

**Fig. 1.** (A) RT-PCR results of *ACOX2* and *ACOX2* intronic RNAs in independent NL, NT (non-B, non-C), and T (non-B, non-C) samples. RT-PCR was performed in triplicate for each sample-primer set from cDNA. The PCR products were semi-quantitatively analyzed with ImageJ software and calculated as levels relative to *polymerase (RNA) II (DNA directed) polypeptide L (POLR2L)*. The bar graph indicates the expression ratio of intronic-*ACOX2-1* to canonical *ACOX2*. The expression pattern of intron 1 was different from that of canonical *ACOX2*. (B) RTD-PCR analysis of *ACOX2* and *ACOX2* intronic RNAs in NL, T (HBV-related, HCV-related, and non-B, non-C), and NT tissues. Quantitative RTD-PCR was performed in duplicate for each sample-primer set from cDNA. Each sample was normalized relative to *POLR2L*. All HCC tissues were pathologically diagnosed as well differentiated HCC (wHCC) or moderately differentiated HCC (mHCC). Kruskal–Wallis tests and Mann–Whitney *U* tests were used for statistical analysis. *ACOX2*, acyl-Coenzyme A oxidase 2; HCC, hepatocellular carcinoma; NL, normal liver; NT, non-tumor; RT-PCR, reverse transcriptase-polymerase chain reaction; RTD-PCR, real-time detection-PCR; T, tumor. \**P* < 0.01, #*P* < 0.05.

were able to clone the intron-origin *ACOX2* RNAs (intronic-*ACOX2-1*, 2) for the first time and found that intronic-*ACOX2-1* was significantly overexpressed in T compared with NT and NL. The ratio of intronic-*ACOX2-1* and canonical *ACOX2* (relative intronic-*ACOX2*) was progressively up-regulated from NL via the background liver to HCC. Importantly, the expression of relative intronic-*ACOX2* was more up-regulated in moderately differentiated HCC than in well-differentiated HCC. The intronic difference in expression might be due to a polymorphism, since the 5'SAGE library for NL and T were from different people. The mechanisms of stepwise increase of intronic-*ACOX2* in the process of hepatocarcinogenesis should be clarified in future.

*ACOX2* is a rate-limiting enzyme of branched-chain acyl-CoA oxidase involved in the degradation of long branched fatty acid and bile acid intermediates in peroxisomes. *ACOX2* expression was associated with the differentiation state of hepatocytes and was repressed under the undifferentiated phase of human hepatoma cell lines [24]. A decreased *ACOX2* expression was also reported in prostate cancer [25]. Here, the expression of canonical *ACOX2* was decreased, while that of intronic-*ACOX2-1* was increased in HCC. The deduced amino acid of intronic-*ACOX2-1* encodes the C-terminal (from 386 to 681 amino acids) of canonical *ACOX2*, lacking the active sites for FAD binding and a fatty acid as the substrate, suggesting that the protein may be functionally departed [26]. The biological role of

the increased intronic-*ACOX2-1* was not clear, but it might be reflected by the activation of peroxisome proliferators-activated receptor alpha (PPARA). It is reported that mice lacking *ACOX1*, another rate-limiting enzyme in peroxisomal straight-chain fatty acid oxidation, developed steatosis and HCC characterized by increased mRNA and protein expression of genes regulated by PPAR $\alpha$  [27]. The importance of PPAR $\alpha$  activation in HCC development has been recently reported using HCV core protein transgenic mice [28]. Moreover, the overexpression of alpha-methylacyl-CoA racemase (AMACR), an enzyme for branched-chain fatty acid beta-oxidation, is reported to be a reliable diagnostic marker of prostate cancer and is associated with the decreased expression of *ACOX2* [25]. Therefore, the expression of intronic-*ACOX2-1* might open the door for further investigations of their potential clinical use, e.g., serving as diagnostic markers of HCC, although the functional relevance of this gene should be further clarified.

In conclusion, we report the first comprehensive transcriptional analysis of non-B, non-C HCC, NT background liver, and NL tissue, based on 5'SAGE. This study offers new insights into the transcriptional changes that occur during HCC development as well as the molecular mechanism of carcinogenesis in the liver. The results suggest the presence of unique intron-origin RNAs that are useful as diagnostic markers and may be used as new therapeutic targets.



## Material and methods

### Samples

Samples were obtained from a 56-year-old man who had undergone surgical hepatic resection for the treatment of solitary HCC. Serological tests for hepatitis B surface (HBs) antigen and anti-HCV antibodies were negative. Tumor (T) and non-tumor (NT) tissue samples were separately obtained from the tumorous parts (diagnosed as moderately differentiated HCC) and non-tumorous parts (diagnosed as mild chronic hepatitis: F1A1) of the resected tissue. We also obtained five normal liver (NL) tissue samples from five patients who had undergone surgical hepatic resection because of metastatic liver cancer. None of the patients was seropositive for both HBs antigen and anti-HCV antibodies. Neither heavy alcohol consumption nor the intake of chemical agents was observed before surgical resection. All laboratory values related to hepatic function were within the normal range. All procedures and risks were explained verbally and provided in a written consent form.

We additionally used independent four NL tissue samples, 19 HBV-HCCs, 20 HCV-HCCs and 4 non-B, non-C HCCs, and their background liver tissue samples for reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time detection (RTD)-PCR (Supplemental Table 1). Four non-B, non-C HCCs were histologically diagnosed as moderately differentiated HCCs, and the adjacent non-cancerous liver tissues were diagnosed as a normal liver, a chronic hepatitis, a pre-cirrhotic liver and a cryptogenic liver cirrhosis, respectively. None of the patients was seropositive for HBs antigen, anti-HBs antibodies, anti-hepatitis B core (HBc) antibodies and anti-HCV antibodies. Neither heavy alcohol consumption nor the intake of chemical agents was observed. Histological grading of the tumor was evaluated by two independent pathologists as described previously [16].

### Generation of the 5' SAGE library

5'SAGE libraries were generated as previously described [14]. Five to ten micrograms of poly(A)+RNA was treated with bacterial alkaline phosphatase (BAP; TaKaRa, Otsu, Japan). Poly(A)+RNA was extracted twice with phenol: chloroform (1:1), ethanol precipitated, and then treated with tobacco acid pyrophosphatase (TAP). Two to four micrograms of the BAP-TAP-treated poly(A)+RNA was divided into two aliquots and an RNA linker containing recognition sites for *EcoRI*/*MmeI* was ligated using RNA ligase (TaKaRa): one aliquot was ligated to a 5'-oligo 1 (5'-GGA UUU GCU GGU GCA GUA CAA CGA AUU CCG AC-3') linker, and the other aliquot was ligated to a 5'-oligo 2 (5'-CUG CUC GAA UGC AAG CUU CUG AAU UCC GAC-3') linker. After removing unligated 5'-oligo, cDNA was synthesized using RNaseH-free reverse-transcriptase (Superscript II, Invitrogen, Carlsbad, CA, USA) at 12 °C for 1 h and 42 °C for the next hour, using 10 pmol of dT adapter-primer (5'-GCG GCT GAA GAC GGC CTA TGT GGC CTT TTT TTT TTT TTT-3'). After first-strand synthesis, RNA was degraded in 15 mM NaOH at 65 °C for 1 h. cDNA was amplified in a volume of 100 µl by PCR with 16 pmol of 5' (5' [biotin]-GGA TTT GCT GGT GCA GTA CAA-3' or 5' [biotin]-CTG CTC GAA TGC AAG CTT CTG-3') and 3' (5'-GCG GCT GAA GAC GGC CTA TGT-3') PCR primers. cDNA was amplified using 10 cycles at 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 2 min. PCR products were digested with the *MmeI* type IIS restriction endonuclease (NEB, Pickering, Ontario, Canada). The digested 5'-terminal cDNA fragments were bound to streptavidin-coated magnetic beads (Dynal, Oslo, Norway). cDNA fragments that bound to the beads were directly ligated together in a reaction mixture containing T4 DNA ligase in a supplied buffer for 2.5 h at 16 °C. The ditags were amplified by PCR using the following primers: 5' GGA TTT GCT GGT GCA GTA CA 3' and 5' CTG CTC GAA TGC AAG CTT CT 3'. The PCR products were analyzed by polyacrylamide gel electrophoresis (PAGE) and digested with *EcoRI*. The region of the gel containing the ditags was excised and the fragments were self-ligated to produce

long concatamers that were then cloned into the *EcoRI* site of pZero 1.0 (Invitrogen). Colonies were screened by PCR using the M13 forward and reverse primers. PCR products containing inserts of more than 600 bp were sequenced with Big Dye terminator ver.3 and analyzed using a 3730 ABI automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). All electrophoretograms were reanalyzed by visual inspection to check for ambiguous bases and to correct misreads. In this study, we obtained 19–20 bp tag information.

### Association of the 5'SAGE tags with their corresponding genes

We attempted to align our 5'tags with the human genome (NCBI build 36, available from <http://www.genome.ucsc.edu/>) using the alignment program ALPS (<http://www.alps.gi.ku-tokyo.ac.jp/>). Only tags that matched in sense orientation were considered in our analysis. The RefSeq database was searched for transcripts corresponding to the regions adjacent to the alignment location of each 5'tag.

### RT-PCR

Total RNA was extracted using a ToTally RNA extraction kit (Ambion, Inc., Austin, TX, USA). Total RNA (500 ng) was reverse-transcribed in a 100-µl reaction solution containing 240 U of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA), 80 U of RNase inhibitor (Promega), 4.6 mM MgCl<sub>2</sub>, 6.6 mM DTT, 1 mM dNTPs, and 2 mM random hexamer (Promega), at 42 °C for 1 h. PCR was performed in a 20-µl volume containing 0.5 U of AmpliTaq DNA polymerase (Applied Biosystems), 16.6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 67 mM Tris-HCl, 6.7 mM MgCl<sub>2</sub>, 10 mM 2-mercaptoethanol, 1 mM dNTPs, and 1.5 µM sense and antisense primers, using an ABI 9600 thermal cycler (Applied Biosystems). The amplification protocol included 28–30 cycles of 95 °C for 45 s, 58 °C for 1 min, and 72 °C for 1 min. Primer sequences are shown in Supplemental Table 2. RT-PCR was performed in triplicate for each sample-primer set. Each sample was normalized relative to *polymerase (RNA) II (DNA directed) polypeptide L (POLR2L)*. *POLR2L* is a housekeeping gene that showed relatively stable gene expression in various tissues [29]. The PCR products were semi-quantitatively analyzed with ImageJ software (<http://rsb.info.nih.gov/ij/>).

### RTD-PCR

Intron-origin transcript expression was quantified using TaqMan Universal Master Mix (Applied Biosystems). The samples were amplified using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Using the standard curve methods, quantitative PCR was performed in duplicate for each sample-primer set. Each sample was normalized relative to *POLR2L*. The assay IDs used were Hs00185873\_m1 for *ACOX2* and Hs00360764\_m1 for *POLR2L*. The specific primers and probe sequence of intronic-*ACOX2-1* were 5'-TTCATAAAGTTGTGAGCA-GAGGAAA-3' (forward), 5'-TGCACCACTTACTGAGCATCTACTC-3' (reverse), and 5'-ACTTCTTACCTCAGAGCTG-3' (probe).

### Analysis of pathway network

MetaCore™ software (GeneGo Inc., St. Joseph, MI) was used to investigate the molecular pathway networks of non-B, non-C HCC, HBV-HCC and HCV-HCC. All genes up-regulated more than five-fold in all HCC libraries subjected to Enrichment analysis in GO process networks by default settings ( $p < 0.05$ ).

### Statistical analysis

Kruskal–Wallis tests were used to compare the expression among normal liver, non-cancerous tissues, and HCC tissues. Mann–Whitney *U* tests were also used to evaluate the statistical significance of *ACOX2*



gene expression levels between two groups. All statistical analyses were performed using R (<http://www.r-project.org/>).

### Acknowledgments

The authors would like to thank Mr. Shungo Deshimaru and Ms. Keiko Harukawa for technical assistance.

### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2010.01.004.

### References

- [1] H.B. El-Serag, K.L. Rudolph, Hepatocellular carcinoma: epidemiology and molecular carcinogenesis, *Gastroenterology* 132 (2007) 2557–2576.
- [2] Y. Yokoi, S. Suzuki, S. Baba, K. Inaba, H. Konno, S. Nakamura, Clinicopathological features of hepatocellular carcinomas (HCCs) arising in patients without chronic viral infection or alcohol abuse: a retrospective study of patients undergoing hepatic resection, *J. Gastroenterol.* 40 (2005) 274–282.
- [3] R.N. Aravalli, C.J. Steer, E.N. Cressman, Molecular mechanisms of hepatocellular carcinoma, *Hepatology* 48 (2008) 2047–2063.
- [4] D.J. Duggan, M. Bittner, Y. Chen, P. Meltzer, J.M. Trent, Expression profiling using cDNA microarrays, *Nat. Genet.* 21 (1999) 10–14.
- [5] V.E. Velculescu, L. Zhang, B. Vogelstein, K.W. Kinzler, Serial analysis of gene expression, *Science* 270 (1995) 484–487.
- [6] T. Yamashita, S. Hashimoto, S. Kaneko, S. Nagai, N. Toyoda, T. Suzuki, K. Kobayashi, K. Matsushima, Comprehensive gene expression profile of a normal human liver, *Biochem. Biophys. Res. Commun.* 269 (2000) 110–116.
- [7] S. Hashimoto, S. Nagai, J. Sese, T. Suzuki, A. Obata, T. Sato, N. Toyoda, H.Y. Dong, M. Kurachi, T. Nagahata, K. Shizuno, S. Morishita, K. Matsushima, Gene expression profile in human leukocytes, *Blood* 101 (2003) 3509–3513.
- [8] H. Okabe, S. Satoh, T. Kato, O. Kitahara, R. Yanagawa, Y. Yamaoka, T. Tsunoda, Y. Furukawa, Y. Nakamura, Genome-wide analysis of gene expression in human hepatocellular carcinomas using cDNA microarray: identification of genes involved in viral carcinogenesis and tumor progression, *Cancer. Res.* 61 (2001) 2129–2137.
- [9] Y. Shirota, S. Kaneko, M. Honda, H.F. Kawai, K. Kobayashi, Identification of differentially expressed genes in hepatocellular carcinoma with cDNA microarrays, *Hepatology* 33 (2001) 832–840.
- [10] T. Yamashita, M. Honda, S. Kaneko, Application of serial analysis of gene expression in cancer research, *Curr. Pharm. Biotechnol.* 9 (2008) 375–382.
- [11] Y. Suzuki, H. Taira, T. Tsunoda, J. Mizushima-Sugano, J. Sese, H. Hata, T. Ota, T. Isogai, T. Tanaka, S. Morishita, K. Okubo, Y. Sakaki, Y. Nakamura, A. Suyama, S. Sugano, Diverse transcriptional initiation revealed by fine, large-scale mapping of mRNA start sites, *EMBO Rep.* 2 (2001) 388–393.
- [12] K. Kimura, A. Wakamatsu, Y. Suzuki, T. Ota, T. Nishikawa, R. Yamashita, J. Yamamoto, M. Sekine, K. Tsuritani, H. Wakaguri, S. Ishii, T. Sugiyama, K. Saito, Y. Isono, R. Irie, N. Kushida, T. Yoneyama, R. Otsuka, K. Kanda, T. Yokoi, H. Kondo, M. Wagatsuma, K. Murakawa, S. Ishida, T. Ishibashi, A. Takahashi-Fujii, T. Tanase, K. Nagai, H. Kikuchi, K. Nakai, T. Isogai, S. Sugano, Diversification of transcriptional modulation: large-scale identification and characterization of putative alternative promoters of human genes, *Genome. Res.* 16 (2006) 55–65.
- [13] T. Shiraki, S. Kondo, S. Katayama, K. Waki, T. Kasukawa, H. Kawaji, R. Kodzius, A. Watahiki, M. Nakamura, T. Arakawa, S. Fukuda, D. Sasaki, A. Podhajski, M. Harbers, J. Kawai, P. Carninci, Y. Hayashizaki, Cap analysis gene expression for high-throughput analysis of transcriptional starting point and identification of promoter usage, *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003) 15776–15781.
- [14] S. Hashimoto, Y. Suzuki, Y. Kasai, K. Morohoshi, T. Yamada, J. Sese, S. Morishita, S. Sugano, K. Matsushima, 5'-end SAGE for the analysis of transcriptional start sites, *Nat. Biotechnol.* 22 (2004) 1146–1149.
- [15] T. Yamashita, S. Kaneko, S. Hashimoto, T. Sato, S. Nagai, N. Toyoda, T. Suzuki, K. Kobayashi, K. Matsushima, Serial analysis of gene expression in chronic hepatitis C and hepatocellular carcinoma, *Biochem. Biophys. Res. Commun.* 282 (2001) 647–654.
- [16] T. Yamashita, M. Honda, H. Takatori, R. Nishino, H. Minato, H. Takamura, T. Ohta, S. Kaneko, Activation of lipogenic pathway correlates with cell proliferation and poor prognosis in hepatocellular carcinoma, *J. Hepatol.* 50 (2009) 100–110.
- [17] J.S. Mattick, Introns: evolution and function, *Curr. Opin. Genet. Dev.* 4 (1994) 823–831.
- [18] J.S. Mattick, I.V. Makunin, Non-coding RNA, *Hum. Mol. Genet.* 15 (Spec No 1) (2006) R17–29.
- [19] R. Louro, A.S. Smirnova, S. Verjovski-Almeida, Long intronic noncoding RNA transcription: expression noise or expression choice? *Genomics* 93 (2009) 291–298.
- [20] S. Yu, S. Rao, J.K. Reddy, Peroxisome proliferator-activated receptors, fatty acid oxidation, steatohepatitis and hepatocarcinogenesis, *Curr. Mol. Med.* 3 (2003) 561–572.
- [21] N. Kondoh, T. Wakatsuki, A. Ryo, A. Hada, T. Aihara, S. Horiuchi, N. Goseki, O. Matsubara, K. Takenaka, M. Shichita, K. Tanaka, M. Shuda, M. Yamamoto, Identification and characterization of genes associated with human hepatocellular carcinogenesis, *Cancer. Res.* 59 (1999) 4990–4996.
- [22] Y. Kobayashi, T. Higashi, K. Nouse, H. Nakatsukasa, M. Ishizaki, T. Kaneyoshi, N. Toshikuni, K. Kariyama, E. Nakayama, T. Tsuji, Expression of MAGE, GAGE and BAGE genes in human liver diseases: utility as molecular markers for hepatocellular carcinoma, *J. Hepatol.* 32 (2000) 612–617.
- [23] A.H. Minn, M. Kayton, D. Lorang, S.C. Hoffmann, D.M. Harlan, S.K. Libutti, A. Shalev, Insulinomas and expression of an insulin splice variant, *Lancet* 363 (2004) 363–367.
- [24] H. Stier, H.D. Fahimi, P.P. Van Veldhoven, G.P. Mannaerts, A. Volkl, E. Baumgart, Maturation of peroxisomes in differentiating human hepatoblastoma cells (HepG2): possible involvement of the peroxisome proliferator-activated receptor alpha (PPAR alpha), *Differentiation* 64 (1998) 55–66.
- [25] S. Zha, S. Ferdinandusse, J.L. Hicks, S. Denis, T.A. Dunn, R.J. Wanders, J. Luo, A.M. De Marzo, W.B. Isaacs, Peroxisomal branched chain fatty acid beta-oxidation pathway is upregulated in prostate cancer, *Prostate* 63 (2005) 316–323.
- [26] K. Tokuoka, Y. Nakajima, K. Hirotsu, I. Miyahara, Y. Nishina, K. Shiga, H. Tamaoki, C. Setoyama, H. Tojo, R. Miura, Three-dimensional structure of rat-liver acyl-CoA oxidase in complex with a fatty acid: insights into substrate-recognition and reactivity toward molecular oxygen, *J. Biochem.* 139 (2006) 789–795.
- [27] K. Meyer, Y. Jia, W.Q. Cao, P. Kashireddy, M.S. Rao, Expression of peroxisome proliferator-activated receptor alpha, and PPARalpha regulated genes in spontaneously developed hepatocellular carcinomas in fatty acyl-CoA oxidase null mice, *Int. J. Oncol.* 21 (2002) 1175–1180.
- [28] N. Tanaka, K. Moriya, K. Kiyosawa, K. Koike, F.J. Gonzalez, T. Aoyama, PPARalpha activation is essential for HCV core protein-induced hepatic steatosis and hepatocellular carcinoma in mice, *J. Clin. Invest.* 118 (2008) 683–694.
- [29] C. Rubie, K. Kempf, J. Hans, T. Su, B. Tilton, T. Georg, B. Brittner, B. Ludwig, M. Schilling, Housekeeping gene variability in normal and cancerous colorectal, pancreatic, esophageal, gastric and hepatic tissues, *Mol. Cell. Probes.* 19 (2005) 101–109.



CLINICAL STUDIES

## dUTP pyrophosphatase expression correlates with a poor prognosis in hepatocellular carcinoma

Hajime Takatori<sup>1</sup>, Taro Yamashita<sup>1</sup>, Masao Honda<sup>1</sup>, Ryuhei Nishino<sup>1</sup>, Kuniaki Arai<sup>1</sup>, Tatsuya Yamashita<sup>1</sup>, Hiroyuki Takamura<sup>2</sup>, Tetsuo Ohta<sup>2</sup>, Yoh Zen<sup>3</sup> and Shuichi Kaneko<sup>1</sup>

1 Department of Gastroenterology, Kanazawa University Graduate School of Medical Science, Ishikawa, Japan

2 Department of Gastroenterologic Surgery, Kanazawa University Graduate School of Medical Science, Ishikawa, Japan

3 Pathology Section, Kanazawa University Hospital, Ishikawa, Japan

### Keywords

dUTP pyrophosphatase – hepatocellular carcinoma – prognosis – serial analysis of gene expression

### Abbreviations

5-FU, 5-fluorouracil; dUTPase, dUTP pyrophosphatase; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; SAGE, serial analysis of gene expression.

### Correspondence

Masao Honda, MD, Department of Gastroenterology, Kanazawa University Graduate School of Medical Science, 13-1 Takara-Machi, Kanazawa, Ishikawa 920-8641, Japan  
Tel: +81 76 265 2233  
Fax: +81 76 234 4250  
e-mail: mhonda@m-kanazawa.jp

Received 13 August 2009

Accepted 26 October 2009

DOI:10.1111/j.1478-3231.2009.02177.x

### Abstract

**Background:** Hepatocellular carcinoma (HCC) is a malignancy with a poor prognosis, partly owing to the lack of biomarkers that support its classification in line with its malignant nature. To discover a novel molecular marker that is related to the efficacy of treatment for HCC and its biological nature, we performed serial analysis of gene expression (SAGE) in HCC, normal liver and cirrhotic liver tissues. **Methods:** Gene expression profiles of HCC tissues and non-cancerous liver tissues were obtained by SAGE. Suppression of the target gene by RNA interference was used to evaluate its role in HCC *in vitro*. The relation of the identified marker and prognosis was statistically examined in surgically resected HCC patients. **Results:** We identified significant over-expression of *DUT*, which encodes dUTP pyrophosphatase (dUTPase), in HCC tissue, and this was confirmed in about two-thirds of the HCC samples by reverse-transcription polymerase chain reaction ( $n=20$ ). Suppression of dUTPase expression using short interfering RNAs inhibited cell proliferation and sensitized HuH7 cells to 5-fluorouracil treatment. Nuclear dUTPase expression was observed in 36.6% of surgically resected HCC samples ( $n=82$ ) evaluated by immunohistochemistry, and its expression was significantly correlated with the histological grades ( $P=0.0099$ ). Notably, nuclear dUTPase expression correlated with a poor prognosis with statistical significance (HR, 2.47; 95% CI, 1.08–5.66;  $P=0.032$ ). **Conclusion:** Taken together, these results suggest that nuclear dUTPase may be a good biomarker for predicting prognosis in HCC patients after surgical resection. Development of novel dUTPase inhibitors may facilitate the eradication of HCC.

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and the third leading cause of cancer-related death worldwide (1). Several risk factors are responsible for HCC development, including alcoholism, aflatoxin and genetic diseases such as haemochromatosis and  $\alpha$ -1 antitrypsin deficiency; however, the major risk factor is chronic hepatitis owing to hepatitis B virus (HBV) or hepatitis C virus (HCV) infection (2–4). Several treatment options are currently available for HCC management, which include liver transplantation, surgical resection, percutaneous ethanol injection, radiofrequency ablation, transcatheter arterial chemoembolization and systemic or local chemotherapy, and optimal treatment is determined based on tumour stage and liver function (5, 6). However, more than 80% of HCC cases develop advanced HCC after initial treatment (7).

Various chemotherapeutic drugs have been investigated for their antitumour activity in advanced HCC. For example, 5-fluorouracil (5-FU), a thymidylate synthase inhibitor, was the first reported drug studied for the treatment of advanced HCC; however, a median survival rate of 3–5 months has discouraged the further use of 5-FU as a single chemotherapeutic agent (8, 9). Interferon- $\alpha$  (IFN- $\alpha$ ) has been reported to have antitumour activity against advanced HCC, and recent reports have suggested the efficacy of a combination of 5-FU/IFN- $\alpha$  for advanced HCC treatment (10–12), although convincing evidence for improved survival rate remains lacking. A recent study has indicated that 16% of advanced HCC patients responded positively to 5-FU/IFN- $\alpha$  treatment with clear and significant survival benefits compared with stable or progressive disease

patients (13). Thus, drug sensitivity appears to be one of the major determinants of the prognosis of advanced HCC patients treated with chemotherapy. Therefore, a hallmark of successful treatment would be the identification of useful biomarkers for determining the survival benefits offered by each treatment strategy.

In this study, we investigated the gene expression profiles of HCCs using serial analysis of gene expression (SAGE) to identify novel molecular markers or targets for the treatment of HCC (14–18). Here, we identified the upregulation of the *DUT* gene that encodes dUTP pyrophosphatase (dUTPase) in HCC. Markedly, HCC with a high nuclear dUTPase expression correlated with a poorly differentiated morphology and a poor prognosis. *DUT* gene knockdown not only suppressed cell proliferation but also sensitized HuH7 cells to low-dose 5-FU.

## Materials and methods

### Samples

All HCC tissues, adjacent non-cancerous liver tissues and normal liver tissues were obtained from 110 patients undergoing a hepatectomy between 1997 and 2006 in Kanazawa University Hospital, Kanazawa, Japan. Five normal liver tissue samples were obtained from patients undergoing surgical resection of the liver for the treatment of metastatic colon cancer. These samples were snap-frozen in liquid nitrogen immediately after resection. One hundred and five HCC and surrounding non-cancerous liver samples were obtained from patients undergoing surgical resection of the liver for HCC treatment, and part of these samples were used for the recent study (19). Three HCC and adjacent non-cancerous liver tissue samples were snap-frozen in liquid nitrogen and later used for SAGE. Twenty HCC tissues and their corresponding non-cancerous liver tissues were also snap-frozen and later used for real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis, as described previously (19). Eighty-two additional HCC samples were formalin-fixed, paraffin-embedded and used for immunohistochemistry (IHC). HCC and adjacent non-cancerous liver tissues were histologically characterized, as reported elsewhere (19).

All strategies used for gene expression analysis as well as tissue acquisition processes were approved by the Ethics Committee and the Institutional Review Board of Kanazawa University Hospital. All procedures and risks were explained verbally to each patient, who then provided written informed consent.

### Serial analysis of gene expression

Total RNA was purified from each homogenized tissue sample using a ToTally RNA extraction kit (Ambion Inc., Austin, TX, USA), and polyadenylated RNA was isolated using a MicroPoly (A) Pure kit (Ambion). A total of 2.5 µg of mRNA per sample was analysed by SAGE (20, 21). SAGE libraries were randomly sequenced at the

Genomic Research Center (Shimadzu-Biotechnology, Kyoto, Japan), and the sequence files were analysed with SAGE 2000 software. The size of each SAGE library was normalized to 300 000 transcripts per library, and the abundance of transcripts was compared with SAGE 2000 software. Monte Carlo simulation was used for selecting genes whose expression levels were significantly different between the two libraries (22). Each SAGE tag was annotated using a gene-mapping website SAGE Genie database (<http://cgap.nci.nih.gov/SAGE/>) and the Source database (<http://smd.stanford.edu/cgi-bin/source/sourceSearch>), as described previously (23).

### Quantitative reverse transcription-polymerase chain reaction

A 1 µg aliquot of each total RNA was reverse-transcribed using SuperScript II reverse-transcriptase (Invitrogen, Carlsbad, CA, USA). Real-time RT-PCR analysis was performed using the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). Using the standard curve method, quantitative PCR was performed in duplicate for each sample–primer set. Each sample was normalized relative to β actin. The assay IDs used were Hs00798995\_s1 for dUTPase and Hs99999903\_m1 for β actin.

### RNA interference targeting *DUT*

Small interfering RNAs (siRNAs) targeting *DUT* or control (scrambled sequence) were synthesized by Dharmacon (Dharmacon Research Inc., Lafayette, CO, USA). The target sequences of *DUT* are 5'-AAGUUGU GAAAACGGACAUC-3' (*DUT*1) and 5'-CGGACAUU CAGAUAGCGCUTT-3' (*DUT*2). Lipofectamine 2000™ reagent (Invitrogen) was used for transfection according to the manufacturer's instructions.

### Cell proliferation assay, soft agar assay and matrigel invasion assay

Cell proliferation assays were performed using a Cell Titer96 Aqueous kit in quintuplicate (Promega, Madison, WI, USA). For the soft agar assay,  $1 \times 10^4$  cells were suspended in 2 ml of 0.36% agar with growth medium and added in each well of a six-well plate containing a base layer of 0.72% agar. The plates were incubated at 37 °C in a 5% CO<sub>2</sub> incubator for 2 weeks. Matrigel invasion assays were performed using BD BioCoat™ Matrigel Matrix Cell Culture Inserts and Control Inserts (BD Biosciences, San Jose, CA, USA), as described in the manufacturer's instruction. 5-FU was obtained from Kyowa Kirin (Kyowa Kirin, Tokyo, Japan). All experiments were repeated at least twice.

### Immunohistochemistry

Mouse monoclonal anti-dUTPase antibody M01 (Abnova Corporation, Taipei, Taiwan) and mouse antiproliferating

cell nuclear antigen (PCNA) monoclonal antibody PC10 (Calbiochem, San Diego, CA, USA) were used to evaluate the immunoreactivity of HCC and adjacent non-cancerous liver samples using a Dako EnVision+™ kit (Dako, Carpinteria, CA, USA), according to the manufacturer's instruction. Immunoreactivity was evaluated by determining the percentage of cells expressing dUTPase in the examined fields, graded as low (0–50%) or high (> 50%). The PCNA index was evaluated as described previously (19).

#### Statistical analysis

Student's *t*-test was used to determine the statistical significance of the differences in cell viability between the two groups. The Mann–Whitney *U*-test was used for the analysis of gene expression between chronic liver disease (CLD) and HCC tissues. The  $\chi^2$ -test was used to evaluate the correlation between clinicopathological characteristics and dUTPase expression status. Univariate and multivariate Cox proportional hazards regression analysis was used to evaluate the association of dUTPase expression and clinicopathological parameters with patient outcome. All statistical analyses were performed using SPSS software (SPSS software package; SPSS Inc., Chicago, IL, USA) and GRAPHPAD PRISM software (GraphPad Software Inc., La Jolla, CA, USA).

#### Results

##### Gene expression profiling identified the overexpression of *DUT* in hepatocellular carcinoma

To overcome the considerable individual variability of transcriptomic characteristics, we constructed a SAGE library of normal human liver using RNAs derived from five normal liver tissues. In addition, we constructed two SAGE libraries derived from three HCC tissues or corresponding non-cancerous liver tissues from patients who developed HCC with a history of chronic hepatitis C. We detected a total of 226 267 tags corresponding to 45 746 unique tags from these SAGE libraries (supporting information Table S1). After excluding the tags detected only once in each library, we selected 15 333 reliable unique transcripts expressed in at least one of the SAGE libraries to avoid contamination of tags derived from sequence errors. Then, we annotated these transcripts using SAGE Genie database and the Source database to identify the potential subcellular localization of transcripts categorized into eight groups in each SAGE library.

The number of nuclear component-related transcripts was increased in the HCC library compared with the normal liver and non-cancerous liver libraries, whereas the other cellular component-related transcripts did not show this tendency (supporting information Fig. S1). Because nuclear component-related genes may closely correlate with cancer cell proliferation and chemosensitivity (24), we further investigated the expression of nuclear component-related tags in

each library, and identified 10 transcripts associated with nucleotide/nucleoside metabolism that are overexpressed in HCC (Table 1). Using Monte Carlo simulation, we evaluated the significance of differentially expressed transcripts in HCC and corresponding CLD libraries or in HCC and normal liver libraries. We identified a *DUT* gene encoding dUTPase (dUTPase) whose expression was significantly altered ( $P=0.01$ ). We also identified a *TS* gene encoding thymidylate synthase in the list, but the difference did not reach statistical significance.

dUTPase is a phosphatase known to maintain a dUMP pool by catalysing the hydrolysis of dUTP to dUMP, and thus provides a substrate of thymidylate synthase. Its role in HCC is unknown; therefore, we examined *DUT* expression in 20 independent HCC and corresponding non-cancerous liver tissues, and identified significant overexpression of *DUT* in HCC tissue ( $P=0.0015$ ) (Fig. 1A). Moreover, we detected more than a two-fold increase in *DUT* expression in 70% of HBV-related and HCV-related HCC cases (14 of 20 HCCs) compared with the non-cancerous liver tissues (Fig. 1B). We further examined the expression of *DUT* in 238 HCC tissues compared with the non-cancerous liver tissues using publicly available microarray data (GSE5975) (Fig. S2). Consistent with the SAGE data, *DUT* was overexpressed more than two-fold in 121 of 238 HCC tissues (median: 2.03), whereas *TS* was overexpressed more than two-fold in 54 of 238 HCC tissues (median: 1.41) compared with the non-cancerous liver tissues.

##### Pivotal role of dUTP pyrophosphatase expression in cell proliferation in hepatocellular carcinoma cell lines

In general, cancer gene signatures discovered by comparison between tumour and non-tumour tissues are more likely to reflect the differences in the control of cell proliferation and growth (25). Accordingly, we investigated the function of dUTPase in cell proliferation in HuH7 cells by *DUT* gene knockdown. *DUT* expression was decreased by 60–70% following the transfection of the siRNA constructs specifically targeting *DUT* 48 h after transfection (*DUT*1 in Fig. 2A and *DUT*2 in Fig. S3A), and cell growth was significantly inhibited compared with the control 72 h after transfection (Fig. 2B and Fig. S3B). Anchorage-independent cell growth was also significantly impaired by *DUT* gene knockdown 14 days after transfection (Fig. 2C). Furthermore, *DUT* gene knockdown decreased the numbers of both migrating and invading cells 72 h after transfection (Fig. 2D and E).

dUTPase is known to be associated with thymidylate synthesis (26), and thus we evaluated the effects of 5-FU, a thymidylate synthase inhibitor, on dUTPase expression in HCC cell lines *in vitro*. When we treated HuH7 cells with low-dose 5-FU (0.25 mg/ml), we could not detect any growth-inhibitory effects (Fig. 2F). Based on this condition, we evaluated the effect of *DUT* gene knockdown on 5-FU sensitivity 72 h after transfection.



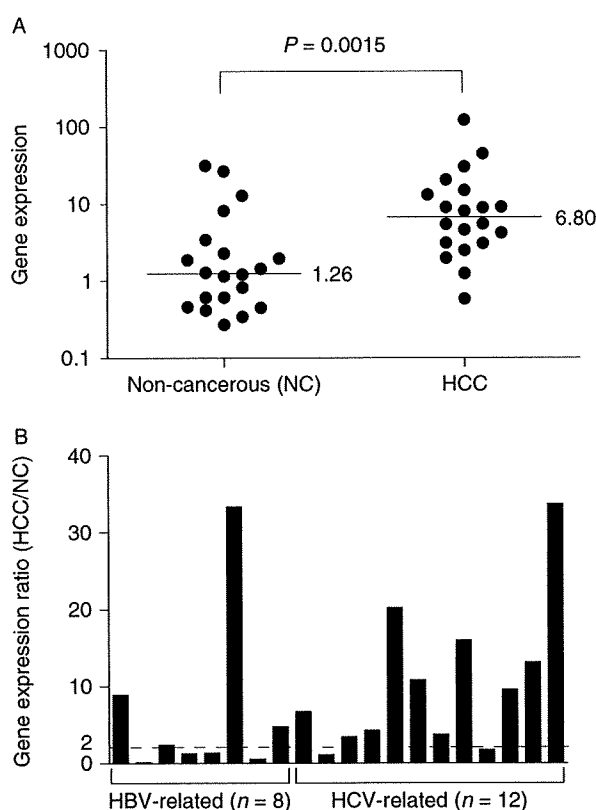
**Table 1.** Genes associated with nucleic acid metabolism overexpressed in hepatocellular carcinoma

Tag sequence	Normal liver	Non-cancerous liver	HCC	Fold*	Gene	P-value†
CAGCTCCGCT	0	2	11	5.5	dUTP pyrophosphatase	0.010
AAAGGATAAT	0	0	3	> 3	General transcription factor II H, polypeptide 2	0.127
ACGGTCCAGG	0	0	3	> 3	Cytidine deaminase	0.127
ATGTAGAGTG	0	0	3	> 3	Thymidylate synthase	0.127
TGGGGATTAC	1	0	3	> 3	Zinc ribbon domain containing, 1	0.127
CACCTGTAC	2	2	6	3	Solute carrier family 29	0.147
GAACGCCTAA	1	1	3	3	Dihydropyrimidinase-like 2	0.308
GCGCTGGTAC	0	1	3	3	2'-5'-oligoadenylate synthetase 3	0.308
CTTAGTGCAA	0	2	4	2	3'-phosphoadenosine 5'-phosphosulphate synthase 2	0.335
TTGTTACATC	0	2	3	1.5	Phosphoribosyl pyrophosphatase synthetase-associated protein 1	0.506

\*Fold increase was calculated by dividing the number of tags in HCC by that of tags in non-cancerous liver. To avoid division by 0, a tag value of 1 was used for any tag that was not detectable in one sample.

†Statistical significance of differentially expressed genes between two groups (HCC and non-cancerous liver libraries) was calculated using Monte Carlo simulation.

HCC, hepatocellular carcinoma.

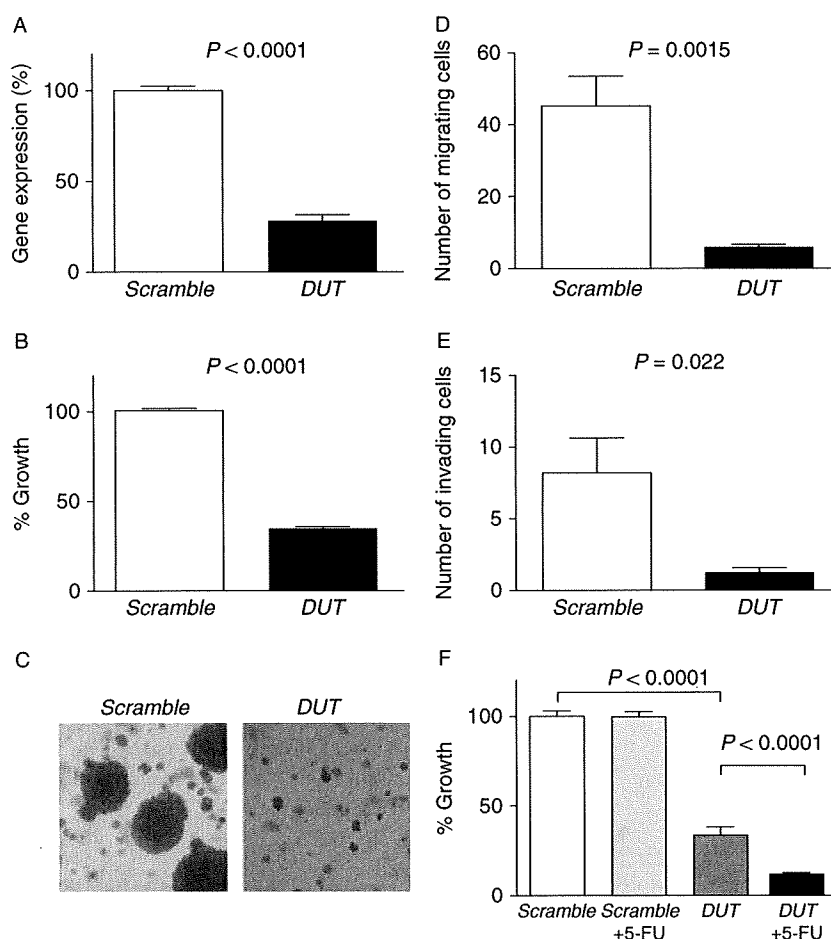


**Fig. 1.** (A) Quantitative reverse transcription-polymerase chain reaction analysis of *DUT* expression in hepatocellular carcinoma (HCC) and corresponding non-cancerous liver tissues. *DUT* was significantly activated in HCC tissues compared with non-cancerous liver tissues ( $P=0.0015$ ). A median value in each group is indicated. (B) *DUT* gene expression ratios of HCC and corresponding non-cancerous liver tissues. Fourteen of 20 HCC tissues expressed *DUT* more than two-fold compared with the background non-cancerous liver tissues. HBV, hepatitis B virus; HCV, hepatitis C virus.

Interestingly, *DUT* gene knockdown not only suppressed cell proliferation but also sensitized HuH7 cells to low-dose 5-FU (Fig. 2F and Fig. S3B). These data suggest that dUTPase overexpression in HCC tissues may be associated with enhanced cell proliferation and 5-FU resistance.

#### Intense dUTP pyrophosphatase expression is correlated with a poor prognosis in hepatocellular carcinoma patients

To characterize the clinicopathological characteristics of dUTPase expression in HCC, we performed IHC using an additional independent HCC cohort. Accordingly, we explored the dUTPase expression in HCC using 82 formalin-fixed paraffin-embedded HCC specimens. All HCC tissues were surgically resected at the Liver Disease Center of Kanazawa University Hospital with full clinical information, and their immunoreactivity to anti-dUTPase antibodies was evaluated by IHC. We noticed that anti-dUTPase antibodies reacted to both nuclear (red arrows) and cytoplasmic (blue arrows) isoforms of dUTPase, as described previously (26) (Fig. 3A and B). We therefore evaluated the nuclear and cytoplasmic expression of dUTPase separately. We stratified HCC tissues and evaluated the dUTPase expression status based on the percentages of dUTPase-positive cells. The frequency of nuclear or cytoplasmic dUTPase-positive cells was highly variable in each HCC tissue, and we defined HCCs with nuclear or cytoplasmic dUTPase expressed in  $\geq 50\%$  of tumour cells as nuclear or cytoplasmic dUTPase-high HCC (Fig. 3C). Nuclear dUTPase overexpression was detected in 36.6% (30 of 82), whereas cytoplasmic dUTPase overexpression was detected in 67.1% (55 of 82) of HCC tissues compared with the corresponding non-cancerous liver tissues



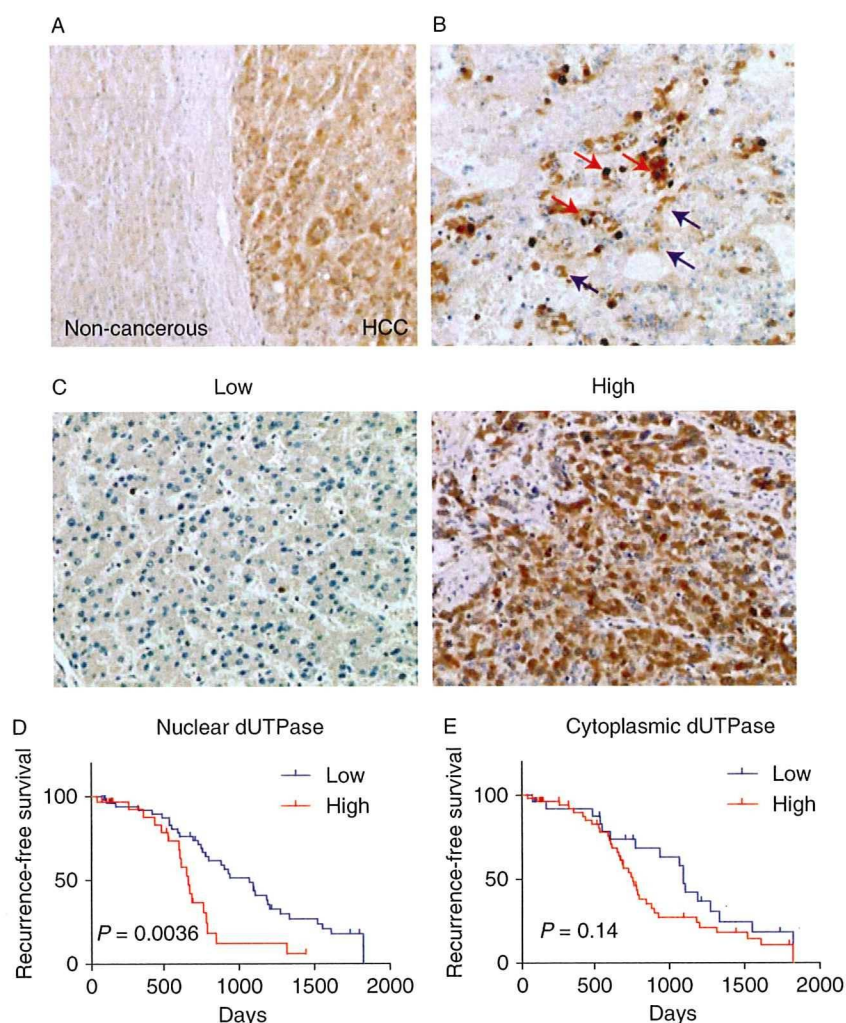
**Fig. 2.** (A) Transfection of small interfering RNAs targeting *DUT* (*DUT1*) decreased *DUT* expression compared with the control (scrambled sequence). Gene expression was evaluated in triplicate 72 h after transfection (mean  $\pm$  SD). (B) *DUT* gene knockdown significantly suppressed cell proliferation ( $P < 0.0001$ ). Cell viability was evaluated in triplicate 72 h after transfection (mean  $\pm$  SD). (C) Soft agar assay. *DUT* gene knockdown suppressed anchorage-independent cell growth. (D and E) Matrigel invasion assay. *DUT* gene knockdown decreased the numbers of both migrating and invading cells. Experiments were performed in triplicate (mean  $\pm$  SD). (F) *DUT* gene knockdown sensitized HuH7 cells to low-dose 5-fluorouracil (5-FU) (0.25  $\mu$ g/ml), which had no effect on the cell proliferation in the control (mean  $\pm$  SD).

(Table 2). In general, non-cancerous hepatocytes rarely expressed nuclear dUTPase (Fig. 3A).

We investigated the clinicopathological characteristics of nuclear or cytoplasmic dUTPase in low/high HCC cases (Table 2). The expression status of nuclear dUTPase showed no correlation with age, gender, virus, presence of cirrhosis,  $\alpha$ -fetoprotein value, tumour size and TNM stages. However, nuclear dUTPase expression was significantly correlated with the histological grades of HCC ( $P = 0.0099$ ), and high frequencies of nuclear dUTPase-positive cells were associated with poorly differentiated cell morphology in the HCC tissue. In contrast, cytoplasmic dUTPase expression was not correlated with the histological grades of HCC ( $P = 0.077$ ). We examined the cell proliferation of these HCC samples by PCNA staining, and PCNA indexes were significantly higher in nuclear dUTPase high HCC than low HCC with statistical significance ( $P = 0.01$ ) (Fig. S4).

We further investigated the prognostic significance of dUTPase expression in HCC. Strikingly, high nuclear dUTPase expression in HCC tissue correlated with a poor survival outcome compared with low nuclear dUTPase expression ( $P = 0.0036$ ), whereas high cytoplasmic dUTPase expression had little effects when evaluated by recurrence-free survival (Fig. 3D). Furthermore, univariate Cox regression analysis showed a significant correlation between high nuclear dUTPase expression and a high risk of mortality (HR, 2.47; 95% CI, 1.08–5.66;  $P = 0.032$ ; Table 3). By multivariate Cox regression analysis, TNM stage (HR, 2.75; 95% CI, 1.11–6.79;  $P = 0.027$ ) and nuclear dUTPase (HR, 2.61; 95% CI, 1.13–6.05;  $P = 0.024$ ) were independent prognostic factors associated with a high risk of mortality, and other clinicopathological features did not add independent prognostic information. These data indicate a significant correlation between the malignant potential of





**Fig. 3.** Immunohistochemistry analysis of dUTP pyrophosphatase (dUTPase) expression in hepatocellular carcinoma (HCC). (A) A representative photomicrograph of dUTPase staining in an HCC and adjacent non-cancerous liver tissue. (B) A representative photomicrograph of dUTPase staining in an HCC. Both nuclear (red arrows) and cytoplasmic (blue arrows) forms of dUTPase were detected. (C) Representative photomicrographs of HCC tissues with low (0–50%) and high ( $\geq 50\%$ ) frequencies of nuclear and cytoplasmic dUTPase-positive cells. (D and E) Kaplan–Meier survival analysis of HCC tissues with nuclear (D) or cytoplasmic (E) dUTPase expression. High percentages of nuclear dUTPase-positive tumour cells significantly correlated with poor clinical outcome in recurrence-free survival.

HCC and nuclear dUTPase expression, implicating the potential effectiveness of nuclear dUTPase level as a biomarker for predicting the survival of HCC patients after surgical resection.

### Discussion

Here, using a global gene expression profiling approach (18), we have identified the activation of the nucleotide/nucleoside metabolism-related gene *DUT* (encoding dUTPase) in HCC. Notably, an intense dUTPase expression was detected in a subset of HCC with a poor prognosis. To the best of our knowledge, this is the first

report describing the correlation between dUTPase activation and poor survival outcome in HCC patients.

In normal cells, dUTPase is known to catalyse the hydrolysis of dUTP to dUMP in order to maintain the dUMP pool at a certain level for thymidylate synthesis (26). Interestingly, dUTPase mutations in *Escherichia coli* increased dUTP levels, leading to dUTP misincorporation into DNA during replication, which resulted in DNA fragmentation and apoptosis (27). Furthermore, introduction of *E. coli* dUTPase into human tumour cells resulted in the induction of resistance to fluorodeoxyuridine cytotoxicity (28), suggesting a pivotal role of dUTPase in the prevention of DNA damage. Thus, dUTPase activation in the nucleus appears to be critical

**Table 2.** Clinicopathological characteristics and dUTP pyrophosphatase expression in hepatocellular carcinoma (n = 82)

dUTPase expression (nuclear)	Low (n = 52)	High (n = 30)	P-value
Age (< 60 years/≥ 60 years)	19/33	8/22	0.36
Sex (male/female)	36/16	23/7	0.47
Virus (HBV/HCV/B+C/NBNC)	15/33/1/3	10/20/0/0	0.48
Cirrhosis (yes/no)	33/19	22/8	0.36
AFP (< 20 ng/ml/≥ 20 ng/ml)	32/20	15/15	0.31
Histological grade*			
I-II	14	3	
II-III	36	20	
III-IV	2	7	0.0099
Tumour size (< 3 cm/≥ 3 cm)	31/21	19/11	0.74
TNM classification† (I, II/III, IV)	43/9	25/5	0.94

dUTPase expression (cytoplasmic)	Low (n = 27)	High (n = 55)	P-value
Age (< 60 years/≥ 60 years)	10/17	17/38	0.58
Sex (male/female)	19/8	40/15	0.82
Virus (HBV/HCV/B+C/NBNC)	8/17/1/1	17/36/0/2	0.56
Cirrhosis (yes/no)	17/10	38/17	0.58
AFP (< 20 ng/ml/≥ 20 ng/ml)	16/11	31/24	0.80
Histological grade*			
I-II	7	10	
II-III	20	36	
III-IV	0	9	0.077
Tumour size (< 3 cm/≥ 3 cm)	17/10	33/22	0.80
TNM classification† (I, II/III, IV)	21/6	47/8	0.39

\*Edmondson–Steiner grades.

†UICC TNM classification of liver cancer, 6th edition (2002).

AFP,  $\alpha$ -fetoprotein; dUTPase, dUTP pyrophosphatase; HBV, hepatitis B virus; HCV, hepatitis C virus.

for preventing DNA damage possibly at the S phase. Specifically, this activation may prevent dUTP misincorporation in various cancers and thus avert DNA damage and apoptosis induction. Indeed, dUTPase activation has recently been reported in colorectal and brain cancer (29, 30), and dUTPase accumulation might correlate with 5-FU-based chemotherapy resistance and poor prognosis in colorectal cancer (26).

If dUTPase activation plays a central role in the development of resistance to thymidylate synthase inhibitors in order to prevent a DNA damage response, dUTPase inhibition may facilitate the eradication of cancer cells by sensitizing these cells to such inhibitors. Indeed, a recent study suggested a drastic sensitization of colon cancer cells to 5-FU by siRNAs-mediated dUTPase suppression (31, 32), which is consistent with our current observation. Because all HCC samples used in this study were surgically resected, we could not evaluate the effect of dUTPase expression on clinical HCC patients' outcome in relation to chemosensitivity to thymidylate synthase inhibitors. Nevertheless, intense nuclear dUTPase expression may be a good biomarker

**Table 3.** Cox regression analysis of recurrence-free survival rate relative to dUTP pyrophosphatase expression and clinicopathological parameters (n = 82)

Variables (n)	Univariate		Multivariate	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Child–Pugh				
A	1			
B	1.73 (0.50–5.97)	0.38		
Tumour size				
< 3 cm (n = 50)	1			
≥ 3 cm (n = 32)	1.58 (0.69–3.63)	0.28		
TNM stage*				
I, II (n = 68)	1		1	
III, IV (n = 14)	2.57 (1.05–6.29)	0.039	2.75 (1.11–6.79)	0.027
Serum AFP				
< 20 ng/ml (n = 49)	1			
≥ 20 ng/ml (n = 38)	1.54 (0.66–3.56)	0.31		
Microvascular invasion				
No	1			
Yes	1.98 (0.89–4.44)	0.095		
BCLC stage				
A	1			
B/C	2.16 (0.93–5.00)	0.07		
Cytoplasmic dUTPase				
Low (n = 27)	1			
High (n = 55)	1.15 (0.50–2.62)	0.73		
Nuclear dUTPase				
Low (n = 52)	1		1	
High (n = 30)	2.47 (1.08–5.66)	0.032	2.61 (1.13–6.05)	0.024

\*UICC TNM classification of liver cancer, 6th edition (2002).

AFP,  $\alpha$ -fetoprotein; CI, confidence intervals; dUTPase, dUTP pyrophosphatase; HR, hazard ratio.

for predicting the response to thymidylate synthase inhibitors, and its usefulness should be further evaluated in the future.

In conclusion, comprehensive gene expression profiling shed new light on the role of dUTPase in HCC. Nuclear dUTPase accumulation is potentially a good biomarker for predicting poor prognosis in HCC patients, and the development of a dUTPase inhibitor may promote the possibility of tumour eradication in HCC patients.

### Acknowledgements

The authors would like to thank Ms Masayo Baba and Nami Nishiyama for technical assistance. This research was supported in part by a Grant-in-Aid for Special Purposes from the Ministry of Education, Culture, Sports, Science and Technology, Japan (no. 20599005).



**Grant support:** Grant-in-Aid for Special Purposes from the Ministry of Education, Culture, Sports, Science and Technology, Japan (no. 20599005).

## References

- Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74–108.
- El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 2007; **132**: 2557–76.
- Farazi PA, Depinho RA. Hepatocellular carcinoma pathogenesis: from genes to environment. *Nat Rev Cancer* 2006; **6**: 674–87.
- Roessler S, Budhu A, Wang XW. Future of molecular profiling of human hepatocellular carcinoma. *Future Oncol* 2007; **3**: 429–39.
- El-Serag HB, Marrero JA, Rudolph L, Reddy KR. Diagnosis and treatment of hepatocellular carcinoma. *Gastroenterology* 2008; **134**: 1752–63.
- Llovet JM, Bruix J. Novel advancements in the management of hepatocellular carcinoma in 2008. *J Hepatol* 2008; **48**(Suppl. 1): S20–37.
- Poon RT, Fan ST, Lo CM, Liu CL, Wong J. Long-term survival and pattern of recurrence after resection of small hepatocellular carcinoma in patients with preserved liver function: implications for a strategy of salvage transplantation. *Ann Surg* 2002; **235**: 373–82.
- Friedman MA. Primary hepatocellular cancer—present results and future prospects. *Int J Radiat Oncol Biol Phys* 1983; **9**: 1841–50.
- Lin DY, Lin SM, Liaw YF. Non-surgical treatment of hepatocellular carcinoma. *J Gastroenterol Hepatol* 1997; **12**: S319–28.
- Nagano H, Miyamoto A, Wada H, *et al.* Interferon-alpha and 5-fluorouracil combination therapy after palliative hepatic resection in patients with advanced hepatocellular carcinoma, portal venous tumor thrombus in the major trunk, and multiple nodules. *Cancer* 2007; **110**: 2493–501.
- Patt YZ, Hassan MM, Lozano RD, *et al.* Phase II trial of systemic continuous fluorouracil and subcutaneous recombinant interferon alfa-2b for treatment of hepatocellular carcinoma. *J Clin Oncol* 2003; **21**: 421–7.
- Urabe T, Kaneko S, Matsushita E, Unoura M, Kobayashi K. Clinical pilot study of intrahepatic arterial chemotherapy with methotrexate, 5-fluorouracil, cisplatin and subcutaneous interferon-alpha-2b for patients with locally advanced hepatocellular carcinoma. *Oncology* 1998; **55**: 39–47.
- Obi S, Yoshida H, Toune R, *et al.* Combination therapy of intraarterial 5-fluorouracil and systemic interferon-alpha for advanced hepatocellular carcinoma with portal venous invasion. *Cancer* 2006; **106**: 1990–7.
- Honda M, Yamashita T, Ueda T, *et al.* Different signaling pathways in the livers of patients with chronic hepatitis B or chronic hepatitis C. *Hepatology* 2006; **44**: 1122–38.
- Nishino R, Honda M, Yamashita T, *et al.* Identification of novel candidate tumour marker genes for intrahepatic cholangiocarcinoma. *J Hepatol* 2008; **49**: 207–16.
- Yamashita T, Honda M, Takatori H, *et al.* Genome-wide transcriptome mapping analysis identifies organ-specific gene expression patterns along human chromosomes. *Genomics* 2004; **84**: 867–75.
- Yamashita T, Kaneko S, Hashimoto S, *et al.* Serial analysis of gene expression in chronic hepatitis C and hepatocellular carcinoma. *Biochem Biophys Res Commun* 2001; **282**: 647–54.
- Yamashita T, Honda M, Kaneko S. Application of serial analysis of gene expression in cancer research. *Curr Pharm Biotechnol* 2008; **9**: 375–82.
- Yamashita T, Honda M, Takatori H, *et al.* Activation of lipogenic pathway correlates with cell proliferation and poor prognosis in hepatocellular carcinoma. *J Hepatol* 2009; **50**: 100–10.
- Velculescu VE, Zhang L, Vogelstein B, Kinzler KW. Serial analysis of gene expression. *Science* 1995; **270**: 484–7.
- Yamashita T, Hashimoto S, Kaneko S, *et al.* Comprehensive gene expression profile of a normal human liver. *Biochem Biophys Res Commun* 2000; **269**: 110–6.
- Polyak K, Xia Y, Zweier JL, Kinzler KW, Vogelstein B. A model for p53-induced apoptosis. *Nature* 1997; **389**: 300–5.
- Misu H, Takamura T, Matsuzawa N, *et al.* Genes involved in oxidative phosphorylation are coordinately upregulated with fasting hyperglycaemia in livers of patients with type 2 diabetes. *Diabetologia* 2007; **50**: 268–77.
- Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer* 2003; **3**: 330–8.
- Whitfield ML, George LK, Grant GD, Perou CM. Common markers of proliferation. *Nat Rev Cancer* 2006; **6**: 99–106.
- Ladner RD, Lynch FJ, Groshen S, *et al.* dUTP nucleotidohydrolase isoform expression in normal and neoplastic tissues: association with survival and response to 5-fluorouracil in colorectal cancer. *Cancer Res* 2000; **60**: 3493–503.
- El-Hajj HH, Zhang H, Weiss B. Lethality of a dut (deoxyuridine triphosphatase) mutation in *Escherichia coli*. *J Bacteriol* 1988; **170**: 1069–75.
- Canman CE, Radany EH, Parsels LA, *et al.* Induction of resistance to fluorodeoxyuridine cytotoxicity and DNA damage in human tumor cells by expression of *Escherichia coli* deoxyuridinetriphosphatase. *Cancer Res* 1994; **54**: 2296–8.
- Fleischmann J, Kremmer E, Muller S, *et al.* Expression of deoxyuridine triphosphatase (dUTPase) in colorectal tumours. *Int J Cancer* 1999; **84**: 614–7.
- Romeike BF, Bockeler A, Kremmer E, *et al.* Immunohistochemical detection of dUTPase in intracranial tumors. *Pathol Res Pract* 2005; **201**: 727–32.
- Koehler SE, Ladner RD. Small interfering RNA-mediated suppression of dUTPase sensitizes cancer cell lines to thymidylate synthase inhibition. *Mol Pharmacol* 2004; **66**: 620–6.

32. Wilson PM, Fazzone W, Labonte MJ, *et al.* Novel opportunities for thymidylate metabolism as a therapeutic target. *Mol Cancer Ther* 2008; 7: 3029–37.

### Supporting Information

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

**Fig. S1.** Subcellular localization of genes detected in each SAGE library.

**Fig. S2.** Microarray analysis of *DUT* and *TS* gene expression in 238 HCC cases publicly available (GSE5975). *DUT* was overexpressed more than 2-fold in 121 of 238 HCC tissues (median: 2.03), whereas *TS* was overexpressed more than 2-fold in 54 of 238 HCC tissues (median: 1.41) compared with the non-cancerous liver tissues.

**Fig. S3.** (A) Transfection of siRNAs targeting *DUT* (*DUT2*) decreased *DUT* expression compared with the control (scrambled sequence). Gene expression was evaluated in triplicates 72 hours after transfection (mean  $\pm$  SD). (B) *DUT* gene knockdown sensitized HuH7 cells to low-dose 5-FU (0.25 mg/ml) (mean  $\pm$  SD).

**Fig. S4.** Nuclear and cytoplasmic dUTPase expression and cell proliferation in HCC. PCNA indexes in nuclear dUTPase-high HCC were higher than those in -low HCC with statistical significance ( $P = 0.01$ ). Cytoplasmic dUTPase expression was not associated with PCNA indexes in HCC.

**Table S1.** A summary of constructed SAGE libraries.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.



## **CD14<sup>+</sup> monocytes are vulnerable and functionally impaired under ER stress in patients with type 2 diabetes**

**A short running title:** Diabetic monocyte is attenuated under ER stress

Takuya Komura<sup>1</sup>, Yoshio Sakai<sup>1</sup>, Masao Honda<sup>1</sup>,  
Toshinari Takamura<sup>1</sup>, Kouji Matsushima<sup>2</sup>, Shuichi Kaneko<sup>1</sup>

<sup>1</sup> Disease Control and Homeostasis, Kanazawa University, Graduate School of Medical Science.

<sup>2</sup> Department of Molecular Prevent Medicine, School of Medicine, The University of Tokyo.

**Corresponding author:**

Shuichi Kaneko, M.D., Ph.D.

Email: [skaneko@m-kanazawa.jp](mailto:skaneko@m-kanazawa.jp)

Additional information for this article can be found in an online appendix at  
<http://diabetes.diabetesjournals.org>

Submitted 13 May 2009 and accepted 16 November 2009.

This is an uncopyedited electronic version of an article accepted for publication in *Diabetes*. The American Diabetes Association, publisher of *Diabetes*, is not responsible for any errors or omissions in this version of the manuscript or any version derived from it by third parties. The definitive publisher-authenticated version will be available in a future issue of *Diabetes* in print and online at <http://diabetes.diabetesjournals.org>.

*Objective*—While patients with diabetes suffer from increased infections and a higher incidence of cancer due to impaired immune function, details on diabetes-induced decrease in immunity are lacking. We assessed how immune-mediating peripheral blood mononuclear cells (PBMCs) are affected in diabetes.

*Research designs and methods*—We obtained PBMCs from 33 patients with type 2 diabetes and 28 healthy volunteers, and investigated their susceptibility to apoptosis and functional alteration.

*Results*—In a subpopulation of PBMCs, monocytes derived from patients with diabetes were more susceptible to apoptosis than monocytes from healthy volunteers. Monocytes from patients with diabetes had decreased phagocytotic activity and were less responsive to Toll-like receptor (TLR) ligands, although the expression of TLRs did not differ significantly between the two groups. Furthermore, monocytes from patients with diabetes had a distinctly different gene expression profile compared to monocytes from normal volunteers as assessed with DNA microarray analysis. Specifically, quantitative real-time detection PCR measurements showed an elevated expression of the markers of endoplasmic reticulum (ER) stress in diabetic monocytes, and electron microscopic examination of monocytes revealed morphological alterations in the ER of cells derived from patients with diabetes. Consistently, the ER stress inducer tunicamycin increased apoptosis of otherwise healthy monocytes and attenuated the pro-inflammatory responses to TLR ligands.

*Conclusions*—These data suggest that monocytes comprise a substantially impaired subpopulation of PBMCs in patients with diabetes and that ER stress is involved in these pathological changes mechanistically. This implies that the affected monocytes should be investigated further to better understand diabetic immunity.



**T**ype 2 diabetes is the most frequent metabolic disease and the leading cause of human morbidity and mortality (1, 2). Based on epidemiological data, patients with diabetes are immunocompromised and have an increased incidence of infections in the respiratory tract, urinary tract, and skin (3–5). The high incidence of colorectal, breast, and pancreatic malignancies in patients with diabetes is also considered to be a consequence of diabetes-associated defects in immune function (6, 7).

Although studies on immune cells and circulating cytokines have shed some light on this diabetic immunological phenomenon, conflicting results have been reported and do not adequately explain the perturbed immune function in patients with diabetes. Controversial results concerning the phagocytotic activity of polymorphonuclear neutrophils and monocytes are in part due to differences in the patients themselves, insufficient numbers in the study populations, or inconsistencies in the collection of the cell populations under investigation (8–11). Therefore, further studies are needed to explain the decreased immune function of patients with diabetes.

We previously investigated the gene expression signatures of peripheral blood mononuclear cells (PBMCs) in patients with diabetes and observed transcriptional expression features that were distinct from those of healthy volunteers (12). Apoptosis-related genes were up-regulated in the PBMCs of patients with diabetes. Based on this result, we investigated apoptotic activity and immunological function in PBMCs from patients with type 2 diabetes.

We observed that the CD14<sup>+</sup> monocyte fraction was the most affected subpopulation of PBMCs from these patients; these cells were especially vulnerable to apoptosis compared to other cell

subpopulations. We also found that CD14<sup>+</sup> monocytes demonstrated attenuated phagocytotic activity and deficient Toll-like receptor (TLR) signaling, both of which are important for innate immunity (13, 14). Transcriptional analysis and electron microscopic examination of monocytes from patients with diabetes showed evidence of endoplasmic reticulum (ER) stress, which may underlie the functional defects in these cells. Collectively, the data presented herein show that CD14<sup>+</sup> monocytes are a vulnerable cell population under ER stress in these patients that could contribute to decreases in immune function in diabetes.

## RESERCH DESIGN AND METHODS

**Patients:** Thirty-three patients with type 2 diabetes (male/female, 15/18; age, 62.0 ± 8.6 years; HbA<sub>1c</sub>, 9.2 ± 2.0%) and 28 healthy volunteers (male/female, 15/13; age, 58.2 ± 10.2 years; HbA<sub>1c</sub>, 5.4 ± 0.7%) were enrolled consecutively for the apoptosis assay (Table 1). The groups were not significantly different in terms of their clinical parameters, except for the fasting plasma glucose (FPG) and HbA<sub>1c</sub> levels. The patients with diabetes (*n* = 16) from whom adequate numbers of monocytes were obtained were enrolled for additional experiments along with 17 other patients with diabetes (male/female, 8/9; age, 60.5 ± 7.2 years; HbA<sub>1c</sub> 8.8 ± 1.8) whose clinical profiles fit the diabetic profile (Table 1). Informed consent for this study was obtained from all subjects. The experimental protocol was carried out in accordance with the Declaration of Helsinki.

**Isolation of subpopulations of PBMCs and flow cytometric analysis:** PBMCs were freshly isolated from heparinized venous blood using Ficoll–Hypaque (Sigma–Aldrich, St. Louis, MO) as previously described (12). CD4<sup>+</sup> T cell and CD14<sup>+</sup> monocyte subpopulations were isolated using a magnetic cell sorting

system in accordance with the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated cells were purified by > 90 % as measured by flow cytometric analysis using FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA). To assess the expression of TLRs on monocytes, PBMCs were incubated with phosphatidylethanolamine (PE)-labeled anti-TLR2, TLR3, or TLR4 (eBioscience, San Diego, CA) and fluorescein isothiocyanate (FITC)-labeled anti-CD14 antibodies (BD Biosciences) and analyzed by flow cytometry. Data were analyzed using CELLQuest™ Software (BD Biosciences).

**Quantitative real-time detection PCR (RTD-PCR):** RTD-PCR was performed as previously described (15). Briefly, total RNA obtained from cells using a MicroRNA isolation kit (Stratagene, La Jolla, CA) was reverse-transcribed using 1 µg oligo (dT) primer and Super Script II Reverse transcriptase (Invitrogen, Carlsbad, CA). The relative quantities of mRNA expression were analyzed by RTD-PCR using ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). All primer pairs and probes were obtained from the TaqMan assay reagents library. Expression levels of genes were calculated with the  $2^{-\Delta\Delta Ct}$  method using either  $\beta$ -actin or GAPDH as internal control genes.

**Apoptotic cell detection assay:** Freshly isolated PBMCs were incubated with AIM-V (Invitrogen) serum-free culture media containing 5 or 30 mM glucose at 37°C with 5% CO<sub>2</sub> for up to 24 h. The cells were incubated with FITC-labeled anti-CD4, CD14, or CD56 antibodies (BD Biosciences) and with PE-labeled Annexin-V and 7-amino-actinomycin D (7-AAD) (BD Biosciences) in PBS containing 2% BSA (Sigma-Aldrich). Apoptotic cells were determined by flow cytometry as the fraction of cells labeled with Annexin-V that were

7-AAD negative. At least 10,000 cells per sample were analyzed.

**Phagocytosis assay:** Phagocytotic activity was assessed using Phagotest Kit (Orpegen Pharma, Heidelberg, Germany) and FITC-labeled opsonized *E. coli* in accordance with manufacturer's protocol. Briefly, heparinized whole blood obtained from the 33 patients with diabetes and 28 healthy volunteers was incubated with FITC-labeled *E. coli* for 10 min at 37°C. After removing the erythrocytes, the remaining cells were incubated with propidium iodide to detect viable leukocytes by flow cytometry. Monocyte populations were assessed based on cellular granularity and size as side scatter and forward scatter, respectively, and FITC-positive cells were assessed as monocytes with phagocytosed FITC-labeled *E. coli*.

**TLR ligand stimuli and expression of pro-inflammatory cytokine genes:** Peptidoglycan (PGN) (1 µg/ml) from *Streptomyces sp.* (Sigma-Aldrich), Poly (I:C) (5 µg/ml) (Sigma-Aldrich), and Lipopolysaccharide (LPS) (2 µg/ml) from *E. coli* (Sigma-Aldrich), which are TLR2, TLR3, and TLR4 ligands, respectively, were added to monocytes ( $3 \times 10^5$  cells) freshly isolated from the 33 patients and 28 healthy volunteers in AIM-V media. Before and 3 h after incubation, the expression of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) was analyzed by RTD-PCR.

**Analysis of gene expression by DNA microarray:** Total RNA was obtained from CD14<sup>+</sup> monocytes using MicroRNA isolation kit (Stratagene, La Jolla, CA), and the mRNA was amplified twice using the Amino-allyl MessageAmp aRNA Kit (Ambion, Austin, TX). The reference RNA sample was isolated from CD14<sup>+</sup> monocytes from a 30-year-old healthy male volunteer and amplified in the same manner. Amplified mRNA was labeled with Cyanine (Cy) 5 or Cy3 (Amersham, Buckinghamshire, UK).

Equal amounts of the amplified mRNAs were hybridized to an oligo-DNA chip (AceGene® Human Oligo Chip 30K; Hitachi Software Engineering Co., Yokohama, Japan) overnight and washed prior to image scanning.

The fluorescence intensity of each spot on the oligo-DNA chip was obtained using cDNA Microarray Scan Array G (PerkinElmer, Wellesley, MA). The obtained images were quantified using DNAsis array v2.6 software (Hitachi Software Engineering Co.). For normalization, the intensity of each spot with oligo DNA was subtracted from that of spots without oligo DNA in the same block. The spot was validated when the intensity was within the intensity plus or minus a 2-fold range of standard deviation within each block. By calibrating the median as the base value, the intensities of all spots were adjusted for normalization between Cy5 and Cy3. Hierarchical clustering of gene expression was calibrated using the method described above using BRB Array Tools (<http://linus.nci.nih.gov/BRB-ArrayTools.htm>). The non-filtered data were log-transformed and applied to the average linkage clustering with centered correlation. For the functional analysis of the 813 upregulated genes, we used GenMAPP (<http://www.genmapp.org>), a computer program designed for viewing and analyzing genome-scale data on MAPPs representing biological pathways and any other groups of genes.

**Electron microscopy:** Monocytes obtained from 3 healthy volunteers and 3 patients with diabetes were fixed with 2.5% glutaraldehyde, and then post-fixed in 1% (v/v) cacodylate-buffered osmium tetroxide. Samples were dehydrated in a graded series of ethanol, transferred to propylene oxide, and embedded in Epon-Araldite. Ultrathin sections were obtained and observed under a Hitachi H-7500 electron microscope (Hitachi High-Technologies Co., Hitachinaka, Japan).

**Caspase-3 assay and enzyme-linked immunosorbent assay (ELISA) of cytokines:** Monocytes from a healthy volunteer were harvested and treated with tunicamycin (1 or 5 µg/ml) in AIM-V media. Every 3 h up to 12 h after tunicamycin treatment, we assessed apoptosis by flow cytometry as described above. After 12 h of incubation, the expression levels of BCL-2, C/EBP homologous protein (CHOP) and (immunoglobulin heavy chain binding protein) BiP were assessed by RTD-PCR. The DEVD-cleaving activity of active caspase-3 was measured using labeled Asp-Glu-Val-Asp-p-nitroanilide (DEVD-pNA) as the substrate and the Caspase-3 Colorimetric Assay Kit (Promega, Madison, WI) in accordance with manufacturer's protocol. The pNA light emission was quantified using a microtiter plate reader at a wavelength of 405 nm. In addition, we measured the production of pro-inflammatory cytokines by RTD-PCR 6 h after treatment of monocytes ( $3 \times 10^5$  cells) with tunicamycin (1 or 5 µg/ml) or the TLR ligands PGN (1 µg/ml), Poly (I:C) (5 µg/ml), and LPS (2 µg/ml). The concentrations of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in the culture supernatants were measured using ELISA kit (eBioscience).

**Statistical analysis:** Data are expressed as means  $\pm$  SEM. The Mann-Whitney *U* test was applied to assess the significant differences between the two groups. Statistical significance was determined as \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

## RESULTS

**Increased apoptosis of CD14<sup>+</sup> monocytes from patients with diabetes:** We first assessed the frequency of apoptosis in the PBMC fractions from 33 patients with diabetes and 28 non-diabetic, healthy volunteers. Apoptosis of the isolated cells was assessed after 3 h incubation in AIM-V



serum-free media containing 5 mM glucose (physiological concentration in blood). As shown in Fig. 1A, a significant difference in the frequency of apoptosis was observed in the PBMCs isolated from patients with diabetes and healthy volunteers. Adding serum to AIM-V serum-free media did not affect the difference in apoptosis (data not shown). The numbers of whole PBMCs, CD4<sup>+</sup>, CD14<sup>+</sup>, and CD56<sup>+</sup> cells were similar in both diabetic and healthy subjects (data not shown). CD14<sup>+</sup> monocytes were observed to be the major contributor to the increased apoptosis measured in the PBMCs. In contrast, apoptosis of CD4<sup>+</sup> T cells and CD56<sup>+</sup> NK cells were not significantly different between the two groups (Fig. 1A). When the incubation period in culture media with or without serum was extended to 24 h, about 20% of the CD56<sup>+</sup> NK cells of both patients with diabetes and healthy volunteers were induced to undergo apoptosis. When incubation period was extended to 5 days, about 5% of CD4<sup>+</sup> T cells of both patients with diabetes and healthy volunteers were induced to undergo apoptosis; there was no significant difference in cell viability of CD56<sup>+</sup> NK cells and CD4<sup>+</sup> T cells between the two groups (data not shown). BCL-2 expression of CD4<sup>+</sup> T cells was not different between the two groups (data not shown). Apoptosis of PBMC subpopulations incubated in culture media containing 30 mM glucose was not different from cells incubated in 5 mM glucose-containing media (data not shown). Moreover, the susceptibility of PBMCs from patients with diabetes to apoptosis was not related to clinical features such as vascular complications, insulin treatment, and FPG concentrations (data not shown).

However, among the 33 patients with diabetes, the frequency of apoptotic CD14<sup>+</sup> monocytes from those with poor glycemic control (HbA<sub>1c</sub> ≥ 9.0) was elevated compared to patients with fair glycemic control (HbA<sub>1c</sub>

< 9.0) (Fig. 1B). Furthermore, after 3 h incubation, the increased ratio of the expression of the anti-apoptotic gene, BCL-2, was substantially lower in monocytes from the 15 patients with HbA<sub>1c</sub> ≥ 9.0 compared to the 18 patients having HbA<sub>1c</sub> < 9.0, as assessed by RTD-PCR (Fig. 1C). These data suggest that the monocytes of patients with diabetes are susceptible to apoptosis, especially under conditions of poor glycemic control.

**Attenuated function of monocytes from patients with diabetes:** To determine if functional alterations exist in monocytes isolated from the 33 patients with diabetes, we cocultured the monocytes with FITC-labeled *E. coli* and counted the number of fluorescent monocytes ingesting the labeled *E. coli* by flow cytometry. The ratio of monocytes that phagocytosed *E. coli* to all monocytes in patients with diabetes was higher than in the healthy volunteers (Fig. 2A and B). No significant correlation was observed between the ratio of phagocytosed *E. coli* and HbA<sub>1c</sub> levels among the patients (data not shown).

Next, we assessed the responsiveness of monocytes to external pathogenic stimuli *in vitro*. Monocytes typically express pattern-recognition molecules such as the TLRs that are important for innate immunity against various pathogens (13, 14). The expression levels of TLR2, TLR3, and TLR4 were not significantly different between monocytes from patients with diabetes and those from healthy volunteers, as assessed by RTD-PCR (Fig. 3A) and flow cytometry (data not shown). We also found that transcriptional expression of TLR signal molecules (MyD88, IRAK1, and TRAF6 for TLR2 and TLR4 signaling and TRIF for TLR3 signaling) was not altered in diabetic monocytes compared to non-diabetic monocytes (data not shown). Next, we exposed the monocytes from the patients with diabetes and healthy volunteers to the TLR ligands, PGN (a TLR2 ligand),

Poly (I:C) (a TLR3 ligand), and LPS (a TLR4 ligand) and measured the expression of the pro-inflammatory cytokine genes, TNF- $\alpha$  and IL-1 $\beta$ . After incubation, the expression of the cytokines was not significantly different between the groups (Fig. 3B), but the responsiveness to PGN, Poly (I:C), and LPS was significantly attenuated in monocytes from patients with diabetes compared to those from healthy volunteers as assessed by RTD-PCR (Fig. 3C and D). These results demonstrate that the monocytes of patients with diabetes are functionally impaired, which implies that they could contribute to immune deficiency in diabetes.

**ER stress is a molecular feature of impaired monocytes:** To elucidate the molecular features of the diabetic monocytes that were distinctly susceptible to apoptosis, DNA microarray analysis was performed on CD14<sup>+</sup> cells isolated from five randomly selected patients with diabetes and five healthy volunteers. These subjects demonstrated clinical features near the median of all study subjects. Unsupervised hierarchical clustering analysis was performed to assess the gene expression profiles of monocytes obtained from patients with diabetes and healthy volunteers; 17,184 filtered genes were evaluated after excluding genes that were not expressed or those with low expression levels that prevented their analysis in 50 % of the cases. As shown in Fig. 4A, two completely discernible clusters formed between the patients with diabetes and the healthy volunteers.

We identified 813 genes that were up-regulated in the monocytes from patients with diabetes compared to those of healthy volunteers ( $P < 0.05$ , student's  $t$  test). Analysis of the biological processes concerning these genes was performed using GenMAPP. The identified genes were shown to be involved in posttranslational protein modification systems occurring in the Golgi-apparatus or were involved in ER stress

(Table 2 and Supplemental Table 1 which can be found in an online appendix at <http://diabetes.diabetesjournals.org>). The elevated expression of genes related to ER stress, such as CHOP and BiP, was confirmed using RTD-PCR; the expression of these genes was significantly higher in the monocytes from the 33 patients with diabetes than in those from the 28 healthy volunteers (Fig. 4B). In contrast, no significant difference in the expression of these genes was observed in CD4<sup>+</sup> T cells from patients with diabetes and healthy volunteers (Fig. 4C).

Electron microscopy further confirmed ER stress in the monocytes derived from patients with diabetes. As shown in Fig. 4D, morphological alterations of the ER such as corruption of concentric, continuous and regular layer structure and a decreased number of ribosomes on the ER membrane were evident from the electron photomicrographic images.

**ER stress-induced apoptosis and attenuation of TLR signaling in human monocytes:** The results described above indicated that the monocytes from patients with diabetes have compromised immunological function and that ER stress is a distinct feature in these cells. To determine whether ER stress could be a mechanism underlying the observed increase in apoptosis and decreased responsiveness to TLR ligands, CD14<sup>+</sup> cells isolated from a healthy volunteer were treated with the ER stress inducer, tunicamycin (1  $\mu$ g/ml), in AIM-V media. As shown in Fig. 5A and B an increased number of apoptotic cells was observed among monocytes treated with tunicamycin compared to untreated monocytes after more than 6 h incubation. Treatment of monocytes with a higher concentration of tunicamycin (5  $\mu$ g/ml) induced more apoptosis (Fig. 5A and B), and when monocytes were treated with tunicamycin for 12 h, the activity of the pro-apoptotic protease, caspase-3,

significantly increased (Fig. 5C). Treatment with tunicamycin coordinately decreased the expression of BCL-2 (Fig. 5D) and increased the expression of the ER stress markers, CHOP and BiP (Fig. 5E). These results suggest that ER stress promotes apoptosis of human monocytes.

Next, we investigated how tunicamycin-induced ER stress affected the responsiveness of human monocytes to TLR ligands. Treatment of monocytes with tunicamycin for 6 h did not affect the transcriptional and translational expression of TLR2 and TLR4 (data not shown). As shown in Fig. 6A–C, however, the expression of the pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 were down-regulated after stimulation with TLR2 and TLR4 ligands. Furthermore, the production of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in media was measured by ELISA and found to decrease after treatment of human monocytes with tunicamycin and after stimulation with TLR2 or TLR4 ligands (Fig. 6D–F). However, tunicamycin-induced ER stress did not affect these expression after treatment of monocytes with the TLR3 ligand, Poly (I:C) (data not shown).

## DISCUSSION

In the present study, we observed that PBMCs from patients with diabetes were more susceptible to apoptosis compared to PBMCs from healthy volunteers and that CD14<sup>+</sup> monocytes comprised the primary PBMC subpopulation undergoing apoptosis. We also found that CD14<sup>+</sup> monocytes from patients with diabetes were hypo-responsive to TLR ligands and that they had attenuated phagocytotic activity. Transcriptional analysis and electron microscopy revealed the presence of ER stress in the affected diabetic monocytes. Consistently, monocytes isolated from non-diabetic patients showed a similar increase in apoptosis and a weakened response to TLR ligands, when they were treated with tunicamycin, indicating that ER

stress may be a pivotal mechanism underlying the decreased immunological function observed in patients with diabetes.

As innate immune-defense mediators, monocytes are capable of ingesting exogenous pathogens to protect the host from infectious diseases. Previous studies have shown that phagocytosis in diabetic neutrophils and monocytes is attenuated (10, 11). Similarly, in our study population, monocytes from patients with diabetes were less capable of phagocytosing *E. coli* pathogens compared to monocytes derived from healthy volunteers. This novel finding might explain, at least in part, the decrease in immune function characteristic of patients with diabetes (16). Nevertheless, the detailed mechanisms underlying diabetes-induced decreases in phagocytotic activity remain unclear, since simple high glucose concentration did neither affect the phagocytotic activity and TLR expression, nor induce ER stress in non-diabetic monocytes *in vitro* (data not shown).

The TLRs are pattern recognition receptors that are important for recognizing pathogens, inducing pro-inflammatory responses, and preventing the host from acquiring infectious diseases (17–20). The expression of TLR2, TLR3, and TLR4 in CD14<sup>+</sup> monocytes was similar between patients with diabetes and healthy volunteers. The administration of a high dose of insulin downregulates TLRs expression (21). Transformed monocyte-lineage blastoma cells showed increased TLR expression under hyperglycemic conditions *in vitro* (22). Type 2 diabetes is characterized as a state of inadequately controlled glycemia associated with hyperinsulinemia due to peripheral insulin resistance (1). Taken together, the TLRs expression may be affected by hyperglycemia and hyperinsulinemia in a complex manner. In contrast to the previous finding that monocytes from patients with diabetes were hypersensitive to the TLR