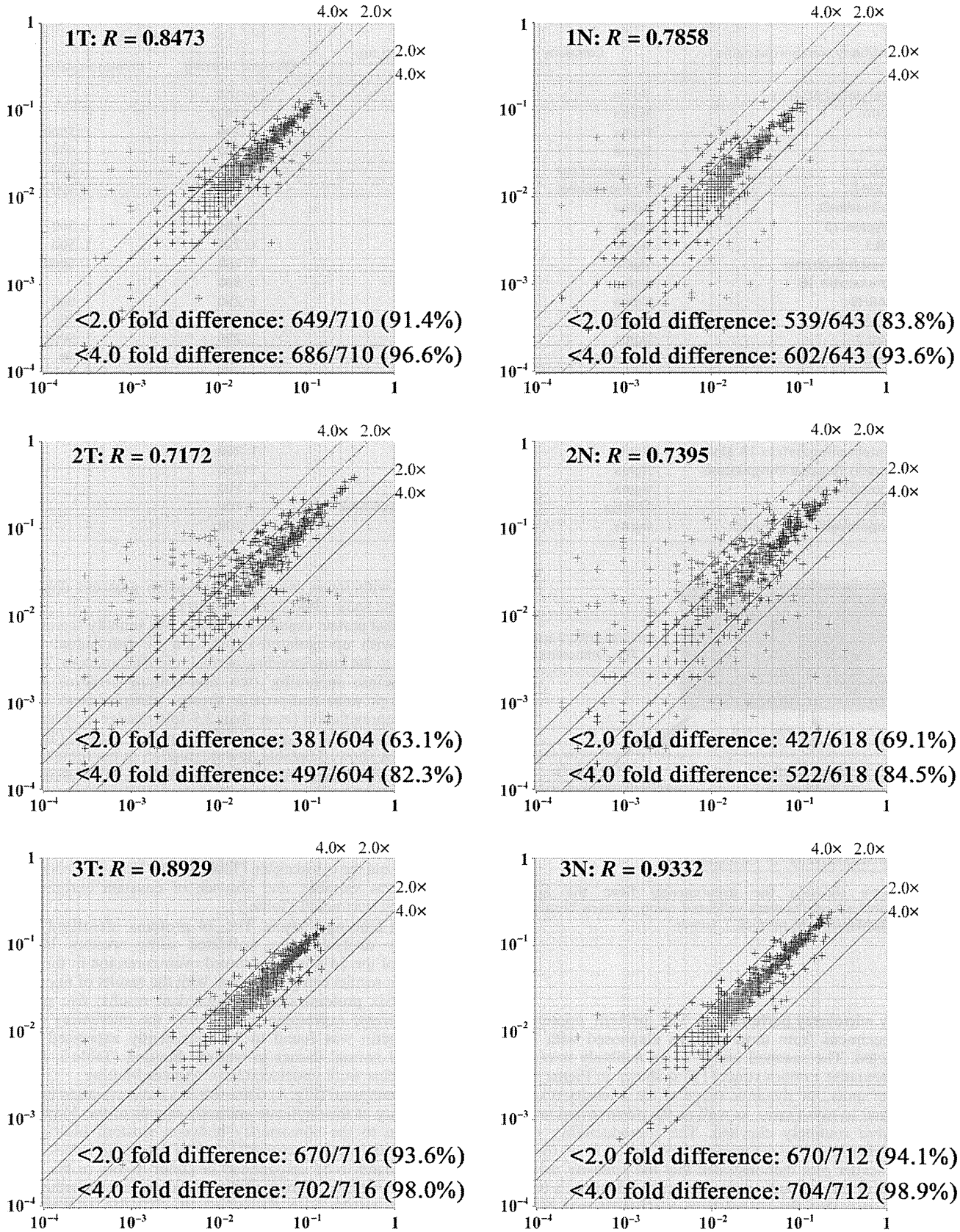

Fig. 2. Reproducibility of the antibody microarray analysis. The scatter plot shows that the intensity values of 63.1–94.1% of the antibodies contained in the microarrays used were scattered within a twofold difference, and that the correlation coefficient (*R*) was 0.7172–0.9332. X-axis, intensity of one spot of duplicate spots; Y-axis, intensity of other spot of duplicate spots.

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Table 2. Proteins found to be upregulated in esophageal squamous cell carcinoma tissues in the antibody microarrays

No.	Commercial antibody name	Official protein name	Gene symbol	Entrez gene ID	Biological function	Locus	Cellular localization
1	Acetylated protein	–	–	–	–	–	–
2	Actin	Actin, alpha 1, skeletal muscle	ACTA1	58	Cytoskeleton	1q42.13	Cytoplasm
3	AP-1	jun oncogene	JUN	3725	Cell cycle	1p32-p31	Cytoplasm, nucleus
4	AP-2a	Transcription factor AP-2 alpha	TFAP2A	7020	Transcription	6p24	Nucleus
5	BAD	Bcl-associated death promoter	BAD	572	Apoptosis	11q13.1	Cytoplasm, membrane, mitochondrion
6	BUBR1	BUB 1 budding uninhibited by benzidazoles 1 homolog beta	BUB1B	701	Cell cycle	15q15	Cytoplasm, nucleus
7	Calbindin-D	Calbindin 1	CALB1	793	Calcium-associated	8q21.3–q22.1	Cytoplasm, nucleus
8	Caspase 10	Caspase 10, apoptosis-related cysteine peptidase	CASP10	843	Apoptosis	2q33–q34	Cytoplasm
9	Cdk1	Cell division cycle 2, G1 to S and G2 to M	CDC2	983	Cell cycle	10q21.1	Cytoplasm, nucleus
10	phosph-beta-catenin	Catenin (cadherin associated protein), beta 1	CTNNB1	1499	Signal transduction	3p21	Cytoplasm, nucleus, membrane
11	Cytokeratin 18	Keratin 18	KRT18	3875	Cytoskeleton	12q13	Cytoplasm
12	HABH3	alkB, alkylation repair homolog 3	ALKBH3	221 120	Oxidation reduction	11p11.2	Cytoplasm, nucleus
13	hnRNP-U	Heterogeneous nuclear ribonucleoprotein U	HNRPU	3192	RNA splicing	1q44	Nucleus, cell surface
14	Mad 2	MAD2 mitotic arrest deficient-like 1	MAD2L1	4085	Cell cycle	4q27	Cytoplasm, nucleus
15	NAK	TANK-binding kinase 1	TBK1	29 110	Immune response	12q14.1	Cytoplasm
16	NG2	Chondroitin sulfate proteoglycan 4	CSPG4	1464	Signal transduction	15q24.2	Membrane, cell surface
17	b-NOS	Nitric oxide synthase 1 (neuronal)	NOS1	4842	Oxidation reduction	12q24.2–q24.31	Cytoplasm
18	NGFb	Nerve growth factor	NGFB	4803	Signal transduction	1p13.1	Extracellular region, Golgi
19	RAB 9	RAB9, member RAS oncogene family	RAB9	9367	Signal transduction	Xp22.2	Golgi, lysosome, membrane
20	Protein phosphatase 2A alpha	Protein phosphatase 2, catalytic subunit, alpha isoform	PPP2CA	5515	RNA splicing	5q31.1	Cytoplasm, nucleus, mitochondrion, membrane
21	Protein tyrosine phosphatase	–	–	–	–	–	–
22	phospho-Pyk2	PTK2 protein tyrosine kinase 2 beta	PTK2B	2185	Apoptosis	8p21.1	Cytoplasm, membrane
23	SKK2	Mitogen-activated protein kinase kinase 3	MAP2K3	5606	Signal transduction	17q11.2	Protein complex
24	Tropomyosin (Sarcimatic)	Tropomyosin 1 (alpha)	TPM1	7168	Cytoskeleton	15q22.1	Cytoplasm, nucleus

experiments may not guarantee concordant results between western blotting and antibody microarray experiments.

Correlation between microarray and western blotting data. The quantitative correlation between the microarray and western blotting data was examined. Each tumor tissue's data were normalized by the normal counterpart's data. The correlation coefficient for AP-1 and AP-2α could not be calculated due to missing microarray values. The 14 proteins overexpressed in tumor tissues in western blotting included proteins with a high correlation coefficient, such as Mad2 ($R = 0.934$) and NAK ($R = 0.763$), as well as proteins with a poor correlative relationship, such as hABH3 ($R = -0.981$) and NG2 ($R = -0.933$) (Table 3; Fig. 5).

Immunohistochemistry. We carried out immunohistochemistry to verify the abundance and cellular localization of the 14 proteins with high expression levels in ESCC as revealed by western blotting (Table 3). The expression of six proteins (AP-1, BUBR1, caspase 10, Cdk1, NAK, and Mad2), either cytoplasmic or nuclear, was stronger in ESCC compared with normal epithelium. Nuclear expression of heterogeneous nuclear ribonucleoprotein U (hn-RNP-U) was observed in all tumor and normal tissues. The expression of Bcl-associated death promoter (BAD) and AP-2α was higher in infiltrating inflammatory cells compared with the tumor stroma (Fig. 6). The expression of p-β-catenin, HABH3, and member RAS oncogene family (RAB) was similar in tumor and normal tissues (data not shown). Nitric oxide synthase 1 (b-NOS) and NG2 expression were not detected immunohistochemically (data not shown).

Of the six proteins with stronger immunohistochemical expression in ESCC, tissue microarray analysis showed that BUBR1, Mad2, AP-1, caspase 10, NAK, and Cdk1 were overexpressed in 80, 86, 73, 73, 63, and 90% of ESCC patients, respectively, compared with normal tissues. There was no association between BUBR1, AP-1, caspase 10, or Cdk1 expression with clinicopathological characteristics. Overexpression of Mad2 and NAK was infrequent in tumors histologically categorized as grade 3 or with lymph node metastases, respectively (Table 4).

mRNA expression. The mRNA expression levels of 22 proteins identified by antibody microarray as overexpressed in tumors

Table 3. Summary of the results of western blotting and immunohistochemistry

No.	Antibody commercial name	WB	R (WB array)	Ihc localization
1	Acetylated protein	Undetectable	ND	ND
2	Actin	T = N	ND	ND
3	AP-1	T > N	†	Cytoplasm, nucleus
4	AP-2a	T > N	†	Inflamatory cells
5	BAD	T > N	0.351	Inflamatory cells
6	BUBR1	T > N	−0.178	Cytoplasm
7	Calbindin-D	T < N	ND	ND
8	Caspase 10	T > N	−0.572	Cytoplasm
9	Cdk1	T > N	0.583	Nucleus
10	phosph-beta-catenin	T > N	−0.699	Cytoplasm
11	Cytokeratin 18	T < N	ND	ND
12	HABH3	T > N	−0.981	Nucleus
13	hnRNP-U	T > N	−0.458	Nucleus
14	Mad 2	T > N	0.934	Cytoplasm
15	NAK	T > N	0.763	Cytoplasm
16	NG2	T > N	−0.933	Undetectable
17	b-NOS	T > N	0.708	Undetectable
18	NGFb	Undetectable	ND	ND
19	RAB 9	T > N	0.805	Cytoplasm
20	Protein phosphatase 2A alpha	T < N	ND	ND
21	Protein tyrosine phosphatase	T < N	ND	ND
22	phospho-Pyk2	Undetectable	ND	ND
23	SKK2	Undetectable	ND	ND
24	Tropomyosin (Sarcimatic)	T < N	ND	ND

†Not calculated due to missing array data. IHC, immunohistochemistry; ND, not done; *R*, correlation coefficiency of the western blotting compared to the microarray results; WB, western blotting.

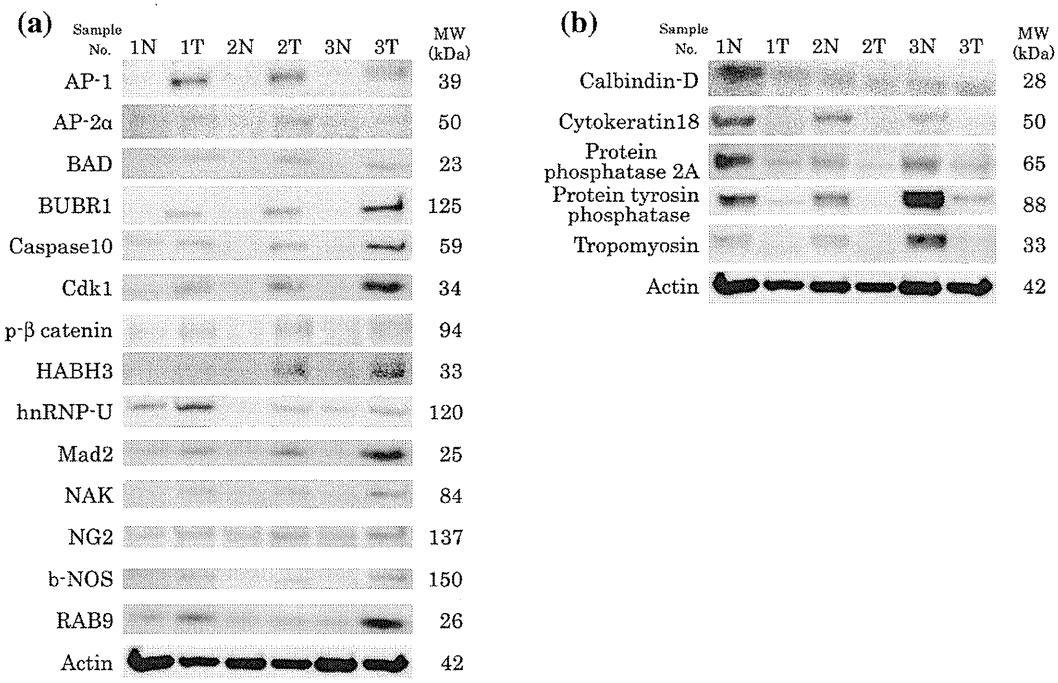

Fig. 3. Western blotting results. (a) Fourteen of the 24 proteins identified in the microarray analysis as being overexpressed in tumor tissues showed concordant results in western blotting analysis. (b) Five proteins showed discordant results.

were examined and compared with western blotting results (Fig. 7). Among the 14 proteins found to be overexpressed in ESCC in western blotting analysis, nine (64%) proteins were also overexpressed at the mRNA level. The expression of two (50%) of four proteins found to be lower in ESCC by western blotting was also lower at the mRNA level. Discordance between the mRNA and western blotting results was observed in approximately 40% of the proteins studied.

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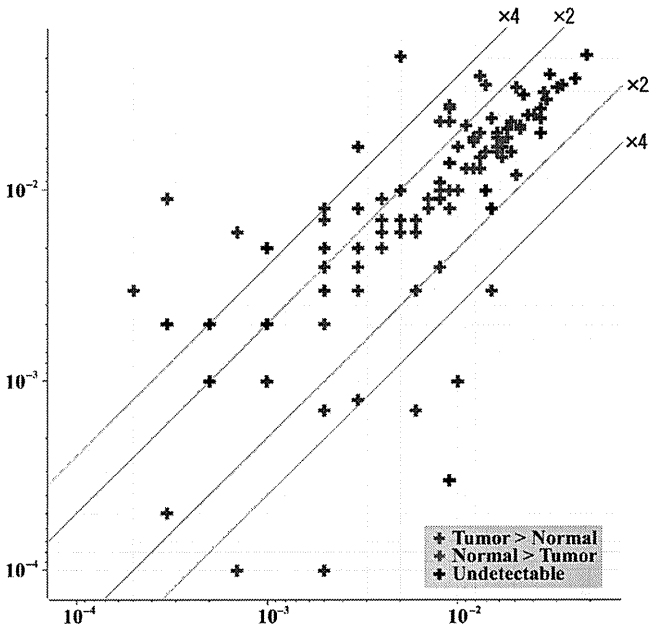

Fig. 4. The scattergram demonstrates the correlation between the intensity of duplicate antibody signals of the 24 proteins found to be overexpressed in the microarray analysis. The intensity values of the proteins found to be overexpressed in tumor tissues in the microarrays showed high reproducibility, irrespective of whether these microarray results were discordant ($R = 0.7666$) or concordant ($R = 0.6921$) with western blotting results, or whether these proteins were not detected by western blotting ($R = 0.6317$). X-axis, intensity of one spot of duplicate spots; Y-axis, intensity of other spot of duplicate spots.

Discussion

The study of cancer through the use of antibody microarrays provides valuable information on protein expression levels, isoforms expressed, and post-translational modifications concerning the particular cancer studied. Furthermore, as many reports have demonstrated discordance between mRNA and protein expression,[15–17] which concerned approximately 40% of proteins in this study, the information provided more closely reflects cancer phenotypes. As well as in separation-based proteomics such as that using two-dimensional gel electrophoresis,[7,18,19] antibody microarrays have been used in the study of colorectal,[20] breast,[21,22] pancreatic,[23,24] bladder,[25] and prostate cancer,[26] squamous cell carcinoma of the oral cavity,[27,28] hepatocellular carcinoma,[29] and gastric cancer.[30] Although several proteomic studies have been reported,[7,8] antibody microarrays have not been used to date in esophageal cancer.

Microarray analysis identified 24 proteins that were overexpressed in the tumor tissues compared with the corresponding normal tissues. Examination of these proteins by western blotting and immunohistochemistry validated these results for six of these proteins. Of the six proteins, Mad2 and BubR1 are crucial components of a functional mitotic checkpoint by organizing spindle assembly.[31] Overexpression of the *Mad2* gene has been reported in nasopharyngeal,[32] gastric,[33] ovarian,[34] colorectal,[35] hepatocellular,[36] and esophageal cancer,[37] whereas BubR1 is overexpressed in colorectal,[38] gastric,[39] and esophageal cancer.[37] Tanaka *et al.* reported that Mad2 and BubR1 are coordinately overexpressed in ESCC, and were overexpressed in 89 and 95% of ESCC, respectively, compared with normal tissues.[37] Although they reported no correlation between any clinicopathological

Table 4. Tissue microarray results

Variable	*n*	BUBR1	Mad2	AP-1	Caspase 10	NAK	Cdk1
All cases	70	56 (80%)	60 (86%)	51 (73%)	51 (73%)	44 (63%)	63 (90%)
Sex							
Male	55	44 (80%)	49 (89%)	40 (73%)	40 (73%)	33 (60%)	49 (89%)
Female	15	12 (80%)	11 (73%)	11 (73%)	11 (73%)	11 (73%)	15 (100%)
Age (years)							
<60	38	29 (76%)	32 (84%)	27 (71%)	25 (66%)	20 (53%)	34 (89%)
≥60	32	27 (84%)	28 (88%)	24 (75%)	26 (81%)	24 (75%)	29 (91%)
Histological grade							
1	16	12 (75%)	16 (100%)*	13 (81%)	9 (56%)	6 (38%)	14 (88%)
2	42	33 (79%)	39 (93%)*	31 (74%)	33 (79%)	29 (69%)	39 (93%)
3	9	8 (89%)	2 (22%)*	4 (44%)	6 (67%)	7 (78%)	8 (89%)
X	3	3 (100%)	3 (100%)	3 (100%)	3 (100%)	2 (67%)	2 (67%)
Tumor stage							
T1	12	10 (83%)	12 (100%)	9 (75%)	9 (75%)	9 (75%)	11 (92%)
T2	7	5 (71%)	6 (86%)	7 (100%)	6 (86%)	4 (57%)	7 (100%)
T3	10	8 (80%)	8 (80%)	8 (80%)	10 (100%)	5 (50%)	9 (90%)
T4	1	0 (0%)	1 (100%)	1 (100%)	0 (0%)	0 (0%)	1 (100%)
Lymph node metastasis							
Negative	17	13 (76%)	15 (88%)	12 (71%)	13 (76%)	14 (82%)*	16 (94%)
Positive	13	10 (77%)	12 (92%)	13 (100%)	12 (92%)	4 (31%)*	12 (92%)
Distant metastasis							
Negative	25	19 (76%)	23 (92%)	21 (84%)	20 (80%)	15 (60%)	23 (92%)
Positive	5	4 (80%)	4 (80%)	4 (80%)	5 (100%)	3 (60%)	5 (100%)
Tumor size (mm)							
<40	14	9 (64%)	12 (86%)	11 (79%)	10 (71%)	10 (71%)	14 (100%)
≥40	16	14 (88%)	15 (94%)	14 (88%)	15 (94%)	8 (50%)	14 (88%)

*Statistically significant differences ($P < 0.05$). BUBR, budding uninhibited by benzidazoles 1 homolog beta; Mad, mitotic arrest deficient-like 1; AP, activator protein; NAK, TANK-binding kinase 1; Cdk, cyclin-dependent kinase.

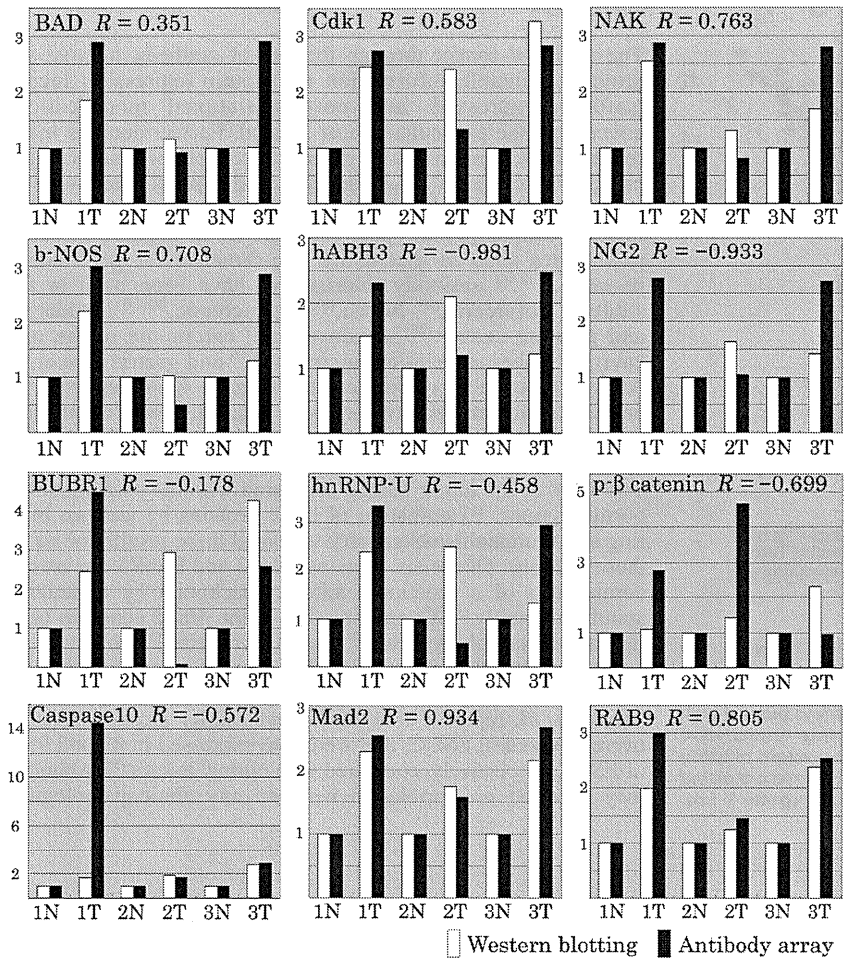

Fig. 5. Comparison of the microarray with the western blotting data. The correlation coefficient of the 14 proteins found to be overexpressed in tumor tissues in western blotting was calculated. Normalization of each tumor tissue's data was carried out using the normal counterpart's data.

data, including the histological grade and the expression of these proteins, we identified a correlation between Mad2 expression and histological grade (Table 4). This correlation has also been reported in gastric cancer[33] and hence is a finding that warrants further investigation. In this study, we detected overexpression of Mad2 and BubR1, which were included in cell cycle regulation, in ESCC in different cohorts. Recently, the relationship between the mitotic checkpoint and drug sensitivity has been the subject of extensive investigations.[40] These proteins may be considered as important determinants of the efficacy of microtubule-targeting drugs in killing cancer cells.

Cdk1 is a member of the CDK family, which regulates phase transitions and checkpoints within the cell cycle.[41,42] Under non-neoplastic conditions, Cdk1 is localized to the nucleus where it regulates cell cycle progression as a subunit of the M-phase promoting factor, together with a cyclin B subunit. Within the normal esophageal mucosa, Cdk1 is localized to the parabasal cell layer, where the majority of normal cellular proliferation occurs.[43] In this study, the fact that these three proteins serving crucial roles in M-phase regulation were concurrently identified may indicate that one or all of them may contribute significantly to the carcinogenesis of ESCC.

The remaining three proteins, AP-1, caspase 10, and NAK, are associated with NF-κB, which is a signal transduction factor that has emerged as an important modulator of altered gene programs and malignant phenotype in the development of cancer.[44] In one esophageal adenocarcinoma study, 61% of resected tumors displayed NF-κB immunoreactivity and 87.5% of the NF-κB-positive tumors were Stage IIb or III.[45] Constitutive activation of the NF-κB and AP-1 signal transduction pathways have been identified as prominent events promoting tumor progression of hemopoietic and solid malignancies.[46–48] Overexpression of a caspase 10 subclass dramatically enhances NF-κB activity in a dose- and time-dependent manner.[49] NAK phosphorylates a NF-κB subunit and increases its activity.[50] In this study, the correlation between underexpression of NAK and the presence of lymph node metastases was revealed (Table 4); although NAK has been reported as a mediator of tumor angiogenesis,[51] this correlation had not been reported to date and, again, additional investigation is necessary. To the best of our knowledge, aberrant expression of these proteins has not been previously reported in ESCC. Our results also suggest that these members of the NF-κB signaling pathway may play an important role in the carcinogenesis of ESCC.

In this study, the reproducibility of the antibody microarray results was poor (Fig. 2) and varied between the experiments, with the correlation coefficient ranging from 0.7172 to 0.9332. It remains a challenge to improve the reproducibility of the results obtained using antibody microarrays such as the ones in our study. To achieve this, Ellmark *et al.* used eight replicates in a microarray and excluded the two highest and two lowest replicates, and each data point represented the mean value of the remaining four replicates.[30] When space allows, the antibody microarrays should therefore be spotted with more replicates in the same microarray to improve reproducibility. Furthermore, as the degree of reproducibility was different based on the signal

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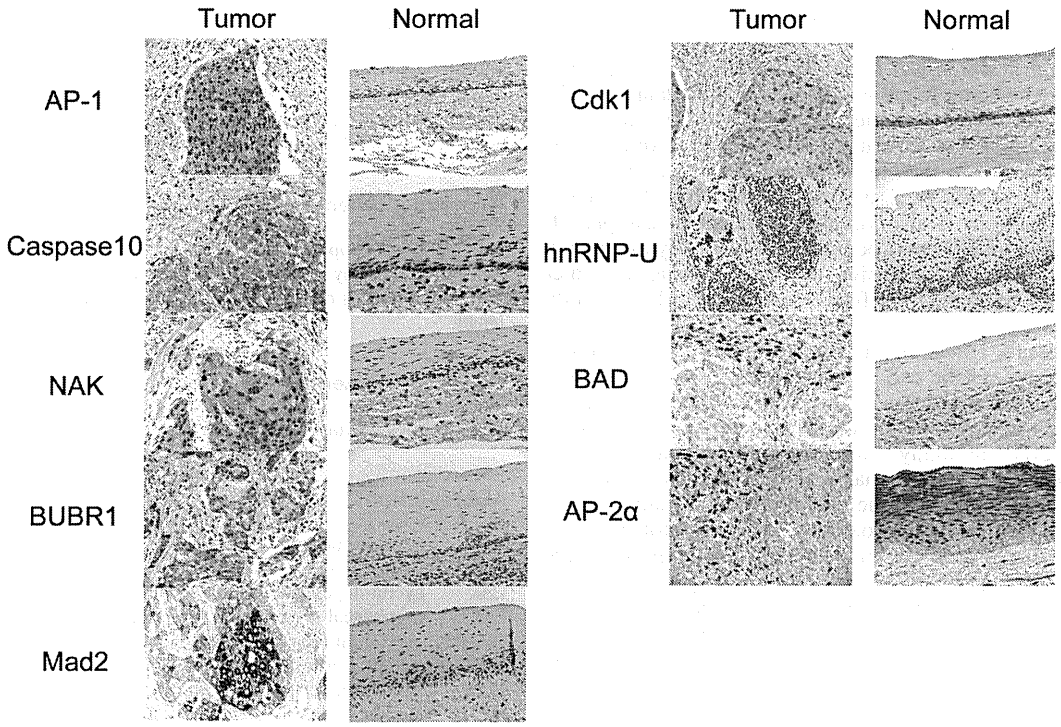

Fig. 6. Immunohistochemistry. The expression of six proteins (activator protein [AP]-1, BUBR1, caspase 10, cyclin-dependent kinase [Cdk] 1, nuclear factor-κB-activating kinase [NAK], and Mad2), either cytoplasmic or nuclear, was stronger in esophageal squamous cell carcinoma compared with normal epithelium. Nuclear expression of hn-RNP-U was observed in all tumor and normal tissues. The expression of BAD and AP-2α was higher in infiltrating inflammatory cells compared with the tumor stroma. BUBR, budding uninhibited by benzidazoles 1 homolog beta; Mad, mitotic arrest deficient-like 1; hn-RNP-U, heterogeneous nuclear ribonucleoprotein U; BAD, Bcl-associated death promoter.

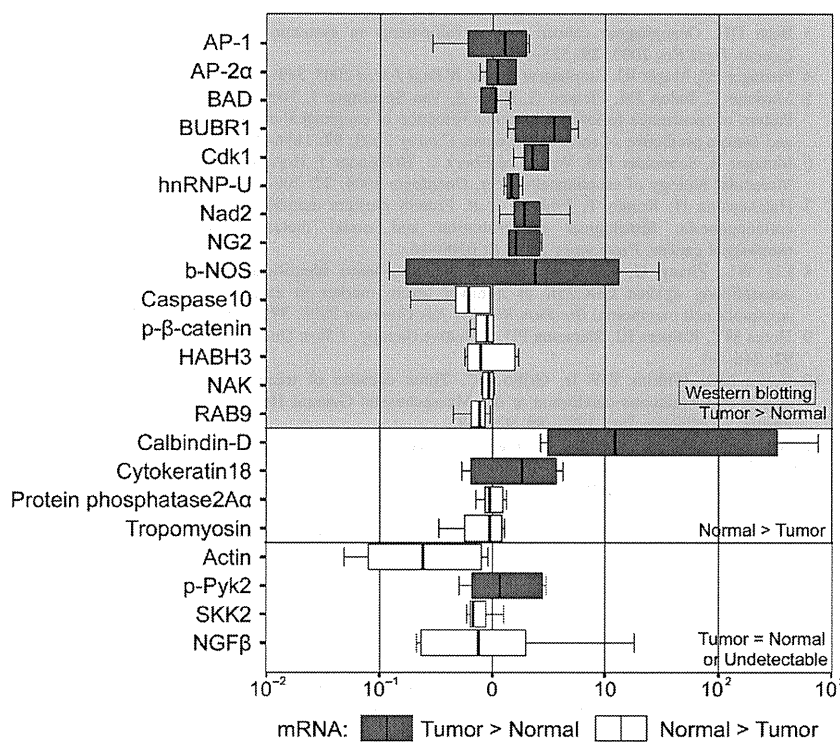

Fig. 7. The mRNA expression levels of the 22 identified proteins. Among the 14 proteins overexpressed in esophageal squamous cell carcinoma (ESCC) by western blotting analysis, nine were also found to be overexpressed at the mRNA level. The expression of two of four proteins found to be lower in ESCC in western blotting was also lower at the mRNA level.

intensity, the threshold may also need to be changed as a function of signal intensity.

As spots with lower signal intensity had poor reproducibility (Fig. 2), it may be better to pre-exclude them from data analysis. The amount and affinity of the antibodies in the microarray should be best optimized so that the dynamic range of all spots is within a linearly measurable range. However, the affinity to antigens is different between antibodies. In addition, the amount of individual antigens is also variable between the samples. It seems that these problems may be inherent to antibody microarray experiments. Therefore, extensive validation studies by other methods should be carried out for results obtained by antibody microarrays.

As tumor tissues contain many types of tumor and non-tumor cells, recovery of specific cell populations by laser microdissection prior to protein extraction is generally required for accurate expression analysis. However, as 100 μg of each protein is required for every antibody microarray experiment, and laser microdissection can collect a small amount of protein, we cannot avoid using homogenized tissue samples. The advantages and limitations of antibody microarrays, western blotting, and immunohistochemistry in the analysis of protein expression are discussed in the Supplemental text. In our study, two proteins identified as being overexpressed in tumor tissues were shown to derive from infiltrating inflammatory cells in the tumor stroma rather than the tumor itself, a finding that stresses the need for immunohistochemical studies in order to localize the identified proteins in the tissues.

In conclusion, through the use of antibody microarrays we identified a novel association of mitotic checkpoint gene products and the NF-κB pathway with esophageal cancer. These proteins will not only be useful in better understanding the molecular background of ESCC but will also be candidates for biomarkers or therapeutic targets. Although antibody microarrays generate expression data in a high-throughput way, validation of the results by other methods is required due to its limited reproducibility.

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Disclosure Statement

The authors declare that they have no competing interests.

Abbreviations

AP	activator protein
BubR	budding uninhibited by benzidazoles 1 homolog beta
Cdk	cyclin-dependent kinase
ESCC	esophageal squamous cell carcinoma
HABH	alkylation repair homolog
Mad	mitotic arrest deficient-like 1
NAK	nuclear factor-κB-activating kinase
NF	nuclear factor
NG	neurite growth

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Supporting Information

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

Supplemental text

We compared the protein expression level of the samples examined using three widely used methods that use antibodies, namely antibody microarrays, western blotting, and immunohistochemistry, as each methodology has advantages and limitations. Antibody microarrays can generate expression data of hundreds or thousands of proteins in a single experiment. However, antibody microarrays measure the total amount of proteins examined, and the expression of isoforms and variants cannot be evaluated. Western blotting separates proteins first according to their molecular weight and the separated proteins then react with antibodies. The advantage of western blotting is that it measures the expression level of protein isoforms and variants even using antibodies that react with all protein forms. Improving throughputness is the challenge of western blotting for proteomic studies. The unique advantage of immunohistochemistry is that it provides detailed data on the localization and distribution of the proteins examined in the cells and tissues. The acquisition quantitative data relies on objective observation in immunohistochemistry. The discordant results observed in this study between these methodologies may be attributable to the reactivity of the antibodies used. The physical and chemical status of proteins may vary in these methods and the antibodies may react with them in varying ways. The use of multiple antibodies against single antigens may be helpful in measuring the expression level of proteins.

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Expression of SLCO1B3 is associated with intratumoral cholestasis and *CTNNB1* mutations in hepatocellular carcinoma

Shigeki Sekine,[1] Reiko Ogawa, Hidenori Ojima and Yae Kanai

Pathology Division, National Cancer Center Research Institute, Tokyo, Japan

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Recent studies have shown that intratumoral cholestasis is a hallmark of *CTNNB1* mutations in hepatocellular carcinomas (HCC). Here, we analyzed the expressions of genes involved in bile acid and bilirubin metabolism and their correlation with the mutational status of *CTNNB1* in a series of HCC. The expressions of *CYP7A1* and *CYP27A1*, which encode rate-limiting enzymes in bile acid synthesis, were unaltered or only marginally increased in *CTNNB1*-mutated HCC compared with those in HCC with wild-type *CTNNB1*. Among the genes involved in bile acid and bilirubin transport, the expression of *SLCO1B3* was significantly elevated in HCC with *CTNNB1* mutations, whereas the expression of *ABCC4* was elevated in HCC with wild-type *CTNNB1*. Immunohistochemistry confirmed the frequent expression of SLCO1B3 in *CTNNB1*-mutated HCC at the protein level, but not in most HCC with wild-type *CTNNB1*. Immunohistochemistry for MRP4 (encoded by *ABCC4*) partly agreed with *ABCC4* expression, but most cases did not express detectable levels of MRP4. Notably, all HCC with bile accumulation, including those without *CTNNB1* mutations, expressed SLCO1B3, suggesting that SLCO1B3 expression, rather than *CTNNB1* mutation, is the critical determinant of intratumoral cholestasis. As SLCO1B3 is involved in the uptake of a number of chemotherapeutic and diagnostic agents, SLCO1B3 expression and the status of *CTNNB1* mutation might need to be considered in the drug delivery to HCC. (*Cancer Sci* 2011; 102: 1742–1747)

Bile acids are major components of bile and the liver plays a central role in their metabolism.[1–3] Bile acids are synthesized from cholesterol in the liver and secreted into bile. Bile plays essential roles in the absorption and excretion of lipid-soluble substances. In addition to widely recognized roles in lipid metabolism, bile acids act as a signaling molecule and modulate proliferation and energy metabolism in hepatocytes.[3,4] Mice deficient in the bile acid receptor FXR exhibit cholestasis and the spontaneous development of hepatocellular carcinomas (HCC), suggesting a potential linkage between bile acid signaling and hepatocarcinogenesis.[5,6]

Interestingly, recent reports have shown that HCC with activating *CTNNB1* mutations frequently exhibit cholestasis.[7,8] This observation implies that β-catenin regulates bile metabolism in HCC. Activating mutations of *CTNNB1*, encoding β-catenin, are present in 30–40% of HCC.[9,10] Physiologically, β-catenin acts as a transducer of the Wnt signaling pathway,[11] and mutation of *CTNNB1* leads to abnormal accumulation of β-catenin and constitutive activation of T-cell factor (TCF)-dependent transcription.[12] This results in the overexpression of β-catenin/TCF-regulated transcriptional targets in *CTNNB1*-mutated tumors and the promotion of tumorigenesis.

Based on these previous reports, we suspected that mutated β-catenin might coordinately induce genes critically involved in bile metabolism in HCC. To elucidate this issue, we examined the expressions of a list of genes that are involved in bile acid synthesis and transport in a series of HCC.

Materials and Methods

Cases. We examined 44 cases of HCC obtained from 42 patients; all of these tumors had been previously analyzed for *CTNNB1* mutations and the presence of intratumoral cholestasis.[8] Mutation analysis was done by direct sequencing of the N-terminal region of *CTNNB1* using cDNA samples. Intratumoral cholestasis was histologically determined by the presence of bile pigments on hematoxylin–eosin and Hall's bile acid staining. Eight non-tumoral liver tissues obtained during the resection of metastatic colorectal cancers were used as normal liver samples for comparison. All tissue samples were obtained at the National Cancer Center Hospital, Tokyo, Japan. The present study was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan.

Quantitative RT-PCR. RNA extraction and reverse-transcription reactions were performed using standard protocols. Quantitative RT-PCR reactions were performed using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA). The expression level of each gene was determined using *GUSB* as a standard, as previously described.[13] The primer sequences are shown in Table 1.

Immunohistochemistry. Among the tumors subjected to RT-PCR analysis, 41 lesions were available for histological analysis. Liver tissue samples were fixed in 10% buffered formalin, embedded in paraffin and cut into 4-μm-thick sections. Antigen retrieval was performed by autoclaving in 10 mmol/L of citrate buffer (pH 6.0) for 10 min. Anti-SLCO1B3 (1:250 dilution; Sigma, St Louis, MO, USA) and anti-ABCC4 antibodies (1:500 dilution; Abnova, Taipei, Taiwan) were used as the primary antibodies and the signals were detected using peroxidase-labeled anti-rabbit and anti-goat polymers (Histofine simple stain; Nichirei, Tokyo, Japan). 3-3′-Diaminobenzidine tetrahydrochloride was used as a chromogen. Normal liver tissue served as a positive control for SLCO1B3 and normal prostatic tissue was used as a positive control for ABCC4. The staining results were evaluated as follows: ++, diffuse (>50%) expression; +, focal (10–50%) expression; and −, no (<10%) expression.

Statistical analysis. For quantitative PCR analysis, statistical significance was confirmed using a two-tailed Mann–Whitney *U*-test. The Fisher–Freeman–Halton exact test was used to analyze each 2 × 3 table. $P < 0.05$ was considered statistically significant.

[1]To whom correspondence should be addressed. E-mail: ssekine@ncc.go.jp

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Table 1. Primers used in the RT-PCR analysis

	Forward primer	Reverse primer
CYP7A1	GCTCTTTACCCACAGTTAATGC	TTGTCTTCCCGTTTTCATCA
CYP27A1	GATTGCAGAGCTGGAGATGC	CTCTTCAACTCCCCCGTCTC
HSD3B7	CTGGGCTGGTAGACGTGTTT	ACACAAGCCTCGATCACGTT
SLC10A1	AATGGACGGTGCAGACGCACT	AGGCCACATTGAGGATGGTGGAA
SLCO1A2	ACCAACGCAGGATCCATCAGAGTGT	ACCCAAAGGCAGGATGGGAGT
SLCO1B1	TGGAGGTGTTTTGACTGCTTTGCCA	ACAAGTGGATAAGGTCGATGTTGAATTTTCT
SLCO1B3	TGGTCCAGTCATTGGCTTTGCACT	AGCTCCAACCCAACGAGAGTCCT
ABCC2	CCAGGACCAAGAGATCCTCTACCAC	AAGGGCCAGCTCTATGGCTGCT
ABCC3	CTCTGGAGGCCTGTGCCTTGCTA	GCTGGCCCCCAGACAGGTTAATGC
ABCC4	GGTGGGCCTCTGGTACTGAAGC	TCCAGCTCCGGTTCTTCCCACAAT
ABCB11	AGATGACATGCTTGCGAGGACCT	AGCGTTGCCGGATGGAAGCC
NR0B2	CCTGAAAGGGACCATCCTCT	ACTTCACACAGCACCCAGTG
AXIN2	ACGCTGGCTCAGCTGGAGGA	ACAGCACCGCTGCTTTGGGG
GUS	GGAATTTTGCCGATTTCATGA	CCGAGTGAAGATCCCCTTTTT

Results

We first determined the expressions of genes encoding enzymes critical for bile acid synthesis, postulating that *CTNNB1*-mutated HCC show increased bile acid production. CYP7A1 and CYP27A1 are the rate-limiting enzymes of the classical and alternative pathways of bile acid synthesis, respectively.[3] HSD3B7 is another critical enzyme in bile acid synthesis and its mutation has been linked to a defect in bile acid synthesis.[14] The results showed that *CYP7A1* expression was increased in the HCC regardless of the *CTNNB1* mutation status (Fig. 1). *CYP27A1* expression was significantly but only marginally elevated in HCC with *CTNNB1* mutations compared with those without mutation. The *HSD3B7* expression level was unaltered between normal liver and the HCC. These results indicate that *CTNNB1* mutations do not induce genes for bile acid synthesis in HCC.

Next we examined four genes involved in bile acid and bilirubin uptake from portal blood. The expressions of three of the genes that were examined, *SLC10A1*, *SLCO1A2* and *SLCO1B1*, tended to be reduced in HCC, and no significant associations with the status of *CTNNB1* mutation were seen (Fig. 1). However, *SLCO1B3* expression was closely correlated with the presence of *CTNNB1* mutations. While HCC without *CTNNB1* mutations showed remarkably reduced *SLCO1B3* expression levels, *CTNNB1*-mutated HCC retained expression levels comparable with that observed in normal liver.

Two genes for canalicular transporters, *ABCC2* (encoding MRP2) and *ABCB11* (encoding BSEP), showed a modest increase in *CTNNB1*-mutated tumors. Between two basolateral efflux transporters, *ABCC3* (encoding MRP3) and *ABCC4* (encoding MRP4), *ABCC4* was significantly elevated in HCC with wild-type *CTNNB1*. Of note, expression of *NR0B2*, which mediates the feedback regulation of bile acid signaling,[15,16] did not differ between HCC with or without *CTNNB1* mutations.

The expressions of SLCO1B3 and MRP4 were further determined at the protein level using immunohistochemistry. SLCO1B3 expression in the HCC was membranous and consistent with the results of the RT-PCR analysis; the expression of SLCO1B3 was significantly correlated with the presence of *CTNNB1* mutations (Fig. 2, Table 2). SLCO1B3 was expressed in pericentral hepatocytes with a membranous pattern in normal liver (Fig. 3).

Immunohistochemistry for MRP4 showed diffuse expression in one case and focal staining in three cases (Figs 4,5). All four cases positive for MRP4 also showed high levels of *ABCC4* expression, indicating concordance between the mRNA and protein expression levels. However, negative or faint expression of MRP4 was observed in the other HCC. Non-neoplastic liver tissue did not express immunohistochemically detectable levels of

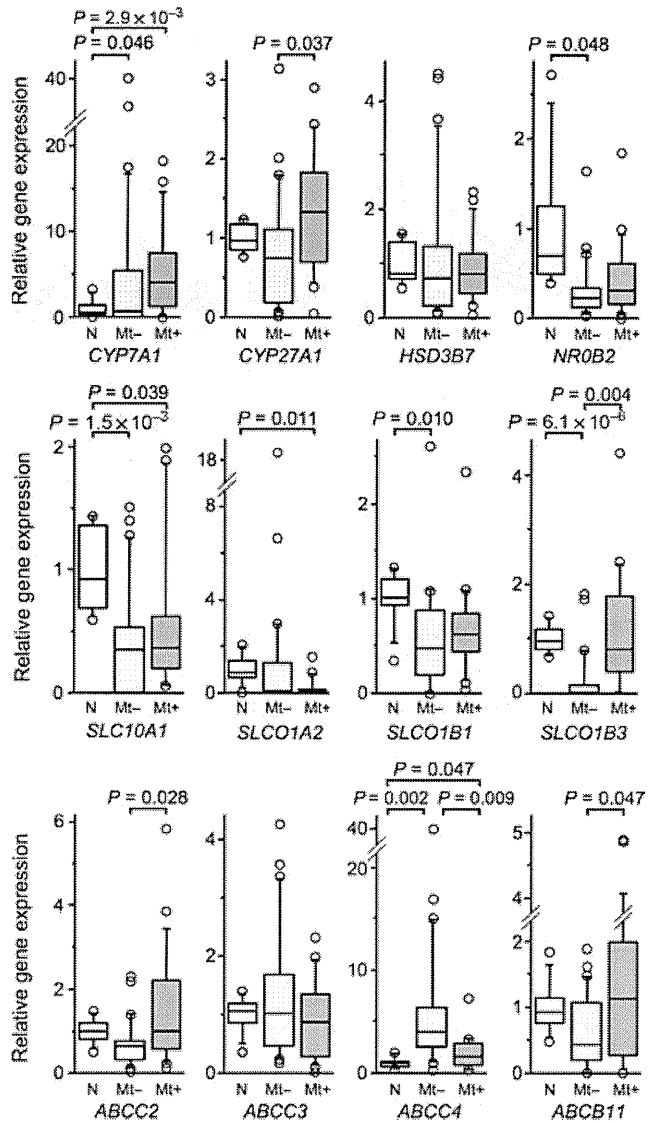

Fig. 1. Expression of genes related to bile acid metabolism. Box plots of the expression of genes related to bile acid metabolism in hepatocellular carcinomas (HCC). The expression of each gene was determined using quantitative RT-PCR with *GUSB* used as a reference. N, normal liver; Mt-, HCC with wild-type *CTNNB1*; Mt+, *CTNNB1*-mutated HCC.

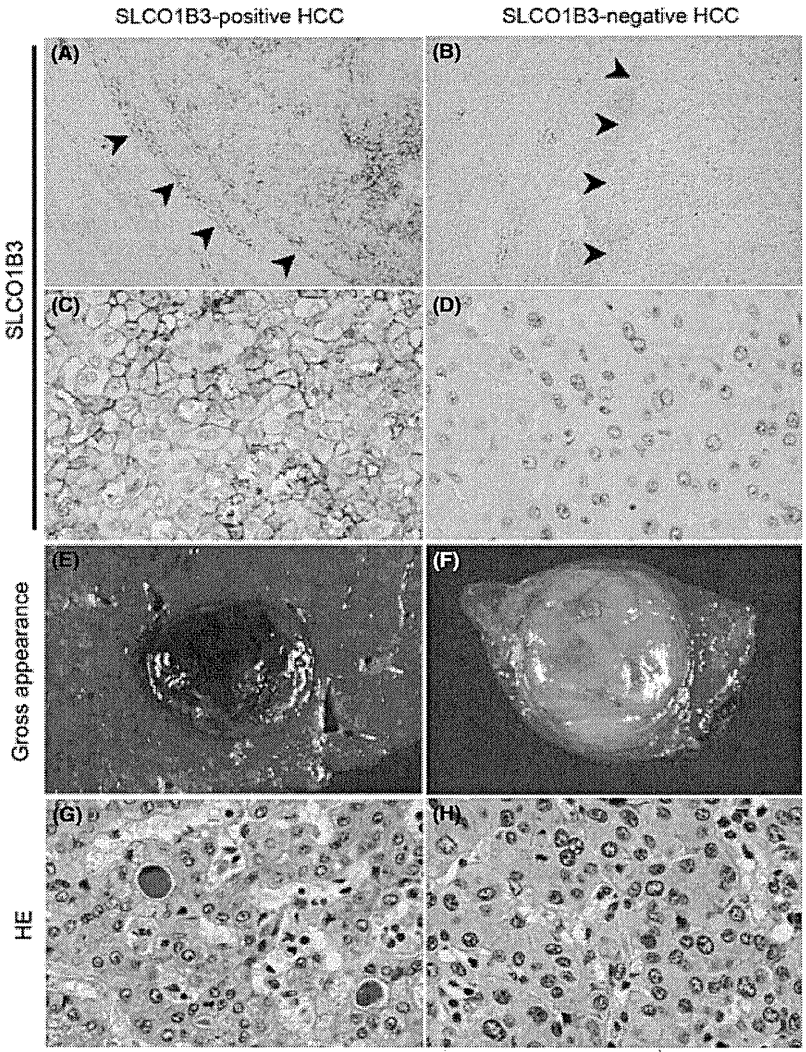

Fig. 2. SLCO1B3 expression, gross morphology and histology in hepatocellular carcinomas (HCC). Immunohistochemistry for SLCO1B3 (A–D), gross morphology (E,F) and histology (G,H) in a SLCO1B3-expressing HCC (A,C,E,G) and a SLCO1B3-negative HCC (B,D,F,H). Low-power views of SLCO1B3 staining (A,B). Areas of the tumor are indicated by arrowheads. Extensive SLCO1B3 expression in a case of cholestatic HCC (A) and almost completely negative staining in a case of non-cholestatic HCC (B). Focal SLCO1B3 expression is observed in non-neoplastic cirrhotic liver on the backgrounds of HCC (A,B). Magnified views showed membranous SLCO1B3 expression (C), and no SLCO1B3 staining (D). A SLCO1B3-expressing HCC has a greenish cholestatic appearance (E), whereas a SLCO1B3-negative HCC has a homogenous whitish appearance (F). A HE-stained section shows bile pigments in a SLCO1B3-expressing HCC (G) but not in a SLCO1B3-negative HCC (H).

MRP4. Prostatic tissues used for positive controls exhibited diffuse and strong membranous expression.

Next, we sought to determine the correlation between SLCO1B3 expression and intratumoral cholestasis. The results showed that HCC with SLCO1B3 expression frequently showed bile accumulation (Figs 2,5, Table 2). Remarkably, all three *CTNNB1* mutation-negative, cholestatic HCC expressed SLCO1B3, implying that the presence of bile accumulation is more closely correlated with SLCO1B3 expression than the mutational status of *CTNNB1*.

Table 2. Correlations among SLCO1B3 expression, *CTNNB1* mutation and cholestasis

	Total	SLCO1B3 immunohistochemistry			*P*-value
		++	+	–	
CTNNB1 mutation					
Present	18	11	4	3	6.8×10^{-4}
Absent	23	2	6	15	
Cholestasis					
Present	15	12	3	0	6.4×10^{-8}
Absent	26	1	7	18	

++, diffuse expression; +, focal expression; –, no expression.

While *CTNNB1*-mutations affecting N-terminal regions of β-catenin is the common cause of activation of β-catenin signaling in HCC, β-catenin signaling could potentially be activated by uncommon genetic alterations such as atypical *CTNNB1* mutations or *APC* mutations.[17,18] To exclude this possibility, we examined the expression of *AXIN2*, a ubiquitous target of β-catenin/TCF.[19,20] As expected, the expression of *AXIN2* was upregulated in *CTNNB1*-mutated HCC, but the levels of *AXIN2* expression were not significantly elevated in any of the *CTNNB1* mutation-negative HCC with SLCO1B3 expression (Fig. 5). This finding indicates that a minor subset of HCC express SLCO1B3 even in the absence of active β-catenin signaling.

Discussion

Based on the association between *CTNNB1* mutations and intratumoral cholestasis,[7,8] we postulated that active β-catenin signaling regulates bile acid metabolism in HCC. While previous analysis in a mouse model suggested that β-catenin induces bile acid synthesis genes under physiological conditions,[21] they were not upregulated in HCC with *CTNNB1* mutations. In contrast, the expression of SLCO1B3, a solute carrier organic anion transporter protein, was associated with the presence of

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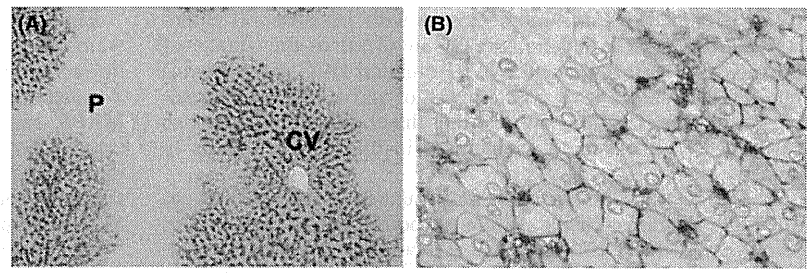

Fig. 3. SLCO1B3 expression in normal liver tissue. Normal liver tissue shows pericentral SLCO1B3 expression (A). A magnified view showing membranous expression of SLCO1B3 in normal hepatocytes (B). CV, central vein; P, portal tract.

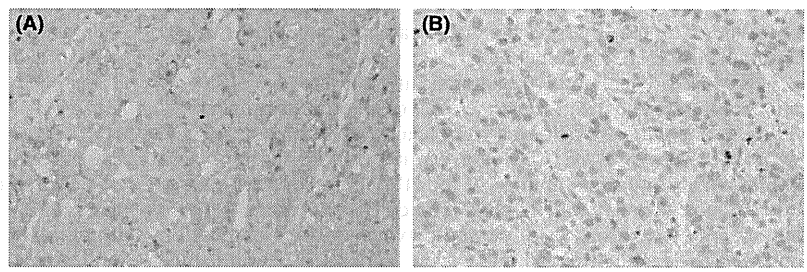

Fig. 4. MRP4 expression in hepatocellular carcinomas (HCC). Most tumor cells show membranous expression of MRP4 with some heterogeneity in this tumor (A), whereas the majority of HCC did not express immunohistochemically detectable levels of MRP4 (B).

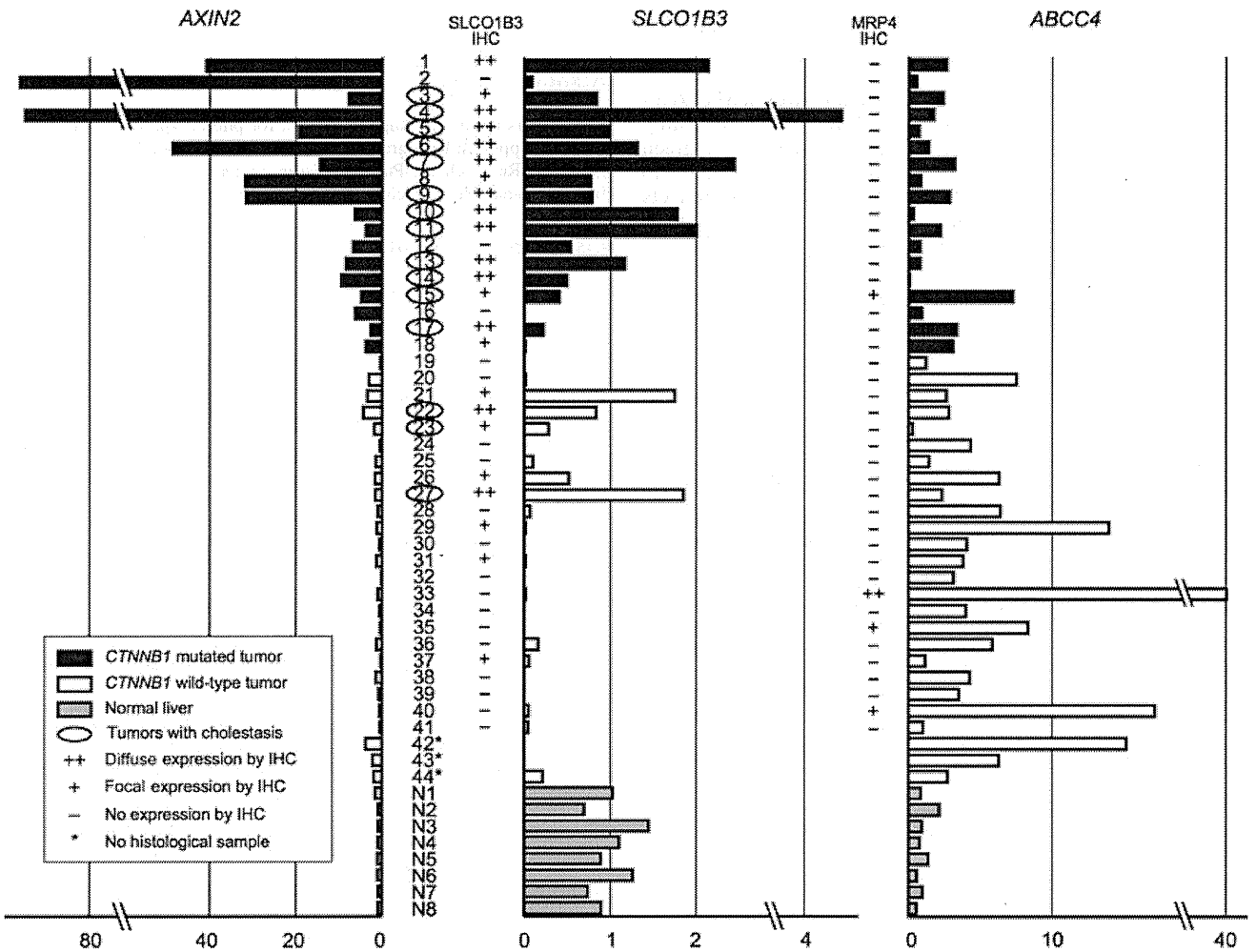

Fig. 5. Expressions of *SLCO1B3*, *ABCC4* and *AXIN2* mRNA, SLCO1B3 and MRP4 protein, *CTNNB1* mutation status and intratumoral cholestasis in each tumor sample. The expressions of *SLCO1B3*, *ABCC4* and *AXIN2* were determined using quantitative RT-PCR. SLCO1B3 and MRP4 expressions were determined using immunohistochemistry (IHC). The results of the *CTNNB1* mutation analysis and for intratumoral cholestasis were obtained in our previous study.[8] The case numbers are identical to those in our previous study.

CTNNB1 mutations and more closely with intratumoral cholestasis. SLCO1B3 is physiologically involved in the uptake of bile acids;[22–26] however, *CTNNB1*-mutated HCC did not exhibit elevated *NR0B2* levels, a hallmark of active bile acid signaling.[15,16] These findings suggest that the cholestatic appearance of HCC is not linked to an increase in bile acid synthesis or uptake.

The exact mechanism by which mutated β-catenin induces *SLCO1B3* remains elusive. While we performed *in vitro* studies using several HCC cell lines, the activation of β-catenin signaling did not induce *SLCO1B3* expression in any of the cell lines (data not shown). Furthermore, some of the HCC expressed high levels of *SLCO1B3* in the absence of *CTNNB1* mutations. These observations imply the presence of β-catenin-independent regulation of SLCO1B3 in some HCC.

MRP4 is a basolateral transporter involved in the efflux of bile acids, steroids and a range of xenobiotic substances.[27] *ABCC4*, encoding MRP4, was significantly upregulated in HCC with wild-type *CTNNB1*. However, the MRP4 protein was expressed at low levels in most of the HCC compared with prostatic tissue, where MRP4 is physiologically expressed. While a significant correlation was observed between *ABCC4* expression and *CTNNB1* mutation in HCC, the functional significance remains to be elucidated.

Bilirubin, the main bile pigment, is another important substrate of SLCO1B3. Previous *in vitro* experiments have shown that the introduction of SLCO1B3 into human cells or xenopus oocytes induced bilirubin uptake.[24,25] Furthermore, two genome-wide association studies identified genetic variations within *SLCO1B3* as being associated with serum bilirubin levels,[28,29] suggesting a physiological role in bilirubin clearance *in vivo*. As the green color of bile is caused by its bilirubin content, it is reasonable to assume that the cholestatic appearance of HCC mainly reflects their ability to uptake bilirubin. While some previous studies reported conflicting results on the correlation between intratumoral cholestasis and SLCO1B3 expression,[30–32] our data suggest that expression of SLCO1B3 is the major determinant of intratumoral cholestasis in HCC.

Eight cases of SLCO1B3-positive HCC without cholestasis were observed. In fact, SLCO1B3 is a bidirectional carrier, and the efflux of bilirubin is reduced by binding to glutathione S-transferase.[33] Furthermore, some transporters, such as MRP3, can export bilirubin. Thus, SLCO1B3 expression is a critical, but not the sole, determinant of bilirubin accumulation in cells. It is expected that some molecules involved in bilirubin transport, other than SLCO1B3, are expressed differently in SLCO1B3-positive HCC without cholestasis.

A number of chemotherapeutic and diagnostic agents, in addition to bile acids and bilirubin, are also known as substrates of SLCO1B3[26,34–36] For example, gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid (Gd-EOB-DTPA), an increasingly used magnetic resonance imaging contrast agent, is also a substrate of SLCO1B3.[35] The majority of HCC are depicted as hypointense areas during the hepatobiliary phase of Gd-EOB-DTPA-enhanced magnetic resonance imaging as HCC generally have a decreased capacity to take up this contrast agent. However, a subset of HCC that express high levels of SLCO1B3 can be detected as iso- or hyperintense masses.[30–32] Considering these previous and current observations, a significant proportion of Gd-EOB-DTPA-accumulating HCC might harbor *CTNNB1* mutations. The present observations suggest that SLCO1B3 expression and the status of *CTNNB1* mutation might need to be considered in drug delivery to HCC.

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Disclosure Statement

The authors have no conflict of interest.

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Overexpression of α-methylacyl-CoA racemase is associated with *CTNNB1* mutations in hepatocellular carcinomas

Shigeki Sekine, Reiko Ogawa, Hidenori Ojima & Yae Kanai
Pathology Division, National Cancer Centre Research Institute, Tokyo, Japan

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Overexpression of α-methylacyl-CoA racemase is associated with *CTNNB1* mutations in hepatocellular carcinomas

Aims: α-Methylacyl-CoA racemase (AMACR) is expressed in the majority of hepatocellular carcinomas (HCCs) at variable levels, but the significance of AMACR overexpression remains elusive. The aim of this study was to investigate the relationship between AMACR expression and the presence of *CTNNB1* mutations in HCCs.
Methods and results: The expression of *AMACR* and *GLUL*, an established downstream target of β-catenin was examined in HCCs, by quantitative reverse transcription polymerase chain reaction (PCR), and the expression of their protein products by immunohistochemistry. The quantitative reverse transcription PCR analysis showed that the expression of *AMACR* was significantly higher in HCCs with *CTNNB1* mutations than in mutation-negative HCCs or normal livers, like the expression of *GLUL*. Immunohistochemistry also showed that strong AMACR protein expression was closely correlated with the presence of *CTNNB1* mutations. HCCs with *CTNNB1* mutations and those with AMACR overexpression frequently exhibited bile production.
Conclusions: The overexpression of AMACR was closely correlated with the presence of *CTNNB1* mutations in HCCs. AMACR is a putative target of β-catenin as well as an excellent immunohistochemically detectable marker of HCCs with *CTNNB1* mutations. As AMACR is physiologically involved in bile acid synthesis, the current observation implies a regulatory role of β-catenin in bile acid metabolism.

Keywords: AMACR, β-catenin, hepatocellular carcinoma

Abbreviations: AMACR, α-methylacyl-CoA racemase; HCC, hepatocellular carcinoma; PCR, polymerase chain reaction

Introduction

α-Methylacyl-CoA racemase (AMACR) is an enzyme that is crucial for the β-oxidation of branched fatty acids and C27 bile acids.[1] Recent studies have shown the utility of AMACR expression in the histological diagnosis of prostatic cancer. AMACR was initially identified as a potential molecular marker of prostatic cancer in a microarray study.[2] As normal prostate expresses very low levels of AMACR, the immunohistochemical detection of AMACR is helpful in the diagnosis of prostatic cancers.[3,4] Furthermore, the inhibition of AMACR expression represses the growth of prostatic cancer cells *in vitro*,[5] suggesting that AMACR is not just a tumour marker, but is also directly involved in tumorigenesis.

On the other hand, the expression of AMACR is not limited to prostatic cancers. AMACR is also expressed in tumours of other organs, including liver, kidney and colorectal cancers.[6,7] With regard to liver cancer, AMACR expression has been reported in 77–100% of hepatocellular carcinomas (HCCs).[6–9] Even though HCCs express AMACR with a frequency comparable to that of prostatic cancers, AMACR expression cannot be directly used as a tumour marker in the liver, as it

Address for correspondence: Y Kanai, Pathology Division, National Cancer Centre Research Institute, 5-1-1, Tsukiji, Chuo-ku, Tokyo, Japan. e-mail: ykanai@ncc.go.jp

Table 1. Clinicopathological features of cases and results of mutational and immunohistochemical analysis

	Age (years)/ Gender	Histology	Viral infection	*CTNNB1* mutation		Immunohistochemistry			
				Nucleotide	Amino acid	AMACR	GS	β-Catenin	Bile
1	52/M	W/D	HCV	A95G	D32G	++	++	+	–
2	65/M	W/D	HCV	del94–141	del32–47	++	++	–	–
3	58/M	M/D	–	A121G	T41A	+	++	+	+
4	77/F	M/D	HCV	A95G	D32G	++	+	–	+
5	63/M	W/D	HCV	T104G	I35S	++	++	+	+
6	55/M	P/D	HCV	T109C	S37P	++	++	+	+
7	63/M	W/D	HCV	del114–125	del39–42	++	++	+	+
8	71/M	M/D	–	A107G/A126G	H36R/T42T	++	++	+	–
9	66/M	M/D	HCV	C134T	S45F	++	++	+	+
10	73/M	M/D	HCV	T104G	I35S	++	++	+	+
11	71/M	W/D	HCV	A107C	H36P	+	+	–	+
12	68/M	M/D	–	G101A	G34E	+	+	–	–
13	66/M	M/D	HBV, HCV	C110G	S37C	++	++	+	+
14	50/M	P/D	HCV	A95G	D32G	++	++	+	+
15	29/M	M/D	HBV	A107G	H36R	+	+	+	+
16	72/M	M/D	–	G101A	G34E	+	+	+	–
17	62/M	W/D	HBV	T133C	S45P	+	+	–	+
18	59/M	M/D	HBV	A121G	T41A	+	+	+	–
19	59/M	P/D	HBV	–	–	–	+	–	–
20	41/M	M/D	HBV	–	–	–	+	–	–
21	54/M	M/D	HBV	–	–	–	+	–	–
22	69/M	M/D	HCV	–	–	+	+	–	+
23	63/M	M/D	HBV, HCV	–	–	+	+	–	+
24	67/M	P/D	HCV	–	–	–	–	–	–
25	58/F	M/D	HBV	–	–	–	+	–	–
26	61/F	P/D	HCV	–	–	–	+	–	–
27	62/M	W/D	HCV	–	–	+	+	–	+
28	61/M	P/D	–	–	–	+	+	–	–
29	66/M	M/D	HBV	–	–	+	+	–	–
30	79/M	M/D	–	–	–	–	–	–	–
31	71/M	M/D	HCV	–	–	++	+	–	–

Table 1. (*Continued*)

	Age (years)/ Gender	Histology	Viral infection	*CTNNB1* mutation		Immunohistochemistry			
				Nucleotide	Amino acid	AMACR	GS	β-Catenin	Bile
32	70/M	M/D	HCV	–	–	+	+	–	–
33	76/M	P/D	–	–	–	+	–	–	–
34	58/M	M/D	HBV	–	–	+	+	–	–
35	56/M	P/D	HBV	–	–	+	+	–	–
36	70/M	P/D	–	–	–	+	+	–	–
37	36/M	P/D	HBV	–	–	–	+	–	–
38	50/M	P/D	HCV	–	–	–	–	+	–
39	59/M	M/D	HCV	–	–	+	+	–	–
40	60/M	P/D	HCV	–	–	–	–	–	–
41	72/F	P/D	HCV	–	–	+	+	–	–
42	68/M	P/D	HCV	–	–	ND	ND	ND	ND
43	68/M	W/D	HCV	–	–	ND	ND	ND	ND
44	75/M	W/D	HCV	–	–	ND	ND	ND	ND

AMACR, α-methylacyl-CoA racemase; F, female; GS, glutamine synthetase; HBV, hepatitis B virus; HCV, hepatitis C virus; M, male; M/D, moderately differentiated; ND, not done; P/D, poorly differentiated; W/D, well differentiated.
Samples 18 and 19 were derived from the same patient, as were samples 42 and 43.

is also expressed in non-neoplastic liver tissue.[6,8] Although some immunohistochemical studies have been performed, the clinicopathological significance of AMACR expression in HCCs remains controversial.[8,9]

CTNNB1 is a major oncogene in HCCs, and is mutated in approximately 30% of all cases.[10,11] β-Catenin, the protein product of *CTNNB1*, is involved in two distinct processes in cells: cell adhesion and the transduction of Wnt signalling. In the absence of active Wnt signalling, β-catenin is localized to the membrane in a complex with cadherins that mediates cell–cell adhesion. When the Wnt signalling pathway is activated, β-catenin is translocated to the nucleus, where it activates TCF-dependent transcription.[12] Oncogenic *CTNNB1* mutations lead to constitutively active T cell factor (TCF)-dependent transcription, and the dysregulated expression of β-catenin/TCF target genes is thought to induce cellular transformation.[13] A number of genes have been hitherto identified as targets of β-catenin-mediated signals in the liver, and are overexpressed in HCCs with *CTNNB1* mutations.[14–19]

Here, we examined the correlation between *CTNNB1* mutations and AMACR expression in a series of HCCs. We also examined the expression of a well-recognized target of β-catenin, glutamine synthetase, which is encoded by *GLUL*.[14,15,20]

Materials and methods

CASES

Forty-four surgically resected HCC samples obtained from 42 patients were analysed in this study. Eight non-neoplastic liver tissues obtained during resection of metastatic colorectal cancers were used as normal liver samples for comparison. All of the tissue samples were obtained from the National Cancer Centre Hospital, Tokyo, Japan. The clinicopathological features of the patients are listed in Table 1. This study was approved by the Ethics Committee of the National Cancer Centre, Tokyo, Japan.

QUANTITATIVE POLYMERASE CHAIN REACTION (PCR)

RNA extraction and the reverse transcription reaction were performed according to standard protocols. Quantitative PCR reactions were performed with SYBR Green PCR master mix (Applied Biosystems, Foster

City, CA, USA). The expression of *GUSB* was used for normalization, as previously described.[21] The primer sequences were as follows: *AMACR*, CGTCTGTGCAAGCGGTCGGA and TGGGCCCAGCTGGAGTTTCT; *GLUL*, GCCATGCGGGAGGAGAAT and ACTGGTGCCGCTTGCTTAGT; and *GUSB*, GGAATTTTGCCGATTTCATGA and CCGAGTGAAGATCCCCTTTTT. A *P*-value of <0.05 (Mann–Whitney *U*-test) was considered to be significant.

CTNNB1 MUTATIONAL ANALYSIS

PCR reactions were performed with the cDNA samples used for quantitative PCR as templates. A pair of primers encompassing the N-terminal region of *CTNNB1*, CCTGTTCCCCTGAGGGTATT and CAGGGAACATAGCAGCTCGT, was used. The PCR products were electrophoresed in an agarose gel and recovered with a QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). Isolated PCR products were sequenced bidirectionally with the same primers used for amplification.

IMMUNOHISTOCHEMISTRY

Liver tissue samples were fixed with 10% formalin, embedded in paraffin and cut into 4-μm-thick sections. Sections were subjected to haematoxylin and eosin and Hall's bile staining for evaluation of bile production in HCCs. Immunohistochemistry was performed using an indirect immunoperoxidase method. Antigen retrieval was performed by autoclaving in 10 mM citrate buffer (pH 6.0). The primary antibodies used were anti-AMACR (Clone 13H4; 1:400 dilution; Dako, Glostrup, Denmark), anti-β-catenin (Clone 14; 1:250 dilution; BD Biosciences, San Jose, CA, USA) and anti-glutamine synthetase (Clone 6; 1:1000 dilution; BD Biosciences). The signals were detected with peroxidase-labelled anti-mouse and anti-rabbit polymers (Histofine simple stain; Nichirei, Tokyo, Japan). 3,3′-Diaminobenzidine tetrahydrochloride was used as a chromogen.

The staining results for AMACR and glutamine synthetase were evaluated as diffuse strong expression (++), heterogeneous and/or weak expression (+), or no expression (−). Diffuse staining was defined as >80% of the cells showing homogeneous staining. Strong expression was defined as a staining intensity comparable to that of pericentral hepatocytes in normal liver. Staining for nuclear/cytoplasmic β-catenin was considered to be positive when more than 5% of the tumour cells exhibited evident nuclear and/or cytoplasmicimmunoreactivity.

Results

Sequencing analysis of *CTNNB1* identified mutations affecting the region encoding the casein kinase 1/glycogen synthase kinase-3β phosphorylation sites of β-catenin in 18 of 44 HCCs (41%; Table 1). These included 18 missense mutations and two in-frame deletions. One tumour with a missense mutation also had a silent mutation.

The quantitative reverse transcription PCR analysis clearly showed that tumours with *CTNNB1* mutations had elevated expression levels of *AMACR* as well as of *GLUL*, a known target of β-catenin,[14,15,20] as compared with normal liver tissues and HCCs without *CTNNB1* mutations (Figure 1). On the other hand, HCCs without *CTNNB1* mutations did not show significantly altered expression levels of *AMACR* or *GLUL*.

To test whether the elevated *AMACR* and *GLUL* expression levels resulted in the overexpression of their protein products, an immunohistochemical analysis of 41 corresponding tumours and seven normal liver tissue samples that were available for histological analysis was perfomed. Immunohistochemistry confirmed that AMACR overexpression was associated with the presence of *CTNNB1* mutations (Figure 2B,C,E,F; Table 2). Eleven of the 12 HCCs with diffuse and intense AMACR staining and seven of the 19 HCCs with heterogeneous and/or weak AMACR expression had *CTNNB1* mutations. In contrast, none

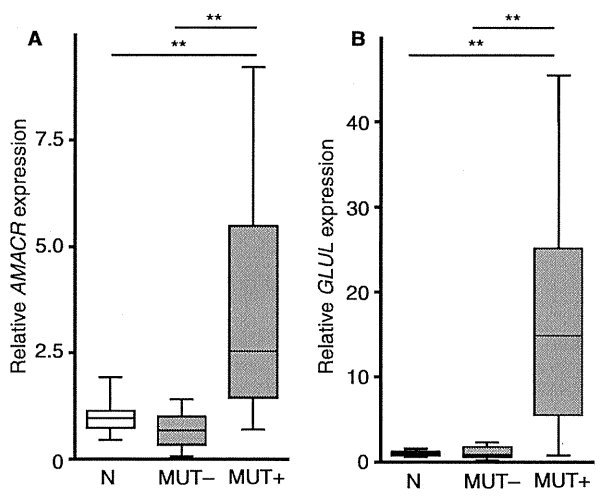

Figure 1. Expression of *AMACR* and *GLUL* in hepatocellular carcinomas. Box plot of *AMACR* and *GLUL* mRNA expression in normal liver (n = 8), hepatocellular carcinomas (HCCs) with wild-type *CTNNB1* (MUT−; n = 26) and HCCs with *CTNNB1* mutations (MUT+; n = 18), as determined using quantitative polymerase chain reaction. **P < 0.001 (Mann–Whitney U-test).

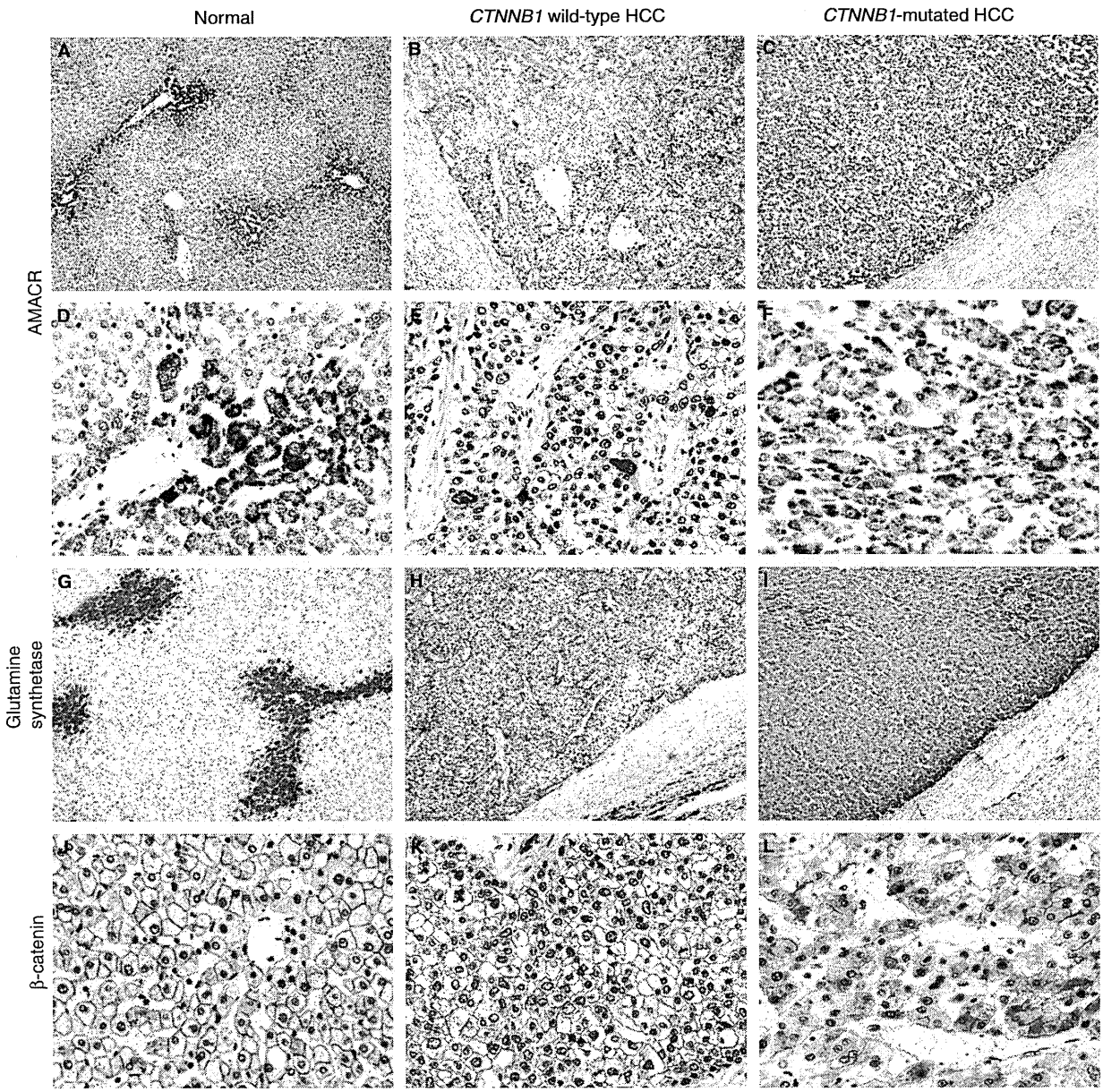

Figure 2. Expression of α-methylacyl-CoA racemase (AMACR), glutamine synthetase and β-catenin in hepatocellular carcinomas (HCCs) and normal liver. Immunohistochemistry for AMACR (A–F), glutamine synthetase (G–I) and β-catenin (J–L) in normal liver (A,D,G,J), HCCs without *CTNNB1* mutation (B,E,H,K) and HCCs with *CTNNB1* mutation (C,F,I,L).

of the AMACR-negative tumours had *CTNNB1* mutations. AMACR was consistently expressed in normal liver tissue: an expression gradient was observed, with the highest expression levels being seen in the proximal pericentral areas within the liver lobule (Figure 2A). The intracellular distributions of AMACR were cytoplasmic and granular, and a significant difference was not observed between the normal liver tissues and the HCCs (Figure 2D–F).

In agreement with previous studies, the diffuse and strong expression of glutamine synthetase, which is encoded by *GLUL*, was observed exclusively in tumours with *CTNNB1* mutations (Figure 2H,I). The staining results for glutamine synthetase were highly concordant with those for AMACR (Table 3). In the normal liver tissues, glutamine synthetase was exclusively expressed in a few layers of hepatocytes surrounding the central veins (Figure 2G). Nuclear/cytoplasmic

Table 2. Correlations among *CTNNB1* mutational status, immunohistochemistry for α-methylacyl-CoA racemase (AMACR), glutamine synthetase and β-catenin expression, and bile production in hepatocellular carcinomas

	Staining intensity	*CTNNB1* mutation (+)	*CTNNB1* mutation (–)	*P*-value
AMACR	++	11	1	6.4×10^{-5}
	+	7	12	
	–	0	10	
Glutamine synthetase	++	11	0	3.5×10^{-5}
	+	7	18	
	–	0	5	
Nuclear/cytoplasmic β-catenin	+	13	1	5.4×10^{-6}
	–	5	22	
Bile production	+	12	3	4.0×10^{-4}
	–	6	20	

The *P*-values indicate the correlation between the *CTNNB1* mutation status and the immunohistochemical expression of each of the proteins or bile production (chi-square test).

Table 3. Correlations among α-methylacyl-CoA racemase (AMACR), glutamine synthetase and β-catenin expression, and bile production in hepatocellular carcinomas

	Staining intensity	AMACR ++	AMACR +	AMACR –	*P*-value
Glutamine synthetase	++	10	1	0	4.2×10^{-7}
	+	2	17	6	
	–	0	1	4	
Nuclear/cytoplasmic β-catenin	+	9	4	1	1.5×10^{-3}
	–	3	15	9	
Bile production	+	8	7	0	5.3×10^{-3}
	–	4	12	10	

The *P*-values indicate the correlations among the immunohistochemical expression of AMACR and that of glutamine synthetase or β-catenin, or bile production (chi-square test).

β-catenin staining was observed in 12 of the 17 *CTNNB1* mutation-positive HCCs. In most cases, the nuclear staining was limited to focal areas within the tumours. In the normal liver tissues and HCCs without *CTNNB1* mutations, β-catenin staining was observed exclusively in the membranes (Figure 2J,K). Positive nuclear/cytoplasmic β-catenin staining was significantly correlated with AMACR expression (Table 3).

A recent study suggested that cholestasis might be useful as a marker for HCCs with *CTNNB1* mutations.[20] As AMACR is involved in bile acid metabolism in the liver, we histologically determined the presence of bile production in HCCs, and examined the correlations with *CTNNB1* mutations and AMACR expression (Figure 3). In agreement with the previous study, HCCs with bile production frequently harboured *CTNNB1* mutations and, as expected, overexpressed AMACR (Tables 2 and 3).

Discussion

Oncogenic mutations of *CTNNB1* result in the stabilization of β-catenin through the inhibition of proper proteosomal degradation. This leads to the abnormal accumulation and nuclear translocation of the protein and the constitutive activation of TCF-dependent transcription. Nuclear/cytoplasmic localization of β-catenin is therefore regarded as a hallmark of active β-catenin signalling.[12] As expected, nuclear and/or cytoplasmic β-catenin staining was closely correlated with the presence of *CTNNB1* mutations. However, in many instances, nuclear and/or cytoplasmic β-catenin was observed only in focal areas within the tumours with *CTNNB1* mutations. Therefore, it might be difficult to use immunohistochemistry for β-catenin to screen for *CTNNB1* mutation-positive HCCs when a limited amount of specimen is available.

So far, several β-catenin-regulated genes have been identified in the liver.[14–19] Among them, the

