Expression of Cancer Stem Cell-associated DKK1 mRNA Serves as Prognostic Marker for Hepatocellular Carcinoma

TOMOHIKO SAKABE1,2, JUNYA AZUMI1, YOSHIHISA UMEKITA2, KAN TORIGUCHI3, ETSURO HATANO3, YASUAKI HIROOKA4 and GOSHI SHIOTA1

1Division of Molecular and Genetic Medicine, Department of Genetic Medicine and Regenerative Therapeutics, Graduate School of Medicine, Tottori University, Yonago, Japan;
2Division of Organ Pathology, Department of Pathology, Faculty of Medicine, Tottori University, Yonago, Japan;
3Department of Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan;
4Department of Pathobiological Science and Technology, School of Health Science, Faculty of Medicine, Tottori University, Yonago, Japan

Abstract. Background/Aim: Cancer stem cells (CSCs) are associated with prognosis of hepatocellular carcinoma (HCC). In our previous study, we created cDNA microarray databases on the CSC population of human HuH7 cells. In the present study, we identified genes that might serve as prognostic markers of HCC by employing existing databases. Materials and Methods: Expressions of glutathione S-transferase pi 1 (GSTP1), lysozyme (LYZ), C-X-C motif chemokine ligand 5 (CXCL5), interleukin-8 (IL8) and dickkopf WNT signaling pathway inhibitor 1 (DKK1), the five most highly expressed genes in the CSC cDNA microarray databases, were examined in 99 patients with HCC by real-time polymerase chain reaction (qRT-PCR), and their clinical significance was analyzed. Results: The Kaplan–Meier analysis showed that both overall and cancer-specific survival were significantly longer in patients with low DKK1 expression than in those with high DKK1 expression. The multivariate analysis revealed that overall survival was negatively associated with albumin and positively associated with alkaline phosphatase (ALP), serosal invasion and stage, and cancer-specific survival was positively associated with ALP, portal vein invasion and DKK1 mRNA. Conclusion: Expression of CSC-associated DKK1 mRNA might be an unfavorable prognostic marker for patients with HCC.

Hepatocellular carcinoma (HCC) is the sixth most common cancer, and the third most frequent cause of death worldwide (1). Since biomarkers are useful for early diagnosis and prediction of prognosis (2), they may provide effective treatment options. Although several biomarkers, including alpha-fetoprotein (AFP), protein induced by vitamin K deficiency or antagonist-II (PIVKAII), and glypican-3, were reported as being useful (2, 3), identification of novel biomarkers for HCC is expected to improve prognosis of patients with HCC.

Cancer stem cells (CSCs) are defined as cells that possess the capacity to self-renew and produce heterogeneous lineages of cancer cells (4). CSCs are involved in development, progression, metastasis, recurrence and prognosis of cancer (5). Indeed, it is reported that patients with HCC having a CSC phenotype have a poor prognosis (6). Dysregulation of several specific signaling pathways in CSCs has been associated with stemness (7). CSCs are also ‘robust’, which encompasses several characteristics including the ability to escape from the effect of cytotoxic agents, resistance to oxidative stress, and a rapid response to and repair of DNA damage (8). Specific genes which confer resistance to chemotherapy and radiotherapy seem to be expressed in CSCs, resulting in poor prognosis for patients with cancer.

In our previous study, we identified CD44 as the best prognostic marker out of four CSC markers, namely CD13, epithelial cell adhesion molecule (EpCAM), CD44 and CD44 variant 9, in HCC (9). In addition, CD44-positive HuH7 HCC cells had CSC properties such as proliferative potential and sphere-forming ability. Importantly, we developed databases for cDNA/miRNA expression of liver CSCs. In the present study, by employing the previously developed cDNA microarray databases, we examined whether the top five most highly expressed genes, glutathione S-transferase pi 1
Table I. Clinical parameters of patients with hepatocellular carcinoma.

| Characteristic                  | Value                                      | Characteristic                  | n  |
|--------------------------------|--------------------------------------------|---------------------------------|----|
| No. of patients                | 99                                         | Capsular invasion (n=95)        |    |
| Gender                         |                                            |                                 |    |
| Male                           | 83                                         | Negative                        | 50 |
| Female                         | 26                                         | Positive                        | 45 |
| Age, years                     | 67 (32-88)                                 | Septum formation                |    |
| Etiology                       |                                            |                                 |    |
| HBV                            | 14                                         | Serosal invasion                 |    |
| HCV                            | 62                                         | Negative                        | 87 |
| HBV/HCV                        | 21                                         | Positive                        | 12 |
| Non HBV/C                      | 2                                          | Portal vein invasion            |    |
| Total bilirubin (mg/dl)        | 0.8 (0.1-3.9)                              | Negative                        | 62 |
| Albumin (g/dl)                 | 3.9 (2.9-5.3)                              | Positive                        | 37 |
| AST (IU/l)                     | 47 (17-166)                                | Hepatic vein invasion           |    |
| ALT (IU/l)                     | 44 (8-311)                                 | Negative                        | 88 |
| ALP (IU/l)                     | 278 (33-1159)                              | Positive                        | 11 |
| γ-GTP (IU/l) (n=97)            | 66 (17-969)                                | Bile duct invasion              |    |
| AFP (ng/ml)                    | 52 (3.2-639256)                            | Negative                        | 87 |
| PIVKA-II (U/ml) (n=98)         | 119 (0.02-30100)                           | Positive                        | 12 |
| Tumor number (n=98)            |                                            | Stage                           |    |
| 1                              | 70                                         | I                               | 6  |
| 2                              | 13                                         | II                              | 43 |
| >3                             | 15                                         | III                             | 28 |
| Tumor size (cm) (n=98)         | 3.5 (1.0-16)                               | IV                              | 22 |
| Survival period (days)         | 1717 (28-4450)                             |                                 |    |
| Differentiation (n=95)         |                                            |                                 |    |
| Well                           | 19                                         |                                 |    |
| Moderate                       | 54                                         |                                 |    |
| Poor/undifferentiated          | 22                                         |                                 |    |

HBV, Hepatitis B virus; HCV, hepatitis C virus; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GTP, gamma-glutamyl transpeptidase; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II. Data are the median (range) for continuous variables and absolute numbers for categorical variables.

(GSTP1), lysozyme (LYZ), C-X-C motif chemokine ligand 5 (CXCL5), interleukin-8 (IL8) and dickkopf WNT signaling pathway inhibitor 1 (DKK1), in the CSC population can serve as prognostic markers for HCC.

Materials and Methods

Patients and clinical samples. The samples for the gene-expression analysis were obtained from 99 patients with HCC who were admitted to Kyoto University Hospital from 1998 to 2008 and agreed to undergo surgical resection with curative intent under informed consent. Clinicopathological parameters of these patients are summarized in Table I. This study conformed with the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of Tottori University Faculty of Medicine and Kyoto University Hospital (approval number: 1619).

Real-time reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA from hNHeps (Lonza, Walkersville, MD, USA), CD44-negative HuH7 (Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan), CD44-positive HuH7 cells (Japanese Collection of Research Bioresources Cell Bank), and 99 HCC clinical specimens were obtained according to the method previously described (9). Total RNA was reverse-transcribed using SuperScript II (Invitrogen, Carlsbad, CA, USA) and oligo (dT) primers. Expression levels of mRNA were measured by Applied Biosystems 7900HT Fast Real Time PCR System using EXPRESS qPCR Supermix with Premixed ROX (Applied Biosystems, Life Technologies, Foster City, CA, USA), universal probes (Roche Applied Science, Basel, Switzerland), and gene-specific primers. Universal probes and gene-specific primers used in this study are summarized in Table II.

Statistical analysis. EXCEL (Microsoft Corporation, Redmond, WA, USA) and PASW statistics (SPSS Inc., Chicago, IL, USA) were used for the statistical calculations in this study. The comparison for statistical differences was performed using Student’s t-test, or Mann–Whitney U-test. The Kaplan–Meier analysis was performed for both overall and cancer-specific survival according to CSC-related genes. The log-rank test was performed to determine the prognostic variables associated with overall survival and cancer-specific survival ratios in patients with HCC. Cox regression model was used for multivariable analysis of variables affecting overall and cancer-specific survival. Diffuses with a value of p<0.05 were considered to be statistically significant.
Table II. Universal probes and primers used in this study.

| Gene          | Encoded protein name                      | Gene ID             | Universal probe ID | Forward     | Reverse                      |
|---------------|-------------------------------------------|---------------------|--------------------|-------------|------------------------------|
| ACTB          | Actin, cytoplasmic 1                      | NM_001101.3         | #64                | ccaacceggagaagatga | ccagggctgatgccagatag        |
| GSTP1         | Glutathione S-transferase P               | NM_000852.3         | #56                | tetcctctctatacacaatcag | aggtgctgctggctgg             |
| LYZ           | Lysozyme C precursor                     | NM_000239.2         | #68                | cgcgctacggtaagatggt    | ccagcgctcgaatgccacag          |
| CXCL5         | C-X-C Motif chemokine 5 precursor        | NM_002994.3         | #71                | ggtctctctctctcttcggt   | gcagctgctcgaatgccacacagacata |
| IL8           | Interleukin-8 precursor                   | NM_000584.3         | #72                | gagctctctctctctctctctcgtt | atgggttgatacgagacacacacata  |
| DKK1          | Dickkopf-related protein 1 precursor     | NM_012242.2         | #4                 | cagggctgatgccagatag    | ccagggctgatgccagatag        |

Table III. List of genes exhibiting differential expression patterns between hNHeps, CD44−, HuH7, and CD44+ HuH7 cells.

| Pattern A (hNHeps <CD44− <CD44+) | Pattern B (CD44− <CD44+ <hNHeps) | Pattern C (CD44− <hNHeps <CD44+) |
|----------------------------------|----------------------------------|----------------------------------|
| Symbol                          | Normalized expression            | Symbol                          | Normalized expression            |
| C17orf45                        | 1343 2364 5311                    | UCHL1                           | 1167 370 4169                    |
| GSTP1                           | 1261 3278                         | PSAP                             | 874 607 1251                     |
| LYZ                             | 1253 2394                         | CLPTM1                           | 1015 563 1155                    |
| CXCL5                           | 28 565 2191                       | MAP2K4                           | 80 74 519                        |
| IL8                             | 331 1767                          | DHRSTB                          | 192 124 505                      |
| DKK1                            | 12 438 1126                       | CEBPD                            | 182 127 420                      |
| USP14                           | 496 1003                          | ZSCAN5C                          | 334 183 368                      |
| USP22                           | 237 901                           | MEST                             | 182 157 344                      |
| TMEM11                          | 174 540                           | XP0I                             | 277 11 355                       |
| MILT1                           | 57 375                            | FBXX2                           | 277 87 301                       |
| PIR                             | 108 316                           | LCP1                             | 202 122 297                      |
| HPGD                            | 34 102 297                        | AC026412.4                       | 237 134 284                      |
| B9D1                            | 44 246                            | CDKN2B                           | 195 102 260                      |
| ZNF18                           | 28 40 225                         | NFU1                             | 123 97 202                       |
| PKIB                            | 50 223                            | KIAA1143                         | 105 79 181                       |

Results

Differential expression patterns of mRNA from CSCs, non-CSCs, and normal hepatocytes. In our previous study, the genes with at least two-fold up-regulation in CD44-positive compared to CD44-negative HuH7 cells were registered in Gene Expression Omnibus (accession number GSE84226) (9). Of these 604 genes, 216 genes in which the global normalization number was over 20 were divided into three patterns of expression as follows: the first pattern included successive increase of expression in the order of normal hepatocytes, CD44-negative cells, and CD44-positive cells. The second pattern included successive increase in the order of CD44-negative cells, normal hepatocytes, and CD44-positive cells. The third pattern included successive increase in the order of CD44-negative cells, normal hepatocytes, and CD44-positive cells, and CD44-negative cells, CD44-positive cells, and CD44-positive cells.
expressed in CD44-positive HuH7 cells (Figure 1).

Overall survival was examined. Both overall and cancer-specific survival. The association of \( \text{Kaplan-Meier analysis of association of CSC-related genes with overall and cancer-specific survival.} \)

The association of CSC-related genes with overall and cancer-specific survival. The association of expression of \( \text{LYZ, CXCL5, DKK1, IL8 and GSTP1 with overall survival was examined. Both overall survival (} p=0.016, \text{ Figure 2A) and cancer specific-survival (} p=0.002, \text{ Figure 2B) were significantly associated with DKK1 mRNA, but not with that for other genes.} \)

Univariate and multivariate analyses of clinical factors and CSC-related genes for overall and cancer-specific survival. Overall survival was significantly associated with AFP; albumin; ALP; tumor size; invasion of serosa, portal vein and bile duct; tumor stage; and DKK1 mRNA by univariate analysis, and was associated with albumin, ALP, serosal invasion and stage by multivariate analysis (Table IV). Cancer-specific survival was significantly associated with hepatitis B surface antigen (HBs-Ag), hepatitis C virus antibody (HCV-Ab); AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; \( \gamma \)-GTP, gamma-glutamyl transferase; GSTP1, glutathione S-transferase pi 1; LYZ, lysozyme; CXCL5, C-X-C motif chemokine ligand 5; IL8, interleukin-8; DKK1, dickkopf WNT signaling pathway inhibitor 1.

Table IV. Univariate and multivariate analyses of clinical variables and cancer stem cells-related mRNA expression for overall and cancer-specific survival.

| Factor                  | Ref. vs. comparator | Overall survival | Cancer-specific survival |
|-------------------------|---------------------|------------------|--------------------------|
|                         |                     | Univariate       | Multivariate             | Univariate       | Multivariate             |
|                         |                     | \( p \)-value    | \( p \)-value         | \( p \)-value    | \( p \)-value         |
| Gender                  | Male vs. female     | 0.776            | 0.447                    | 0.776            | 0.876                    |
| Age                     | ≥67 vs. >67 years   | 0.209            | 0.327                    | 0.209            | 0.327                    |
| HBs-Ag                  | Negative vs. positive | 0.128            | 0.202                    | 0.128            | 0.202                    |
| HCV-Ab                  | Negative vs. positive | 0.161            | 0.127                    | 0.161            | 0.127                    |
| AFP                     | ≤57 vs. >57 ng/ml   | 0.004            | N/A                      | 0.054            | (0.121-0.494)            |
| PIVKA-II                | ≤140 vs. >140 U/ml  | 0.275            | 0.201                    | 0.275            | 0.201                    |
| Total bilirubin         | ≤0.8 vs. >0.8 mg/dl | 0.525            | 0.203                    | 0.525            | 0.203                    |
| Albumin                 | ≤3 vs. >3 g/dl      | 0.015            | 0.083                    | 0.015            | 0.083                    |
| AST                     | ≤51 vs. >51 IU/l    | 0.454            | 0.205                    | 0.454            | 0.205                    |
| ALT                     | ≤45 vs. >45 IU/l    | 0.457            | 0.209                    | 0.457            | 0.209                    |
| ALP                     | ≤283 vs. >283 IU/l  | <0.001           | 2.796 (1.592-4.009)      | <0.001           | 2.796 (1.592-4.009)      |
| γ-GTP                   | ≤74 vs. >74 IU/l    | 0.069            | 0.204                    | 0.069            | 0.204                    |
| Tumor number            | 1 vs. ≥2            | 0.071            | 0.128                    | 0.071            | 0.128                    |
| Tumor size              | ≤3 vs. >3.5 cm      | 0.023            | N/A                      | 0.297            | 0.297                    |
| Capsular invasion       | Negative vs. positive | 0.141            | 0.141                    | 0.141            | 0.141                    |
| Septum formation        | Negative vs. positive | 0.187            | 0.187                    | 0.187            | 0.187                    |
| Serosal invasion        | Negative vs. positive | 0.012           | 2.182 (1.020-4.671)      | 0.044            | 2.182 (1.020-4.671)      |
| Portal vein invasion    | Negative vs. positive | 0.004           | N/A                      | 0.338            | 0.338                    |
| Bile duct invasion      | Negative vs. positive | 0.023           | N/A                      | 0.604            | 0.604                    |
| Hepatic vein invasion   | Negative vs. positive | 0.066           | 0.066                    | 0.066            | 0.066                    |
| Stage                   | I/II vs. III/IV     | 0.007            | 1.840 (1.054-3.212)      | <0.001           | 1.840 (1.054-3.212)      |
| GSTP1 mRNA              | Low vs. high        | 0.842            | 0.744                    | 0.842            | 0.744                    |
| LYZ mRNA                | Low vs. high        | 0.664            | 0.266                    | 0.664            | 0.266                    |
| CXCL5 mRNA              | Low vs. high        | 0.207            | 0.638                    | 0.207            | 0.638                    |
| IL8 mRNA                | Low vs. high        | 0.797            | 0.819                    | 0.797            | 0.819                    |
| DKK1 mRNA               | Low vs. high        | 0.016            | 0.016                    | 0.016            | 0.016                    |

Ref.: Referent; HR, hazard ratio; CI, confidence interval; N/A, not applicable; HBs-Ag, hepatitis B surface antigen; HCV Ab, hepatitis C virus antibody; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; \( \gamma \)-GTP, gamma-glutamyl transferase; GSTP1, glutathione S-transferase pi 1; LYZ, lysozyme; CXCL5, C-X-C motif chemokine ligand 5; IL8, interleukin-8; DKK1, dickkopf WNT signaling pathway inhibitor 1.

normal hepatocytes. Each pattern included 60, 57 and 99 genes, respectively. The top 15 genes in each group are listed in Table III. On the assumption that the genes showing a successive increase of expression in the order of normal hepatocytes, CD44-negative cells, and CD44-positive cells are associated with CSCs of HCC, the individual mRNA expressions of the top five genes were examined in normal hepatocytes, CD44-negative cells, and CD44-positive cells by qRT-PCR. C17orf45 was omitted since this gene is a non-protein coding RNA (10). Expression of \( \text{GSTP1, LYZ, CXCL5, IL8, and DKK1 was confirmed to be highly expressed in CD44-positive HuH7 cells (Figure 1).} \)

Kaplan–Meier analysis of association of CSC-related genes with overall and cancer-specific survival. The association of expression of LYZ, CXCR5, DKK1, IL8 and GSTP1 with overall survival was examined. Both overall survival (p=0.016, Figure 2A) and cancer specific-survival (p=0.002, Figure 2B) were significantly associated with DKK1 mRNA, but not with that for other genes.
Association of DKK1 mRNA with clinicopathological variables. Expression of DKK1 mRNA was negatively associated with albumin, but was positively associated with AFP, GSTP1 mRNA, CXCL5 mRNA, and IL8 mRNA (Figure 3A). Expression of DKK1 mRNA was higher in patients with HBsAg-positive, stage III/IV, or capsular invasion-positive tumor than HBsAg-negative, stage I/II or capsular invasion-negative, respectively (Figure 3B).
Discussion

In the present study, we showed that DKK1 mRNA is significantly associated with cancer-specific survival in patients with HCC. A meta-analysis of prognostic significance in solid tumors reported that DKK1 overexpression predicted poor overall survival in HCC, ovarian cancer, and other cancer types (11-15). On the other hand, serum DKK1 was reported to be a biomarker useful for diagnosis of HCC by a large-scale and multicenter study (16). These data suggest that DKK1 plays an important role in HCC from viewpoints of biology and clinical settings.

DKK gene family comprise an evolutionary conserved small genes (17). DKK genes encode secreted proteins that antagonize WNT/β-catenin signaling by binding to the WNT co-receptors LRP5 and -6. The human DKK family consists of five members, DKK1, DKK2, DKK3, DKK4, and a unique DKK3-related gene, DKKL1 (18). Dysregulation of WNT signal activation is thought to play a causative role in several types of cancer and is involved in the acquisition of stem cell-like properties of CSCs. DKK1 is an antagonist of the WNT signaling pathway, and plays crucial roles in tumor growth and progression. DKK1 levels are elevated in a wide variety of cancer types including HCC (11-15), and breast (19), colorectal (20), and pancreatic (21) cancer. Although how DKK1 regulates WNT signaling remains to be solved, one important fact is that DKK1 is a downstream target of WNT signaling, allowing for a negative feedback loop (18). Indeed, activation of canonical WNT signaling causes an increase in DKK1 mRNA and protein (22). These data suggest that DKK1 overexpression is a result of WNT/β-catenin pathway expression.

In the present study, by employing the previously developed databases of cDNA microarray in CSCs population, we identified DKK1 mRNA expression as a prognostic marker. Since liver CSCs have been reported to be associated with increased chemo/radioresistance, earlier recurrence after surgical or locoregional treatment, increased invasiveness, metastasis, and poor prognosis (23), it is clinically useful to identify novel prognostic genes which are highly expressed in liver CSCs.

In conclusion, by employing our previously developed databases of cDNA microarray in CSCs, we identified DKK1 mRNA expression in HCC tissues as representing cancer-specific survival of patients with HCC. Therefore it is expected to serve as a novel prognostic marker in HCC.

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Figure 3. Comparison of the relationships between dickkopf 1 (DKK1) expression and clinicopathological parameters. A: Scatter diagrams showing the results of Spearman’s rank correlation analysis between DKK1 expression and albumin (ALB), alpha-fetoprotein (AFP), and mRNA expression of cancer stem cell-related GSTP1, CXCL5, and IL8. mRNA expression levels were normalized by actin beta (ACTB) expression and converted into log2 values. (B) Relative expression level of DKK1 mRNA was analyzed in HCC patients classified into two groups based on their clinicopathological variables. DKK1 expression level was normalized by ACTB expression and converted into log2 values. Mann-Whitney U-test was performed to determine the statistical significance: significantly different at *p<0.05 and **p<0.01.
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