Identification of overexpressed genes in hepatocellular carcinoma, with special reference to ubiquitin-conjugating enzyme E2C gene expression

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This study consisted of 2 aims: (i) to determine genes associated with hepatocellular carcinoma (HCC) by microarray analysis; and (ii) to evaluate the clinicopathological significance of human ubiquitin-conjugating enzyme E2C (Ube2c) found to be overexpressed in HCC from microarray analysis. Laser microdissection and cDNA-microarray were performed to identify genes associated with HCC. We then focused on the Ube2c gene. Using real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR), Ube2c expression status and clinicopathological significance were studied in 65 clinical HCC samples. A number of genes upregulated in HCC cells compared to noncancerous liver cells were identified, one of which was the Ube2c gene. Ube2c gene expression in the cancer tissue was higher than in the correspondingly noncancerous tissue in 62 of the 65 cases (95.4%, p < 0.01). Tumors with high Ube2c expression showed higher frequencies of tumor invasion to capsular formation (fc-inf), invasion to portal vein (vp) and tumor de-differentiation (p < 0.05). Patients with high Ube2c expression also showed significantly worse disease-free survival rates than those with low Ube2c expression (p < 0.01). In addition, Ube2c expression was found to be an independent prognostic factor for disease-free survival rate in multivariate analysis. We identified differentially expressed genes between HCC and normal liver tissues. Of those, the Ube2c gene appeared to be associated with HCC progression, and may be useful as a prognostic indicator for HCC patients.

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Key words: hepatocellular carcinoma; microarray; laser microdissection (LMD); ubiquitin-conjugating enzyme E2C (Ube2c)

Hepatocellular carcinoma (HCC) is one of the most common cancers in Japan, and its prevalence is increasing in America. Most Japanese HCC cases develop from liver cirrhosis that is almost entirely due to chronic hepatitis C or B.1-3 Although recent advances in molecular biology have elucidated the developmental pathway of HCC from liver cirrhosis, few studies have determined the differences in gene expression profiles between HCC and normal liver tissues. Therefore, we analyzed for differentially expressed genes between cancerous tissues from HCC patients and noncancerous liver tissues without liver cirrhosis from patients with metastatic liver tumors using laser microdissection (LMD) and cDNA-microarray. As a result, we detected 123 genes that were overexpressed by more than 2-fold in HCC compared to normal liver in at least 4 of the 6 samples examined. In the past, the ubiquitin-conjugating enzyme E2C (Ube2c) gene due to its involvement in the ubiquitination pathway.

The Ube2c gene, located on chromosome 20q13, belongs to the E2 gene family and codes for a 19.6 kDa protein involved in ubiquitin-dependent proteolysis. Rape and Kirschner showed that cyclin A degradation was highly sensitive to the concentration of Ube2c, and self-degradation of Ube2c is an autonomous sensor of mitotic completion and provides the molecular switch that allows cells to proceed from DNA segregation and cell division to a new round of DNA duplication.7,8 Ube2c has also been reported to be highly expressed in various types of cancers.9-13 However, the relationship between Ube2c expression and clinicopathological factors in HCC has not yet been investigated.

In the present study, we report the identification of overexpressed genes in HCC by LMD and cDNA-microarray analysis, and then examine the clinicopathological significance of the Ube2c gene, detected as an overexpressed gene in HCC patients.

Material and methods

Clinical samples

Sixty-five patients with HCC were enrolled into this study. All patients underwent resection of the primary tumor at the Kyushu University Hospital at Beppu and affiliated hospitals between 2001 and 2003. Resected tumor and paired nontumor tissue specimens were immediately cut from the resected liver and placed in RNA Layter (TaKaRa, Japan) or embedded in Tissue Tek OCT medium (Sakura, Tokyo, Japan), frozen in liquid nitrogen and kept at −80°C until RNA extraction. Written informed consent was obtained from all patients. The follow-up period ranged from 0.1 to 4.3 years with a median of 2.5 years.

Identification of overexpressed genes in HCC

Samples. For the identification of overexpressed genes in HCC, 6 randomly selected HCC cases were used (hepatitis B virus (HBV) (+): 2 cases, hepatitis C virus (HCV) (+): 2 cases, HBV (−) HCV (−): 2 cases). As a control, the samples of noncancerous liver tissues were obtained from another 6 cases. These 6 were the reference to ubiquitin-conjugating enzyme E2C (Ube2c), and self-degradation of Ube2c is an autonomous sensor of mitotic completion and provides the molecular switch that allows cells to proceed from DNA segregation and cell division to a new round of DNA duplication.7,8 Ube2c has also been reported to be highly expressed in various types of cancers.9-13 However, the relationship between Ube2c expression and clinicopathological factors in HCC has not yet been investigated.

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patients with metastatic liver tumor, and the liver showed normal appearance without cirrhosis or infection with HCV or HBV.

** Laser microdissection and RNA extraction.** Cancer tissues were microdissected using the LMD system (Leica Laser Microdissection System, Leica Microsystems, Wetzlar, Germany) as previously described.\(^{12}\) Total RNA was extracted using an RNasey Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Total RNA from noncancerous samples was obtained from 6 normal liver samples. Purity and concentration of the RNA samples were determined with a NanoDrop (NanoDrop Technologies, Wilmington) and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto) as previously described.\(^{15}\)

**cDNA-microarray.** T7-based RNA amplification was performed using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies, USA). Total RNA (100 ng) was reverse transcribed to cDNA using MMLV-RT and oligo dT primers, and used as a template for *in vitro* transcription reactions in the presence of Cyanine labeled CTP (NEN Life Science, Boston, MA) and T7 RNA polymerase. cRNA from noncancerous tissues was labeled with Cyanine 3-CTP (Cy3), while cancer tissue cRNA was labeled with Cyanine 5-CTP (Cy5). After purification using an RNasey Mini Kit, aliquots of the amplified cRNA were validated on an Agilent 2100 Bioanalyzer. The Cy3 and Cy5 labeled cRNA were mixed and hybridized to a cDNA-microarray (Agilent Human Microarray, Agilent Technologies, USA). A standard curve on this cDNA microarray is available from http://www.agilent.com/chem/genelist. Scanning of the array slides was performed using an Agilent dual laser DNA microarray scanner (Agilent Technologies, USA).

**Data analysis.** The intensity of each hybridization signal was evaluated using Feature Extraction software (Agilent Technologies, USA). The common logarithm of the Cy5/Cy3 ratio for each sample was calculated by averaging the spots. A cutoff value for expression data. Candidate genes which fulfilled the following criteria were selected: the fold changes were **p** <0.01. Moreover, within the selected genes that met these criteria, genes that were upregulated in 4 or more samples (6 total samples) were analyzed.

**Evaluation of Ube2c gene expression**

**Clinical samples and cell lines.** For evaluation of *Ube2c* gene expression, all 65 tumors and nontumor samples, as described above, were used. Human HCC cell lines (HuH-7, Hep-G2, Hep-3B) were provided by the Cell Resource Center of Biomedical Research, Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan) and maintained in RPMI 1640 medium or DMEM containing 10% fetal bovine serum (FBS) and antibiotics at 37°C in a 5% humidified CO₂ atmosphere.

**Oligonucleotide primers for Ube2c gene amplification by RT-PCR.** Total RNA was extracted from each clinical sample and cDNA synthesized from 8.0 μg total RNA as previously described.\(^{14}\)

**Ube2c-specific oligonucleotide primers were designed to give a 165 bp PCR product:** sense primer 5’-GGATTCTGCTCTTCCA-GAA-3’; antisense primer 5’-GATAGCGGGCAGGAGA-3’.\(^{12}\) Primers were also designed for *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) (270 bp): sense primer 5’-CTG-GTATCCGGAAGGACTCA-3’; antisense primer 5’-TTGCTAT- CATATTTGGCAGGT-3’. To avoid amplification of contaminating genomic DNA, the primers spanned more than 2 exons. Amplification was performed for 29 cycles (22 cycles for *GAPDH*) of 1 min at 95°C, 1 min at 60°C (56°C for *GAPDH*) and 1 min at 72°C. An 8.0 μl aliquot of each PCR-amplified DNA was electrophoresed on 2% agarose gels containing ethidium bromide. **Real-time quantitative RT-PCR.** PCR amplification for quantification of *Ube2c* and *GAPDH* mRNA in 65 clinical samples was performed using the LightCycler system (Roche Applied Science, IN) and the LightCycler-FastStart DNA Master SYBR Green I kit (Roche Applied Science, IN) as previously described.\(^{17}\) Amplification conditions consisted of initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 64°C (60°C for *GAPDH*) for 10 s and elongation at 72°C for 10 s. Melting curve analysis and electrophoresis on 2% agarose gels were performed to ensure that the expected PCR products were generated. To quantify specific mRNA in the samples, a standard curve was produced for each run based on 3 points from diluted HuH-7 cDNA. Relative *Ube2c* expression levels were then obtained by normalizing the amount of *Ube2c* mRNA divided by that of *GAPDH* mRNA as an endogenous control in each sample.

**Western blot analysis.** Total protein was extracted from 4 representative pairs of samples and cell lines using protein extraction solution (PRO-PREP, nNITRON Biotecnology, Korea). Aliquots of total protein (60 μg for clinical samples or 20 μg for cell lines) were electrophoresed in 12.5% concentrated READY GELS J (Bio-Rad Laboratories, Japan) and then electroblotted onto pure nitrocellulose membranes (Trans-Blot Transfer Medium; Bio-Rad Laboratories, Japan) at 0.2 A for 120 min at 4°C. *Ube2c* protein was detected using goat polyclonal antibody (AB3935, Abcam, USA) diluted 1:200. *Ube2c* protein levels were normalized to the level of β-actin protein (Cytoskeleton, Denver, CO) diluted 1:1,000. Blots were developed with horse-radish peroxidase-linked anti-goat immunoglobulin (Promega, Madison, WI) diluted 1:1,000. ECL Detection Reagents (Amer sham Biosciences, Piscataway, NJ) were used to detect antigen-antibody reactions.

**Immunohistochemistry.** Immunohistochemical studies of *Ube2c* were performed on surgical specimens from representative HCC patients. Formalin-fixed, paraffin-embedded tissues were deparaffi nized, blocked, incubated with specific antibodies overnight at 4°C, and detected using ENVISION reagents (ENVISION+ Dual Link/HRP, Dako Cytomation, Denmark). All sections were counterstained with hematoxylin. Primary goat polyclonal anti-*Ube2c* antibody (AB3935, Abcam, USA) was used at a dilution of 1:200.

**Ube2c RNA interference.** *Ube2c*-specific siRNA (Silencer™ Predesigned siRNA) and negative control siRNA (Silencer™ Negative Control siRNA) were purchased from Ambion, USA. Logarithmic growth-phase Hep3B cells were seeded at 1.5 × 10⁵ or 2 × 10⁵ cells/well in a final volume of 2 ml or 100 μl, respectively, in 6 or 96 well flat bottom microtitre plates, respectively, and then cultured overnight to allow adherence. siRNA-Lipofectamine™ 2000 complexes were then added to each well as previously described,\(^{13}\) and *in vitro* proliferation assay were performed after 48 hr incubation from siRNA addition.

**In vitro proliferation assay.** Proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Roche Diagnostics Corp., GmbH). After 48 hr incubation from siRNA addition, cells were further cultured for 0–96 hr. After 0–96 hr culture, spectrophotometric absorbance of the samples was measured as previously described.\(^{18}\) Each independent experiment was performed 3 times.

**Statistical analysis.** For continuous variables, data were expressed as the means ± SD. Differences between groups were estimated using Student’s *t* test, *p* value of 0.05 by univariate analysis were used in subsequent multivariate analyses based on Cox’s proportional hazards model. All differences were deemed significant at the level of *p* <0.05.
Evaluating the expression of Ube2c mRNA and protein in representative HCC cases.

**Figure 1** – (a) Expression of Ube2c mRNA as assessed by RT-PCR in representative HCC cases. (T, cancer tissue; N, noncancerous tissue; n, negative control; p, positive control; m, marker) (b) Ube2c mRNA expression in cancer and noncancerous tissues from HCC patients as assessed by real-time quantitative PCR (n = 65). Horizontal lines indicate mean value of each group. (T, cancer tissue; N, noncancerous tissue) (c) Expression of Ube2c protein by western blot in representative HCC patient tissues. Ube2c protein was detected as a band of ~20 kDa. (T, cancer tissue; N, noncancerous tissue; p, positive control; m, marker) (d) Immunohistochemistry with Ube2c antibody on HCC patient samples. The majority of staining occurred in cancer cells. (a): cancer tissue, original magnification ×200, Ube2c stain. (b): noncancerous tissue, original magnification ×200, Ube2c stain.

Statistical analyses were performed using the JMP 5 for Windows software package (SAS Institute, Cary, NC).

**Results**

Identification of overexpressed genes in HCC

Using a human cDNA-microarray, we determined differentially expressed genes between HCC and noncancerous liver cells. We identified 123 upregulated genes in the cancer cells compared to the noncancerous cells (Supplementary 1). These genes were overexpressed more than 2-fold in at least 4 of 6 cases, and included genes previously reported to be associated with HCC, such as FAT10, GPC3, MDK, SPINK1, ROBO1, PLA2G2A and PEG10.19–28 Genes involved in cell cycle regulation or cell adhesion, as well as growth factors and proteinases were also overexpressed and so could be considered as candidate cancer-related genes. One of the overexpressed genes was Ube2c, associated with ubiquitination.

Evaluation of Ube2c gene expression

Expression of Ube2c mRNA in cell lines and clinical tissue specimens. Ube2c mRNA expression in cell lines was examined by reverse transcription-polymerase chain reaction (RT-PCR) and revealed that all 3 cell lines tested, HuH-7, Hep-G2 and Hep-3B, highly expressed Ube2c mRNA. Ube2c mRNA expression in cancerous and noncancerous tissues of HCC patients was examined by RT-PCR and real-time quantitative PCR, with quantified values used to calculate Ube2c/GAPDH expression ratios. Results indicated that Ube2c mRNA expression levels were higher in cancer tissues (0.074 ± 0.066) than in noncancerous tissues (0.012 ± 0.014) in 62 of the 65 cases (95.4%). This resulted in a significant difference in mRNA expression level between cancer and normal tissues (p < 0.01) (Figs. 1a and 1b). We classified the 65 HCC cases into 2 groups according to median Ube2c mRNA expression level in tumor tissues, as determined by quantitative RT-PCR, to give high (n = 33) and low (n = 32) expression groups.

The clinicopathological significance of Ube2c mRNA expression in HCC. Clinicopathological features were analyzed in relation to Ube2c expression status (Table I). The incidence of tumor invasion to capsular formation (fc-inf) was significantly higher (p < 0.05) in the high expression group (23 of 33 fc-inf positive, 69.7%) than in the low expression group (13 of 32 fc-inf positive, 40.6%). Likewise, the incidence of invasion to portal vein (vp) was higher (p < 0.05) in the high expression group (23 of 33 vp positive, 69.7%) than in the low expression group (13 of 32 vp positive, 40.6%), and poorly-differentiated HCC showed higher Ube2c expression levels than well-differentiated HCC (p < 0.05).

No other significant differences were observed with respect to age, gender, tumor size, capsular formation (fc), invasion to hepatic vein (vv), invasion to bile duct (b) and number of tumors.

Analysis of disease-free survival curves showed that patients in the high expression group had a significantly poorer prognosis than those in the low expression group (p < 0.01) (Fig. 2). However, overall survival rates between the 2 groups were not statistically different (data not shown).

Univariate analysis identified Ube2c expression (low or high expression), tumor size (≤ or >3 cm) and number of tumors (soli- tary or multiple) as adverse prognostic factors for disease-free survival after hepatic resection. Variables with a p value of less than 0.15 by univariate analysis were selected for multivariate analysis using Cox’s proportional hazards model. Ube2c expression (relative risk (RR): 1.51, confidence interval (CI): 1.06–2.22, p = 0.02) was found to be a significant factor affecting disease-free survival rate following hepatic resection (Table II).

Ube2c protein expression in clinical tissue specimens. Ube2c protein expression in tumor and normal tissues from representative
TABLE I – Ube2c GENE EXPRESSION AND CLINICOPATHOLOGICAL FEATURES FOR 65 HCC PATIENTS

| Clinicopathologic variable | High expression group (n = 33) | Low expression group (n = 32) | p value |
|----------------------------|-------------------------------|-------------------------------|---------|
| Age                        | 65.3 ± 11.2                   | 66.7 ± 9.1                    | 0.59    |
| Gender                     |                               |                               |         |
| Male                       | 20                            | 23                            | 0.34    |
| Female                     | 13                            | 9                             |         |
| Tumor size                 | 4.2 ± 3.8                     | 3.2 ± 1.9                     | 0.21    |
| Capsular formation (fc)    |                               |                               |         |
| Absent                     | 8                             | 11                            | 0.37    |
| Present                    | 25                            | 21                            |         |
| Invasion to capsular formation (fc-inf) |                   |                               |         |
| Absent                     | 10                            | 19                            | 0.02    |
| Present                    | 23                            | 13                            |         |
| Invasion to portal vein (vv) | 10                            | 19                            | 0.02    |
| Absent                     | 23                            | 13                            |         |
| Invasion to hepatic vein (vp) | 30                            | 29                            | 0.68    |
| Absent                     | 2                             | 2                             |         |
| Invasion to bile duct (b)  |                               |                               |         |
| Absent                     | 32                            | 31                            | 0.75    |
| Present                    | 1                             | 1                             |         |
| Number of tumors           |                               |                               |         |
| Solitary                   | 23                            | 21                            | 0.87    |
| Multiple                   | 10                            | 10                            |         |
| Histology                  |                               |                               |         |
| Poor                       | 7                             | 1                             | 0.01    |
| Moderate                   | 24                            | 24                            |         |
| Well                       | 1                             | 7                             |         |
| HBV/HCV                    |                               |                               |         |
| HBV                        | 6                             | 3                             | 0.47    |
| HCV                        | 24                            | 24                            |         |
| Non-B/C                    | 3                             | 5                             |         |
| Liver tissue               |                               |                               |         |
| LC                         | 12                            | 15                            | 0.26    |
| Non-LC                     | 20                            | 14                            |         |

High expression group (Ube2c/GAPDH ≥ median value), Low expression group (Ube2c/GAPDH < median value). Well, well differentiated; poor, poorly differentiated; moderate, moderately differentiated; HBV, hepatitis B virus; HCV, hepatitis C virus; LC, liver cirrhosis; fc-inf, invasion to capsule or outside of capsule. Cases with no capsule formation were included in fc-inf (–). Ube2c expression was localized to cell nuclei (Fig. 1d). Expression of Ube2c in poorly differentiated HCC was detected in nuclei and cytoplasm.

**Effect of Ube2c gene silencing on an HCC cell line.** As described earlier, Hep-3B cells showed high Ube2c expression levels. Suppression of Ube2c mRNA was confirmed by real-time quantitative PCR (50% suppression) (Fig. 3a). Protein expression was suppressed by Ube2c-specific siRNA in western blots (Fig. 3b). As shown in Figure 3c, suppression of Ube2c inhibited the proliferation rate of Hep3B HCC cells (96 hr Ube2c siRNA: 1.92 ± 0.40, negative siRNA: 2.97 ± 0.36, control: 3.29 ± 0.29) (p < 0.01).

**Discussion**

This study identified differentially expressed genes between HCC and normal liver tissues (Supplementary 1). Diubiquitin (FAT10) was the most upregulated gene and belongs to the ubiquitin-like modifier (UBL) family. It has been reported that upregulation of FAT10 expression in tumors was observed in 90% of HCC patients. Another highly upregulated gene was glypican-3 (GPC3), a member of the glypican family of glycosyl-phosphatidylinositol-anchored cell-surface heparin sulfate proteoglycans. GPC3 is also reported to be expressed in most HCC cells, but not in normal hepatocytes and benign liver lesions, and serum GPC3 protein levels are elevated in a large proportion of HCC patients. Midkine (MDK) is a member of the heparin-binding growth factor family and increased MDK expression has been reported in various human cancers including HCC, and significantly elevated serum MDK levels are observed in cancer patients. It is thought that MDK acts as an antiapoptotic factor in HepG2 cells through the down-regulation of caspase-3 activity. Other genes reported to be associated with HCC (SPINK1, ROBO1, PLA2G2A, PEG10, and so on) were also included in the list of upregulated genes.
The function of the Ube2c gene product is closely linked to cell cycle progression and the destruction of mitotic cyclins. Rape and Kirschner showed that the decision between cyclinA degradation and APC inactivation is determined by Ube2c availability. Our study demonstrated that siRNA-mediated suppression of Ube2c expression inhibited the growth rate of an HCC cell line, which was consistent with reports that NIH3T3 cells stably transfected with Ube2c exhibited a more malignant phenotype than the parental NIH3T3 cells, such that Ube2c gene silencing by siRNA inhibited cell proliferation without inducing cell death, with cell cycle analysis by FACS following Ube2c siRNA treatment showing arrest at the G2/M phase. Furthermore, when combined with agonists for the DR5/TNF-related apoptosis inducing ligand (TRAIL) receptor, inhibition of Ube2c by siRNA enhanced tumor cell killing. Therefore, in HCC patients, high Ube2c expression may lead to increased malignant potential of the tumor, such that the Ube2c gene may have some utility as a therapeutic target.

The Ube2c gene was found to be overexpressed in gastrointestinal cancer, with chromosomal amplification at the Ube2c locus, 20q13.1, shown. 20q amplification is common among various carcinomas, including HCC, so it is likely that this amplification would induce Ube2c overexpression, thereby increasing the malignant potential of HCC cells.

In conclusion, our study identified upregulated genes in HCC compared to normal liver cells, and also showed that one of the upregulated genes, Ube2c, may play an important role in HCC, and may prove useful as a novel prognostic marker for patients with HCC.

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