamma-carboxy prothrombin

Table 4. Risk factors in tumor recurrence: multivariate analysis

	Multivariate		
	H.R.	**95% C.I.**	***p*-value**
vp (+)	2.907	0.119-1.000	0.0499
im (+)	1.996	0.160-1.570	0.2352
Stage III, IV	1.002	0.338-2.946	0.9965
CD44: high	2.963	1.111-7.907	0.0300

H.R – Hazard Ratio C.I - Confidence interval

Figure 1

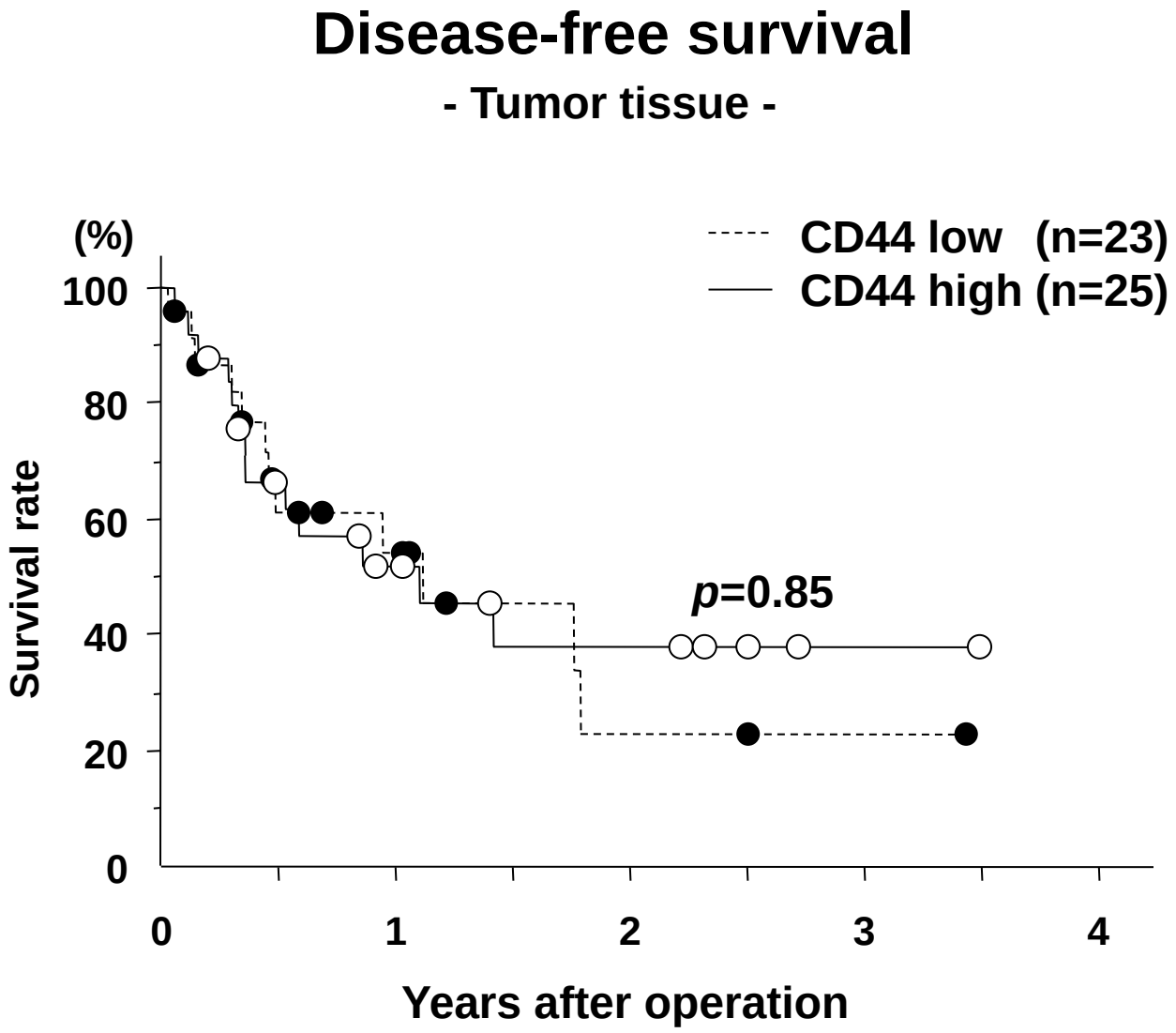
Disease-free survival
- Tumor tissue -
(%)
100
80
60
40
20
0
Survival rate
CD44 low (n=23)
CD44 high (n=25)
p=0.85
0
1
2
3
4
Years after operation

Figure 2

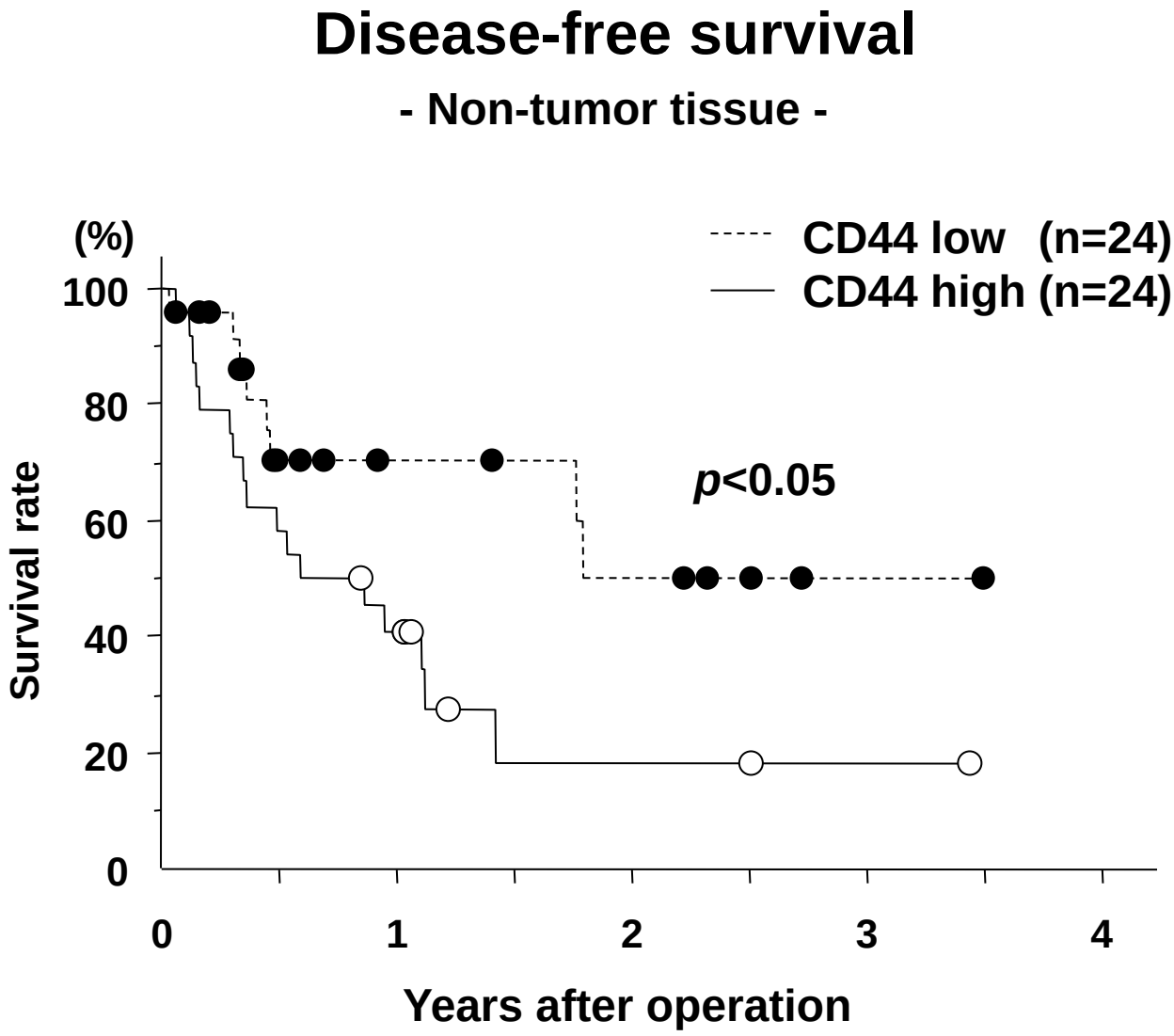
Disease-free survival
- Non-tumor tissue -
(%)
100
80
60
40
20
0
Survival rate
CD44 low (n=24)
CD44 high (n=24)
p<0.05
0
1
2
3
4
Years after operation

Figure 3

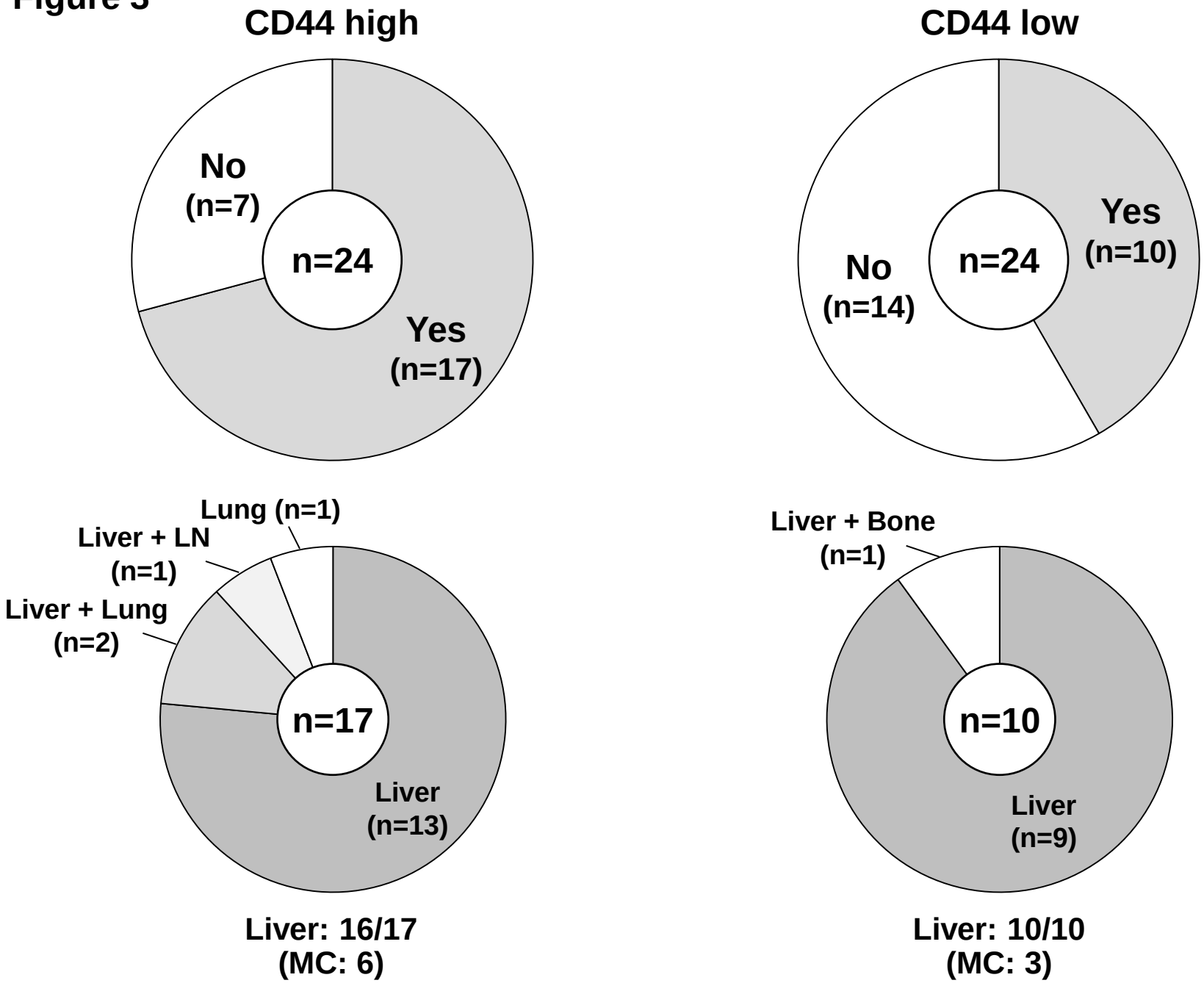
CD44 high
No
(n=7)
n=24
Yes
(n=17)
CD44 low
No
(n=14)
n=24
Yes
(n=10)
Lung (n=1)
Liver + LN
(n=1)
Liver + Lung
(n=2)
n=17
Liver
(n=13)
Liver: 16/17
(MC: 6)
Liver + Bone
(n=1)
n=10
Liver
(n=9)
Liver: 10/10
(MC: 3)

Figure 4