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Hepatocellular Carcinoma-associated microRNAs Induced by Hepatoma-derived Growth Factor Stimulation

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Abstract. *Background/Aim: Hepatoma-derived growth factor (HDGF) is involved in the progression of hepatocellular carcinoma (HCC). The present study assessed the epigenomic changes in hepatoma-derived cells through HDGF stimulation. Materials and Methods: We used two hepatoma-derived cell lines (HepG2 and SK-Hep1) and searched for microRNAs whose expression commonly changed in response to HDGF administration. We further explored a genetic database to investigate the association of the candidate microRNAs with the survival of HCC patients. Results: Despite both HepG2 and SK-Hep1 cells being categorized as hepatoma-derived cells, the microRNA profile differed between these two lines. However, HepG2 and SK-Hep1 cells shared 30 up-regulated and 2 down-regulated microRNAs. Of these, miR-6072 and miR-3137 were significantly associated with a poor prognosis in HCC patients. Conclusion: We identified two candidate microRNAs whose expression increased in response to HDGF stimulation. Both these molecules were associated with a poor prognosis of HCC patients.*

Hepatocellular carcinoma (HCC) is a common malignant disease with an unfavorable prognosis. Although several new drugs have been developed, there is no definitive therapy against HCC, and new treatment strategies for HCC are warranted (1, 2).

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The hepatoma-derived growth factor (HDGF) was recently identified as a novel factor involved in HCC growth (3, 4). We have previously shown that HDGF promotes the proliferation of hepatoma cells *in vitro* and *in vivo* (4, 5). In addition, a high expression of HDGF was shown to be related to a poor clinical outcome for HCC patients (6-9). HDGF is considered to translocate to the nucleus and regulate the expression of target genes by binding to their promoter regions in the genomic DNA (10).

Recently, several non-genomic/epigenetic factors, such as microRNAs, have been suggested to play an important role in various diseases (11, 12). MicroRNAs are known to be non-coding short RNA molecules that bind to complementary sequences and influence the function of target genes.

Concerning HCC changes in microRNAs of hepatoma-derived cells and the importance of HDGF signaling have not been adequately clarified. For this reason, in the present study, we investigated which microRNAs showed a change in expression in response to HDGF stimulation and were associated with a poor prognosis of HCC patients.

Materials and Methods

Cell culture. We selected HepG2 and SK-Hep1 cells, as we previously confirmed the *in vivo* tumor growth promotion by HDGF in experiments with these cell lines (4). Cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Sigma-Aldrich Japan, Tokyo, Japan). Cells were plated into 12 well-dishes (Corning International, Tokyo, Japan) at a density of 1×10^5 cells/well. Twenty-four hours after the inoculation, the cells were exogenously stimulated with the administration of recombinant HDGF (Abcam, Tokyo, Japan; product code: ab132259) for 48 h (final concentration: 100 ng/ml). At the same time, cells without HDGF administration were also cultured and used as the control cells.

RNA extraction and microRNA analysis. Total RNA was extracted from cultured cells using the miRNeasy Mini Kit (Qiagen-Japan, Tokyo, Japan) according to the manufacturer's instructions. The isolated RNA samples from the HDGF-stimulated HepG2 cells and control

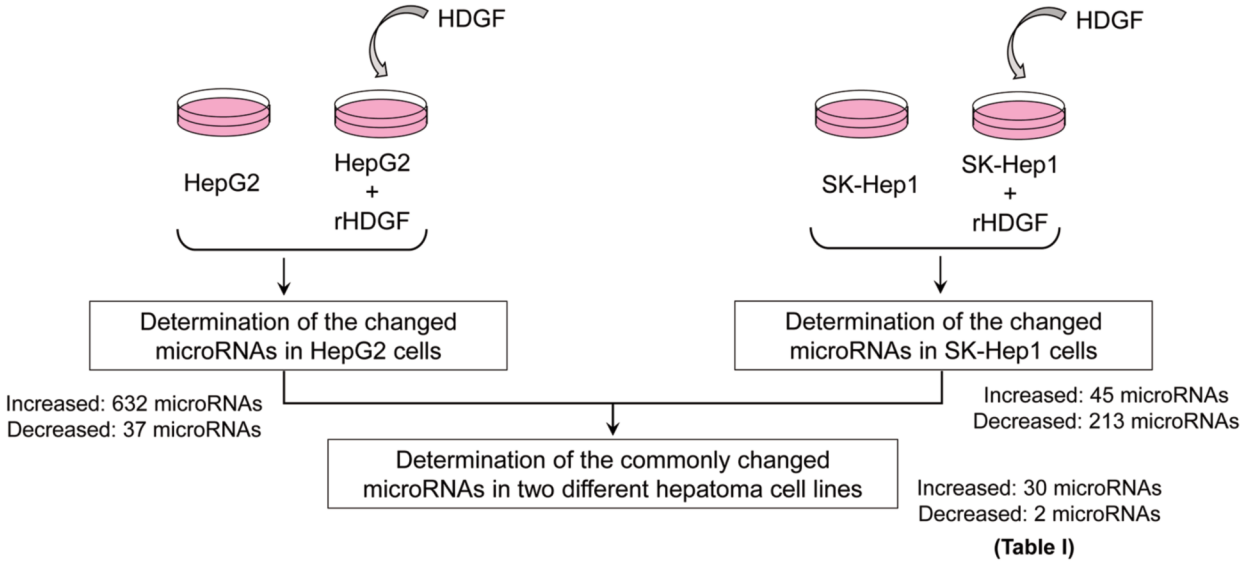

Figure 1. *Flowchart to determine the microRNAs whose expression changed in response to hepatoma-derived growth factor (HDGF).*

(untreated) HepG2 cells were used to search for the microRNAs whose expression changed in response to HDGF administration. We screened for microRNAs with an increased (≥1.5-fold) or a decreased expression (≤0.67-fold) following HDGF administration using the microarray system Toray 3D Gene, Human miRNA Ver. 21 (Toray, Kamakura, Japan) (13). We also used two RNA samples of SK-Hep1 cells with and without HDGF administration and compared the expression levels of microRNAs. Finally, we determined which microRNAs with changes in expression were commonly observed among the two RNA sets and considered them potentially functional microRNAs in response to HDGF (Figure 1).

Association of the microRNAs with the survival of primary liver cancer. We assessed the association of the microRNAs with the prognosis of patients with liver cancer in the UCSC Xena platform, which provides cancer genomics datasets (14). Based on the data from the system (GDC TCGA Liver Cancer), a total of 351 patients were divided into 2 groups according to the median copy number of each microRNA, and Kaplan-Meier survival curves were generated. MicroRNAs with a *p*-Value less than 0.05 according to the log-rank test was considered to have a significantly different prognosis.

Results

From the two RNA sample sets we assessed the changes in the microRNA expression in response to HDGF treatment (Figure 1). For HepG2 cells, we identified an increased expression of 632 microRNAs and a decreased expression of 37 microRNAs. For SK-Hep1 cells, we identified an increased expression of 45 microRNAs and a decreased expression of 213 microRNAs. Despite both HepG2 and SK-Hep1 cells being categorized as hepatoma-derived cells, the microRNA profile differed markedly between these two lines. However,

Table I. *MicroRNAs suggested to commonly increase or decrease in HepG2 and SK-Hep1cells in response to exogenously supplied HDGF.*

Commonly increased microRNAs	Commonly decreased microRNAs
hsa-miR-6072	hsa-miR-133b
hsa-miR-3689b-3p/hsa-miR-3689c	hsa-miR-1292-5p
hsa-miR-362-5p	
hsa-miR-5010-5p	
hsa-miR-7850-5p	
hsa-miR-6752-3p	
hsa-miR-6831-5p	
hsa-miR-19b-1-5p	
hsa-miR-1301-5p	
hsa-miR-1293	
hsa-miR-675-3p	
hsa-miR-1304-3p	
hsa-miR-5187-5p	
hsa-miR-4762-3p	
hsa-miR-3124-5p	
hsa-miR-4534	
hsa-miR-4271	
hsa-miR-6872-5p	
hsa-miR-6511b-5p	
hsa-miR-6068	
hsa-miR-4290	
hsa-miR-936	
hsa-miR-4725-5p	
hsa-miR-6840-5p	
hsa-miR-6514-3p	
hsa-miR-6750-3p	
hsa-miR-6767-5p	
hsa-miR-3064-3p	
hsa-miR-3137	
hsa-miR-206	

HDGF: Hepatoma-derived growth factor.

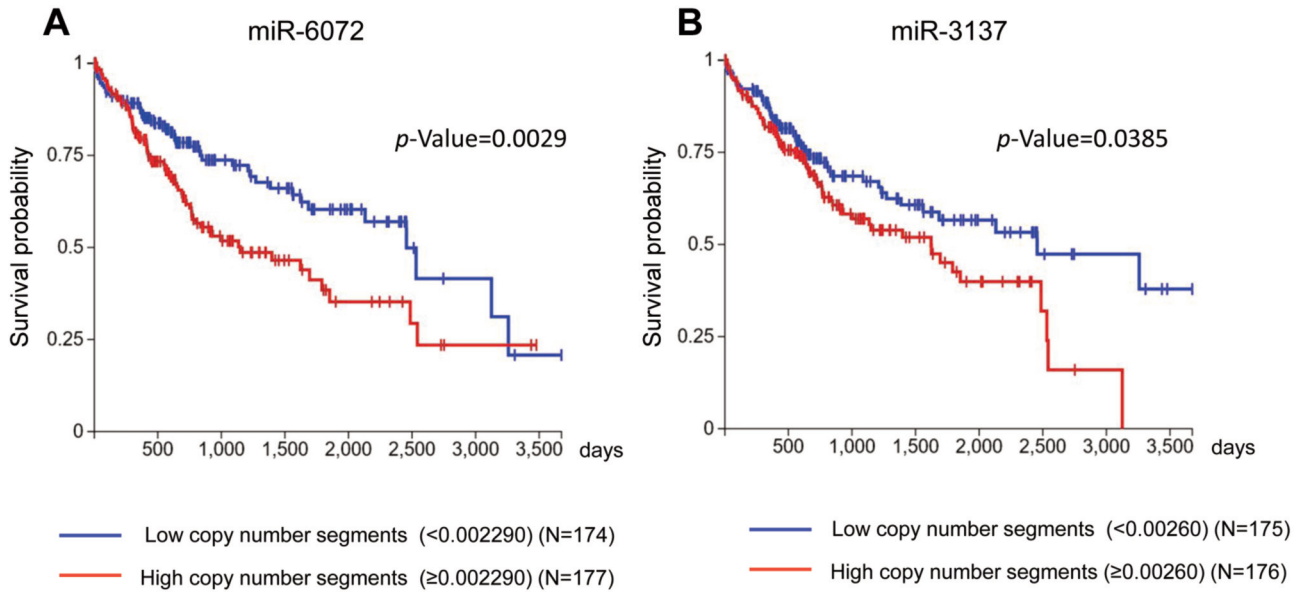

Figure 2. *Association of two HDGF-related microRNAs with the 10-year survival of patients with primary liver cancer. Using the UCSC Xena platform, we found that the copy numbers of two microRNAs in the cancerous tissues were related to the prognosis of the HCC patients. The Kaplan-Meier survival curves of miR-6072 (A) and miR-3137 (B) are shown.*

HepG2 and SK-Hep1 cells shared 30 up-regulated microRNAs and 2 down-regulated microRNAs, suggesting that these microRNAs may be involved in the mechanism underlying cellular proliferation stimulated by HDGF. These 32 micro RNAs were considered potentially involved in HDGF-associated HCC growth (Table I).

We next assessed the association of these 32 candidates with the prognosis of patients with liver cancer. Among the microRNAs, increased copy numbers of miR-6072 and miR-3137 were related to the overall survival of HCC patients, suggesting that increases in these two microRNAs are associated with a poor clinical course in patients with a high HDGF expression (Figure 2).

Discussion

HCC is a major health concern worldwide, and we have previously reported that the HDGF protein as a novel mitogenic factor for HCC. In the present study, we identified 32 microRNAs whose expression was commonly changed in response to HDGF administration in two different hepatoma cell lines (HepG2 and SK-Hep1), suggesting that they may contribute to HDGF-related HCC growth.

MicroRNAs are considered to affect clinical features in various diseases (11, 12), and several of them have been proposed as candidates that are potentially involved in the malignant characteristics caused by the HDGF expression (15-20). However, the reported microRNAs vary between studies, depending on the cell lines used. In order to detect commonly observed changes in hepatoma cells, we used two different cell lines, as we previously confirmed the *in vivo* tumor growth promotion by HDGF in experiments with these cell lines (4). Since we identified 32 microRNAs commonly altered between the different hepatoma-derived cell lines, we hoped that some of these microRNAs would be relevant for the clinical course of HCC patients. Two of these microRNAs were in fact associated with a poor prognosis of HCC patients, suggesting that these commonly increased microRNAs might be involved in HDGF-related cellular growth. Interestingly, to our knowledge, the association of these two microRNA molecules with a malignant disease has not been previously reported, though recent studies showed that some microRNAs and their target genes were suggested to relate to the prognosis of the HCC patients (21-23). In addition, the use of two different cell lines detecting common changes in the expression of these microRNA molecules make are results reproducible. A high HDGF expression is known to be associated with various malignant diseases (24, 25), which highlights the importance of our approach as a research on this topic.

Several limitations associated with the present study warrant mention. First, we selected two microRNAs based on the *in vitro* study; however, the association of the prognosis in the HCC patients was evaluated in a cohort from a public genetic database (14). This evaluation using this type of patient cohort may have helped obtain independent and reliable results (26), however, this meant we were unable to include detailed clinical data in the current

study. Second, our results were obtained using two cell lines and one cohort. A re-evaluation with additional cell lines and a different cohort should be conducted to confirm the results. Third, the function of the determined microRNAs and how these contribute to the poor survival of HCC patients remain unclear and should be explored in a subsequent study.

In conclusion, we identified two microRNAs that were induced by HDGF and were potentially associated with the poor survival of HCC patients.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

HE: Study design, data analysis, funding acquisition and the original draft writing; HN (Hideji Nakamura): data analysis and manuscript writing; HN (Hiroki Nishikawa), TN, YI: performed the experiments and data analysis; SN: study design, funding acquisition and supervising the study. HI: study design, data interpretation, review and editing the manuscript. All Authors read and edited the manuscript and approved the final version of the manuscript.

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References

1 Bruix J, da Fonseca LG and Reig M: Insights into the success and failure of systemic therapy for hepatocellular carcinoma. Nat Rev Gastroenterol Hepatol *16(10)*: 617-630, 2019. PMID: 31371809. DOI: 10.1038/s41575-019-0179-x

2 Llovet JM, Montal R, Sia D and Finn RS: Molecular therapies and precision medicine for hepatocellular carcinoma. Nat Rev Clin Oncol *15(10)*: 599-616, 2018. PMID: 30061739. DOI: 10.1038/s41571-018-0073-4

3 Nakamura H, Izumoto Y, Kambe H, Kuroda T, Mori T, Kawamura K, Yamamoto H and Kishimoto T: Molecular cloning of complementary DNA for a novel human hepatoma-derived growth factor. Its homology with high mobility group-1 protein. J Biol Chem *269(40)*: 25143-25149, 1994. PMID: 7929202.

4 Enomoto H, Nakamura H, Liu W and Nishiguchi S: Hepatoma-derived growth factor: Its possible involvement in the progression of hepatocellular carcinoma. Int J Mol Sci *16(6)*: 14086-14097, 2015. PMID: 26101867. DOI: 10.3390/ijms160614086

5 Enomoto H, Nakamura H, Liu W, Iwata Y, Nishikawa H, Takata R, Yoh K, Hasegawa K, Ishii A, Takashima T, Sakai Y, Aizawa N, Ikeda N, Iijima H and Nishiguchi S: Down-regulation of HDGF inhibits the growth of hepatocellular carcinoma cells *in vitro* and *in vivo*. Anticancer Res *35(12)*: 6475-6479, 2015. PMID: 26637859.

6 Yoshida K, Tomita Y, Okuda Y, Yamamoto S, Enomoto H, Uyama H, Ito H, Hoshida Y, Aozasa K, Nagano H, Sakon M, Kawase I, Monden M and Nakamura H: Hepatoma-derived growth factor is a novel prognostic factor for hepatocellular carcinoma. Ann Surg Oncol *13(2)*: 159-167, 2006. PMID: 16411141. DOI: 10.1245/ASO.2006.11.035

7 Hu TH, Huang CC, Liu LF, Lin PR, Liu SY, Chang HW, Changchien CS, Lee CM, Chuang JH and Tai MH: Expression of hepatoma-derived growth factor in hepatocellular carcinoma. Cancer *98(7)*: 1444-1456, 2003. PMID: 14508832. DOI: 10.1002/cncr.11653

8 Zhou Y, Zhou N, Fang W and Huo J: Overexpressed HDGF as an independent prognostic factor is involved in poor prognosis in Chinese patients with liver cancer. Diagn Pathol *5*: 58, 2010. PMID: 20846397. DOI: 10.1186/1746-1596-5-58

9 Min X, Wen J, Zhao L, Wang K, Li Q, Huang G, Liu J and Zhao X: Role of hepatoma-derived growth factor in promoting *de novo* lipogenesis and tumorigenesis in hepatocellular carcinoma. Mol Oncol *12(9)*: 1480-1497, 2018. PMID: 30004626. DOI: 10.1002/1878-0261.12357

10 Enomoto H, Nakamura H, Nishikawa H, Nishiguchi S and Iijima H: Hepatoma-derived growth factor: an overview and its role as a potential therapeutic target molecule for digestive malignancies. Int J Mol Sci *21(12)*: E4216, 2020. PMID: 32545762. DOI: 10.3390/ijms21124216

11 Kristensen LS, Andersen MS, Stagsted LVW, Ebbesen KK, Hansen TB and Kjems J: The biogenesis, biology and characterization of circular RNAs. Nat Rev Genet *20(11)*: 675-691, 2019. PMID: 31395983. DOI: 10.1038/s41576-019-0158-7

12 Xue M, Zhuo Y and Shan B: MicroRNAs, long noncoding RNAs, and their functions in human disease. Methods Mol Biol *1617*: 1-25, 2017. PMID: 28540673. DOI: 10.1007/978-1-4939-7046-9_1

13 Yamamoto Y, Kondo S, Matsuzaki J, Esaki M, Okusaka T, Shimada K, Murakami Y, Enomoto M, Tamori A, Kato K, Aoki Y, Takizawa S, Sakamoto H, Niida S, Takeshita F and Ochiya T: Highly sensitive circulating microRNA panel for accurate detection of hepatocellular carcinoma in patients with liver disease. Hepatol Commun *4(2)*: 284-297, 2019. DOI: 10.1002/hep4.1451

14 Goldman MJ, Craft B, Hastie M, Repečka K, McDade F, Kamath A, Banerjee A, Luo Y, Rogers D, Brooks AN, Zhu J and Haussler D: Visualizing and interpreting cancer genomics data *via* the Xena platform. Nat Biotechnol, 2020. PMID: 32444850. DOI: 10.1038/s41587-020-0546-8

15 Yin R, Liu J, Zhao D and Wang F: Long non-coding RNA ASB16-AS1 functions as a mir-760 sponge to facilitate the malignant phenotype of osteosarcoma by increasing HDGF expression. Onco Targets Ther *13*: 2261-2274, 2020. PMID: 32214826. DOI: 10.2147/OTT.S240022

16 Zhang Z, Li W, Jiang D, Liu C and Lai Z: MicroRNA-139-5p inhibits cell viability, migration and invasion and suppresses tumor growth by targeting HDGF in non-small cell lung cancer. Oncol Lett *19(3)*: 1806-1814, 2020. PMID: 32194674. DOI: 10.3892/ol.2020.11296

17 Fu Q, Song X, Liu Z, Deng X, Luo R, Ge C, Li R, Li Z, Zhao M, Chen Y, Lin X, Zhang Q and Fang W: miRomics and proteomics reveal a miR-296-3p/PRKCA/FAK/Ras/c-Myc feedback loop modulated by HDGF/DDX5/β-catenin complex in lung adenocarcinoma. Clin Cancer Res *23(20)*: 6336-6350, 2017. PMID: 28751441. DOI: 10.1158/1078-0432.CCR-16-2813

18 Liu X, Ma W, Ma J, Xiao L and Hao D: Upregulation of miR-95-3p inhibits growth of osteosarcoma by targeting HDGF. Pathol Res Pract *215(8)*: 152492, 2019. PMID: 31257090. DOI: 10.1016/j.prp.2019.152492

19 Wei Y, Chen X, Liang C, Ling Y, Yang X, Ye X, Zhang H, Yang P, Cui X, Ren Y, Xin X, Li H, Wang R, Wang W, Jiang F, Liu S, Ding J, Zhang B, Li L and Wang H: A noncoding regulatory RNAs network driven by Circ-CDYL acts specifically in the early stages hepatocellular carcinoma. Hepatology *71(1)*: 130-147, 2020. PMID: 31148183. DOI: 10.1002/hep.30795

20 Kim MK, Moon YA, Song CK, Baskaran R, Bae S and Yang SG: Tumor-suppressing miR-141 gene complex-loaded tissue-adhesive glue for the locoregional treatment of hepatocellular carcinoma. Theranostics *8(14)*: 3891-3901, 2018. PMID: 30083268. DOI: 10.7150/thno.24056

21 Sakabe T, Azumi J, Umekita Y, Toriguchi K, Hatano E, Hirooka and Shiota G: Expression of cancer stem cell-associated DKK1 mRNA serves as prognostic marker for hepatocellular carcinoma. Anticancer Res *37(9)*: 4881-4888, 2017. PMID: 28870909. DOI: 10.21873/anticanres.11897

22 Ashida R, Okamura Y, Ohshima K, Kakuda Y, Uesaka K, Sugiura T, Ito T, Yamamoto Y, Sugino T, Urakami K, Kusuhara M and Yamaguchi K: CYP3A4 gene is a novel biomarker for predicting a poor prognosis in hepatocellular carcinoma. Cancer Genomics Proteomics *14(6)*: 445-453, 2017. PMID: 29109094. DOI: 10.21873/cgp.20054

23 Chen YL, Huang WC, Yao HL, Chen PM, Lin PY, Feng FY and Chu PY: Down-regulation of RASA1 is associated with poor prognosis in human hepatocellular carcinoma. Anticancer Res *37(2)*: 781-785, 2017. PMID: 28179330. DOI: 10.21873/anticanres.11377

24 Bao C, Wang J, Ma W, Wang X and Cheng Y: HDGF: a novel jack-of-all-trades in cancer. Future Oncol *10(16)*: 2675-2685, 2014. PMID: 25236340. DOI: 10.2217/fon.14.194

25 Bao CH, Liu K, Wang XT, Ma W, Wang JB, Wang C, Jia YB, Wang NN, Tan BX, Song QX and Cheng YF: Prognostic role of hepatoma-derived growth factor in solid tumors of Eastern Asia: a systematic review and meta- analysis. Asian Pac J Cancer Prev *16(5)*: 1803-1811, 2015. PMID: 25773828. DOI: 10.7314/apjcp.2015.16.5.1803

26 Lehrer S, Rheinstein PH and Rosenzweig KE: Glioblastoma multiforme: Fewer tumor copy number segments of the SGK1 gene are associated with poorer survival. Cancer Genomics Proteomics *15(4)*: 273-278, 2018. PMID: 29976632. DOI: 10.21873/cgp.20085

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