

Reduction of fatty acid oxidation and responses to hypoxia correlate with the progression of de-differentiation in HCC

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Abstract. The prognosis of patients with hepatocellular carcinoma (HCC) may be improved by novel treatments focusing on the characteristic metabolic changes of this disease. Therefore, we analyzed the biological interactions of metabolic features with the degree of tumor differentiation and the level of malignant potential in 41 patients with completely resectable HCC. The expression levels in resected samples of mRNAs encoded by genes related to tumor metabolism and metastasis were investigated, and the correlation between these expression levels and degrees of differentiation was analyzed. Of the 41 patients, 2 patients had grade I, 27 had grade II, and 12 had grade III tumors. Reductions in the levels of 3-hydroxyacyl-CoA dehydrogenase (HADHA) and acyl-CoA oxidase (ACOX)-2 mRNAs, and increases in pyruvate kinase isoenzyme type M2 (PKM2) mRNA were significantly correlated with the progression of de-differentiation. Analysis of partial correlation coefficients showed that the level of PKM2 mRNA expression was significantly correlated with those of pro-angiogenic genes, vascular endothelial growth factor (VEGF) and ETS-1. Moreover, the levels of VEGF-A and ETS-1 mRNA expression were independently correlated with that of the epithelial-mesenchymal transition (EMT)-related gene SNAIL. These findings suggest that reductions in fatty acid oxidation and responses to hypoxia may affect the progression of malignant phenotypes in HCC.

Introduction

Although local ablation and surgical resection are effective in the management of hepatocellular carcinoma (HCC), satisfactory outcomes have not yet been achieved (1-4). Sorafenib, a multikinase inhibitor that suppresses intracellular growth signaling and tumor angiogenesis, has been shown to prolong overall survival time and delay the time to progression, although its effectiveness remains limited (5,6). Since the metabolism of cancer cells differs from that of normal cells, these metabolic processes may constitute additional therapeutic targets. Hepatocytes maintain systemic nutrition; thus, metabolic changes during carcinogenesis may be extensive. Therefore, investigating the correlations between metabolic features and the levels of malignant potential is useful in understanding how cancer cell metabolism affects HCC progression.

The metabolism of glucose and fatty acids is re-organized in cancer cells (7,8). Although cancer cells take up more glucose compared with normal cells, they metabolize less by oxidative phosphorylation while metabolizing more by aerobic glycolysis (9,10). This conversion in energy production is adaptive to the hypoxic conditions of cancer cells, with pyruvate kinase isoenzyme type M2 (PKM2) believed to play a critical role in this conversion (11,12). Enhanced fatty acid synthesis is another feature characteristic of cancer cell metabolism. For instance, in various types of cancer including HCC, there is an increase in the levels of mRNA-encoding enzymes involved in fatty acid synthesis (13,14). Cancer cells are also characterized by a reduction in fatty acid oxidation. In mature hepatocytes, fatty acids are metabolized in mitochondria and peroxisomes to generate energy resources for gluconeogenesis. In HCCs, however, the expression of fatty acid oxidation enzymes is reduced, accompanied by a concomitant reduction in the number of peroxisomes (15).

The degrees of tumor differentiation have been found to be significantly correlated with tumor growth rate, metastasis, vascular invasion and, consequently, with patient prognosis (16-19). In well-differentiated HCC, slow growth

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rate and infrequent metastasis result in a favorable prognosis after complete tumor resection. De-differentiation allows cancer cells to grow more rapidly and in an invasive manner, occasionally detaching from the primary tumor site, and establishing intra- and extra-hepatic metastases. Epithelial-mesenchymal transition (EMT) is a key step towards cancer metastasis (20,21). EMT regulates the detachment of tumor cells from their matrix and their migration and invasion of blood vessels. An additional risk factor for HCC metastasis is increased microvessel density (MVD) (22). The positive correlation between MVD and levels of vascular endothelial growth factor (VEGF) suggests that VEGF-dependent tumor angiogenesis facilitates HCC metastasis (23,24).

To analyze the changes in metabolism occurring in HCCs, as well as the correlation between metabolic enzymes and the degree of tumor differentiation, we investigated the levels of expression of mRNAs encoded by genes involved in glucose and lipid metabolism, hepatocyte differentiation, EMT and angiogenesis. We analyzed the correlation between these levels of expression and the degree of tumor differentiation, and estimated the impact of cancer metabolism on the malignant potential of HCC. It was found that increased PKM2 expression and reduced expression of fatty acid oxidation enzymes were significantly correlated with the de-differentiation of HCC. The positive correlation of pro-angiogenic factors with PKM2 and SNAIL suggested that hypoxia in tumor parenchyma may regulate the progression of HCC through the induction of hypoxia-related gene expression.

Materials and methods

Patients. Between April 2004 and February 2008, 41 HCC patients, without distant metastasis or lymphatic or vascular invasion, underwent partial hepatic resection at Kyushu University Hospital, Japan. The 41 patients included 31 males and 10 females, aged 42–83 years with a mean age of 68.6 years. Written informed consent was obtained from all patients. Background liver diseases were hepatitis C virus infection in 26 patients, hepatitis B virus infection in 10, alcoholic liver disease in 3 and undetermined in 2 patients. Of the 41 patients, 36 had been diagnosed with liver cirrhosis, with 31, 5 and 0 patients classified as Child-Pugh class A, B and C, respectively. Patients had a mean 1.6 ± 0.9 tumors (range, 1–4), with a mean preoperative tumor size of 3.5 ± 1.9 cm (range, 1–7.0). The study protocol conformed to the ethical guidelines of 1975 Declaration of Helsinki and was approved by the ethics committees at our institutions.

Evaluation of HCC differentiation. Resected tissues were fixed in 10% formaldehyde solution, paraffin-embedded and sectioned at a thickness of 5 μ m, followed by hematoxylin and eosin staining (H&E) for histological evaluation. The degree of differentiation of each tumor was assessed by an experimental pathologist and 2 hepatologists, and classified into 4 categories (25). It was found that 2 patients had grade I differentiation, 27 had grade II, 12 had grade III and 0 had grade IV.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from the resected tumor using TRIzol

reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized from 1.0 μ g RNA with GeneAmp™ RNA PCR (Applied Biosystems, Branchburg, NJ, USA). RT-PCR was performed using LightCycler-FastStart DNA Master SYBR-Green 1 (Roche, Basel, Switzerland). The level of expression of target mRNAs in each sample was normalized relative to the expression of β -actin mRNA. The PCR primers used were: PKM2 forward, 5'-AGATCCGAAGTGGGCTCATCA-3' and reverse, 5'-TAGATCTTGCTGCCCCACTTCAC-3'; 3-hydroxyacyl-CoA dehydrogenase (HADHA) forward, 5'-CTAGACCGAGGACAGCAACAAGT G-3' and reverse, 5'-CCAGTCAAGTTGCTGAAGATGGA A-3'; acyl-CoA oxidase (ACOX)-2 forward, 5'-AAACATCCAGATCATCGCAACGTA-3' and reverse, 5'-TGCGTCATAGGTGCTTCAGTC-3'; acetyl-CoA carboxylase (ACC)-1 forward, 5'-AGTGAGGATGGCAGCTCTGGA-3' and reverse, 5'-TGAGATGTGGGCAGCATGAAC-3'; fatty acid synthase (FAS) forward, 5'-TGAACGCCGGCACCAATA-3' and reverse, 5'-GACTGGTACAACGAGCGGATGA-3'; VEGF-A forward, 5'-GAGCCTTGCTTGCTGCTCTAC-3' and reverse, 5'-CACCAGGGTCTCGATTGGATG-3'; ETS-1 forward, 5'-GTCATTCCTGCTGCTGCCCTA-3' and reverse, 5'-AGTTTGAATTCCAGCCATCTCC-3'; SNAIL forward, 5'-GACCACTATGCCGCGCTCTT-3' and reverse, 5'-TCGCTGTAGTTAGGCTTCCGATT-3'; hepatocytes nuclear factor (HNF)-1 α forward, 5'-ACCAGAAAGCCGTGGTGAG-3' and reverse, 5'-ATGTTGTGCTGCTGCAGGTAGG-3'; HNF-1 β forward, 5'-CGCTCTGTACACCTGGTACGTCA-3' and reverse, 5'-CCAGAACTCTGGACTGTCTGGTTG-3'; HNF-4 α forward, 5'-GGGTGTCCATACGCATCCTTG-3' and reverse, 5'-CATACTGGCGGTCTGTTGATGTAG-3'.

Statistical analysis. To confirm the reciprocal effects between the examined parameters, the partial correlation coefficient for each pair of factors was calculated. Logistic regression analysis was used to determine the influence of the examined parameters on the degree of HCC differentiation. Statistical comparisons between the two groups were performed using Wilcoxon's rank sum tests. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Reduction of fatty acid oxidation and increase of aerobic glycolysis correlate with the de-differentiation of HCC. To identify factors that may affect the degree of tumor differentiation, we investigated the levels of mRNA-encoding metabolic enzymes and proteins involved in EMT, angiogenesis and hepatocyte maturation. The metabolic markers included PKM2, an enzyme involved in aerobic glycolysis; HADHA, a mitochondrial functional protein that catalyzes the third step of β oxidation; ACOX-2, which catalyzes the β oxidation of branched chain acyl-CoA in peroxisomes; as well as ACC-1 and FAS, enzymes involved in fatty acid synthesis that catalyze the carboxylation of acetyl-CoA to form malonyl-CoA and the condensation of malonyl-CoA to produce palmitate, respectively. We also analyzed the expression of SNAIL, a major transcriptional factor that regulates EMT by directly repressing E-cadherin expression and facilitating the migra-

Table I. Correlations between the levels of expression of genes associated with tumor metabolism and metastasis and the degree of tumor differentiation.

Genes	Odds ratio	SE	χ^2	P-value	Lower 95%	Upper 95%
HADHA	0.0993	0.9953	5.38	0.0203	0.4481	4.5868
ACOX-2	0.3664	0.5452	3.39	0.0356	-0.01	2.1032
PKM2	1.1207	0.0652	3.05	0.0405	-0.2819	-0.015
ACC-1	1.0229	0.155	0.02	0.8836	-0.3402	0.2856
FAS	1.2204	0.1911	1.09	0.2975	-0.6087	0.183
VEGF-A	0.999	0.002	0.23	0.6316	-0.0025	0.0046
ETS-1	1.0474	0.2801	0.03	0.8687	-0.5931	0.5279
SNAIL	0.9292	0.1323	0.31	0.5791	-0.1756	0.3595
HNF-1 α	1.2989	0.2947	0.79	0.3748	-0.8626	0.3365
HNF-1 β	1.0481	0.2079	0.05	0.8211	-0.4534	0.3717
HNF-4 α	1.0007	0.0093	0.01	0.9382	-0.7052	0.0001

SE, standard error.

Table II. Pairwise partial correlation coefficients between the levels of expression of genes related to hepatic metabolism, maturation, angiogenesis and epithelial-mesenchymal transition (EMT).

	HADHA	ACOX-2	PKM2	ACC-1	FAS	VEGF-A	ETS-1	SNAIL	HNF-1 α	HNF-1 β	HNF-4 α
HADHA		0.3712	0.0098	0.0779	-0.0855	-0.5845	0.1417	-0.0644	-0.1433	0.4994	0.0945
ACOX-2	0.3712		-0.2588	0.1297	-0.1157	0.329	0.1501	-0.1255	0.3718	-0.2295	-0.0284
PKM2	0.0098	-0.2588		0.5023	-0.2586	0.4097	0.6051	-0.4558	-0.0803	-0.0148	0.0144
ACC-1	0.0779	0.1297	0.5023		0.4781	-0.3257	-0.35	0.668	0.1157	-0.1539	0.0438
FAS	-0.0855	-0.1157	-0.2586	0.4781		0.1298	0.1276	-0.4065	0.1609	0.2364	-0.0726
VEGF-A	-0.5845	0.329	0.4097	-0.3257	0.1298		-0.4944	0.4585	-0.1069	0.3748	0.0742
ETS-1	0.1417	0.1501	0.6051	-0.35	0.1276	-0.4944		0.4057	0.012	0.1389	-0.0266
SNAIL	-0.0644	-0.1255	-0.4558	0.668	-0.4065	0.4585	0.4057		-0.0761	0.0053	-0.1209
HNF-1 α	-0.1433	0.3718	-0.0803	0.1157	0.1609	-0.1069	0.012	-0.0761		0.298	-0.051
HNF-1 β	0.4994	-0.2295	-0.0148	-0.1539	0.2364	0.3748	0.1389	0.0053	0.298		0.0212
HNF-4 α	0.0945	-0.0284	0.0144	0.0438	-0.0726	0.0742	-0.0266	-0.1209	-0.051	0.0212	

tion and invasion of cancer cells (26,27). In addition, we assayed the expression of the angiogenesis marker VEGF-A, which induces the proliferation and migration of endothelial cells and promotes vasculogenesis and angiogenesis (28,29), and ETS-1, a transcriptional factor that promotes angiogenesis by inducing the expression of matrix metalloproteinases and is associated with the invasive behavior of cancer cells (30,31). We also analyzed the expression of hepatocyte nuclear factor (HNF) family members that play a pivotal role in liver development, including HNF-1 α / β , which regulates the transcription of liver-specific genes, such as albumin, fibrinogen and anti-trypsin (32), and HNF-4 α , which regulates liver organogenesis and is required to maintain a liver phenotype (33,34).

Statistical analyses demonstrated significant correlations between the expression of three metabolic genes and the degree of differentiation of HCCs (Table I). Reductions in HADHA [odds ratio (OR)=0.0993, P=0.0203] and ACOX-2 mRNAs (OR=0.3664, P=0.0356) and increases in PKM2 mRNA (OR=1.1207, P=0.0405) were each correlated with the progression of HCC de-differentiation. The levels of expression of the

remaining mRNAs analyzed were not significantly correlated with tumor differentiation.

PKM2 expression levels correlate with genes involved in angiogenesis and EMT. To confirm the impact of metabolic features on the malignant potential of HCCs, the correlations among the genes were statistically analyzed (Table II). Analyses of partial correlation coefficients showed strong positive correlations between the levels of PKM2 mRNA and mRNAs encoding both pro-angiogenic genes, VEGF-A (correlation coefficient, 0.4097) and ETS-1 (correlation coefficient, 0.6051) as well as a strong negative correlation with mRNA encoded by the EMT-related gene, SNAIL (correlation coefficient, -0.4558). Due to the significance of PKM2 in the degree of tumor differentiation, angiogenesis and EMT may be involved in the progression of de-differentiation through a mechanism regulating PKM2 transcription.

Expression levels of pro-angiogenic genes correlate with SNAIL expression. Analyses of partial correlation coefficients

also showed positive correlations between SNAIL mRNA and mRNAs encoded by the pro-angiogenic genes VEGF-A (correlation coefficient, 0.4585) and ETS-1 (correlation coefficient, 0.4057) (Table II). However, the level of expression of VEGF-A mRNA was negatively correlated with that of ETS-1 mRNA (correlation coefficient, -0.4944). These findings suggest that the simultaneous progression of tumor angiogenesis and EMT may cooperatively promote the metastasis of HCC, while the pathways involving VEGF-A and ETS-1 may differ.

Discussion

To understand the impact of metabolic changes in HCC on malignant potentiality, we analyzed the correlations between the levels of expression of mRNAs encoded by genes involved in fatty acid synthesis, oxidation and aerobic glycolysis with pathologically estimated degrees of differentiation of resected tumor specimens. It was found that de-differentiation was associated with the downregulation of HADHA and ACOX-2 mRNAs and the upregulation of PKM2 mRNA. Analyses of the correlations among genes involved in metabolism, angiogenesis, EMT and hepatocyte maturation demonstrated that the levels of expression of PKM2 mRNA were positively correlated with the levels of expression of mRNAs encoding the pro-angiogenic molecules VEGF-A and ETS-1, and that the latter were correlated with the levels of expression of SNAIL mRNA.

HADHA was one of 11 proteins shown to be significantly downregulated in HCC lesions compared with non-cancerous tissues (35). In addition, the level of HADHA protein was found to be dependent on the degree of tumor differentiation, in that 5/8 HCCs with Edmondson's grade I were immunohistochemically positive for HADHA, whereas 31/37 HCCs with grade II-IV were negative (36). Those findings are consistent with our observation. Reductions in the level of ACOX-2 mRNA during de-differentiation provide further evidence that fatty acid oxidation is reduced in HCCs. Changes in ACOX-2 expression levels during de-differentiation have not been reported, however, pathological examination has shown a reduction in the number of peroxisomes during de-differentiation (15). Fat deposition into tumor parenchyma, which is frequently observed in HCC, could be induced by impaired fatty acid oxidation (37). Due to the fact that fatty acid oxidation produces a large amount of reactive oxygen species (ROS), loss of this physiological function may be an adaptation by HCCs to avoid further accumulation of ROS (38,39).

PKM2, an embryonic isoform of pyruvate kinase, is expressed in tumor tissues and is necessary for tumor cells to survive hypoxic conditions (40,41). Under conditions of hypoxia, cells expressing PKM2 showed a higher proliferation rate and lactate production compared with cells expressing PKM1, an adult isoform (11). A functional and transcriptional shift from PKM1 to PKM2 has been observed in human HCCs and experimental hepatocarcinogenesis (42,43). To date, correlations between the levels of expression of PKM2 and the degree of tumor differentiation have not been observed in HCCs. However, patients with a high Ki-67 labeling index, with advanced tumor stages and low survival rates, had a higher level of PKM2 expression compared with patients with

a low labeling index, suggesting that PKM2 levels increase during de-differentiation (44). These findings, together with the results of this study, suggest that the levels of PKM2 expression may reflect the progression of de-differentiation and, therefore, constitute a potential biological marker to predict patient prognosis.

The levels of PKM2 expression were correlated with the degree of tumor differentiation, as well as with the levels of expression of the pro-angiogenic genes, VEGF-A and ETS-1. Hypoxia-inducible factor (HIF)-1, a master regulator of cell responses under hypoxic conditions, has been reported to transactivate VEGF and PKM2 (45,46) and may be a transcriptional regulator of ETS-1. Genetic deletion of the HIF-1 binding region of the ETS-1 gene, reducing the expression of ETS-1, suggests that HIF-1 is involved in ETS-1 transcription (47). However, in the present study that the levels of VEGF-A and ETS-1 mRNA expression were negatively correlated, suggesting that HIF-1 is not a common transcriptional regulator of VEGF-A and ETS-1 in HCCs. In addition, TX-402 induced the downregulation of HIF-1 but did not reduce ETS-1 expression under hypoxic conditions in hepatoma cells (48). Despite these contrary findings, the coordinated regulation of these genes is necessary for tumor cells to adapt to hypoxia. HIF-1-dependent hypoxia-inducible genes have been shown to be transactivated in the presence of ETS-1 (49). Additionally, direct binding of PKM2 to HIF-1 has been shown to promote the further upregulation of HIF-1 target genes (46). Taken together, these findings indicate that hypoxia is a common inducer of genes associated with the malignant potential of HCC, with PKM2 being a downstream target, since its expression was correlated with the degree of differentiation.

Multiple pathways are involved in the transcriptional regulation of SNAIL. Growth factors, including fibroblast growth factor (FGF), epidermal growth factor (EGF), and transforming growth factor (TGF)- β , increase SNAIL expression (27,50,51). VEGF has also been reported to stimulate SNAIL expression, followed by a reduction of E-cadherin (52). Hypoxia also upregulates SNAIL expression via an HIF-1-mediated pathway (53,54). Although the interaction between ETS-1 and SNAIL remains to be elucidated, ETS-1 expression under conditions of hypoxia suggests that increased SNAIL expression in HCC is likely induced by a coordinated response to hypoxia involving HIF-1.

In conclusion, findings of the present study have shown that metabolic changes, especially reduction of fatty acid oxidation and induction of the aerobic glycolysis gene PKM2, were significant features during the progression of de-differentiation of HCC. Other factors associated with malignant potential may be induced by a coordinated cell response to tumor hypoxia. These observations suggest that targeting these metabolic changes and tumor hypoxia may be useful in developing novel treatment options of HCC.

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