Abstract. Metformin is a commonly used oral anti-hyperglycemic agent of the biguanide family. Recent studies suggest that metformin may reduce cancer risk and improve prognosis. However, the antitumor mechanism of metformin in several types of cancers, including hepatocellular carcinoma (HCC), has not been elucidated. The goal of the present study was to evaluate the effects of metformin on HCC cell proliferation in vitro and in vivo, and to study microRNAs (miRNAs) associated with the antitumor effect of metformin in vitro. We used the cell lines Alex, HLE and Huh7, and normal hepatocytes to study the effects of metformin on human HCC cells. In an in vivo study, athymic nude mice bearing xenograft tumors were treated with metformin or left untreated. Tumor growth was recorded after 4 weeks, and the expression of cell cycle-related proteins was determined. Metformin inhibited the proliferation of Alex, HLE and Huh7 cells in vitro and in vivo. Metformin blocked the cell cycle in G0/G1 in vitro and in vivo. This blockade was accompanied by a strong decrease of G1 cyclins, especially cyclin D1, cyclin E and cyclin-dependent kinase 4 (Cdk4).

In addition, microRNA (miRNA) expression was markedly altered by the treatment with metformin in vitro and in vivo. In addition, various miRNAs induced by metformin also may contribute to the suppression of tumor growth. Our results demonstrate that metformin inhibits the growth of HCC, possibly by inducing G1 cell cycle arrest through the alteration of microRNAs.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common and frequently occurring tumors worldwide (1). Although the clinical diagnosis and management of early-stage HCC have improved significantly, HCC prognosis is still extremely poor (2). Furthermore, advanced HCC is a highly aggressive tumor with poor or no response to common therapies. Thus, there is a strong demand for new curative approaches to advanced HCC.

Metformin is an oral biguanide drug introduced into clinical practice in the 1950s for the treatment of type 2 diabetes (3). It lowers hyperglycemia by inhibiting hepatic glucose production. According to a recent gastroenterological study, metformin has significant effects on tumorigenesis (4). For instance, it has been reported that patients with type 2 diabetes who are prescribed metformin have a lower risk of pancreatic cancer compared to patients who do not take metformin (5). In basic investigations, metformin was shown to inhibit the proliferation of various human cancer cell types, such as those of the prostate (6), breast (7), colon (8,9), stomach (10), esophagus (11) and HCC (12). However, the mechanism underlying the growth suppression of various cancers, including HCC, by metformin remains relatively unknown.

The relatively recent investigation into miRNAs has identified a new layer of gene regulation mechanisms. It has become apparent that aberrant miRNA expression is a common feature of various human malignancies. In terms of the relationship between miRNAs and HCC, many studies
have shown that specific miRNAs are expressed aberrantly in malignant HCC cells or tissues compared to non-malignant hepatocytes and tissues (13-15). These previous studies have also shown that the modulation of non-coding RNAs, specifically miRNAs, may act to modulate HCC formation. However, although miRNAs are becoming increasingly recognized as regulatory molecules in HCC, their involvement in the responses to environmental changes, such as exposure to drugs, remains unknown.

Here we show that metformin is effective for the treatment of HCC, and have also studied the expression of cell cycle-related molecules, tyrosine kinases of receptor type and angiogenesis molecules to explore the mechanism underlying the antitumor effect of metformin. In addition, we have identified miRNAs associated with the antitumor effect of metformin.

Materials and methods

**Chemicals.** Metformin (1,1-dimethylbiguanide monohydrochloride) was purchased from Dainippon Sumitomo Pharma (Tokyo, Japan). A Cell Counting Kit (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan), and all other chemicals were obtained from Sigma Chemical (Tokyo, Japan).

**Antibodies.** In this study, the following antibodies were used: anti-β-actin monoclonal antibody (Sigma-Aldrich, St. Louis, MO; A5441, used at 1:3,000), cyclin D1 (Thermo Fisher Scientific, Waltham, MA; RB-9041, used at 1:1,000), cyclin E (BD Biosciences, Franklin Lakes, NJ; used at 1:1,000), Cdk6 (Santa Cruz Biotechnology, Santa Cruz, CA; sc-177, used at 1:1,000), Cdk4 (Cell Signaling Technology, Danvers, MA; #2906, used at 1:1,000), Cdk2 (Santa Cruz Biotechnology, sc-163, used at 1:2,000), and secondary horseradish peroxidase-linked anti-mouse and anti-rabbit IgG antibodies (GE Healthcare UK, Buckinghamshire, UK; used at 1:2,000).

**Cell lines and culture.** The human HCC cell lines Alex, HLE, and Huh7 were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Cells of a normal human liver cell line, Hc-Cells, were obtained from DS Pharma Biomedical (Osaka, Japan). The human HCC cells were grown in RPMI-1640 (Gibco Invitrogen, Carlsbad, CA, USA) and human normal liver cells were grown in a CS-C Complete Medium Kit R (DS Pharma Biomedical, Osaka, Japan). Both media contained 10% fetal bovine serum (533-69545; Wako, Osaka, Japan) and penicillin-streptomycin (100 µg/ml; Invitrogen, Tokyo, Japan).

**Cell proliferation assay.** Cell proliferation assays were performed using the Alex, HLE, and Huh7 cells according to the manufacturer’s instructions. Cells from each line (1x10^4) were seeded into a well of a 96-well plate and cultured in 100 µl of RPMI-1640 supplemented with 10% FBS. After 24 h seeding cells were treated by addition of 1 or 5 mM metformin into the culture medium or were left untreated. At the indicated time points, the medium was exchanged for 100 µl of RPMI-1640 with the cell lines reagent (10 µl CKK-8 and 100 µl RPMI-1640), and the cells were incubated for 2 h. The absorbance was measured for each well at a wavelength of 450 nm using an auto-microplate reader.

**Cell lysate and tissue lysate.** The cell lysate assay was performed according to the methods described in our previous reports (16-18). All steps were carried out at 4°C. Protein concentrations were measured using a dye-binding protein assay based on the Bradford method (19).

**Flow cytometry analysis.** To evaluate the mechanism of growth inhibition by metformin, the cell cycle profile was analyzed after treatment with metformin. Huh7 cells (1.0x10^6 cells in a 6-well plate dish) were treated with 10 mM metformin or without metformin for 24-72 h. After treatment, the cells were harvested and fixed in 80% ethanol. The fixed cells were washed with PBS and then stored at -20°C until flow cytometric analysis was performed. On the day of analysis, cells were washed and centrifuged using cold PBS, suspended in 100 µl PBS and 10 µl RNase A solution (250 µg/ml) and incubated for 30 min at 37°C. Then, 110 µl propidium iodide (PI) stain (100 µg/ml) was added to each tube, and the tubes were incubated at 4°C for at least 30 min prior to analysis.

Flow cytometric analysis was performed using a Cytomics FC 500 flow cytometer (Beckman Coulter, Brea, CA) appointed with an argon laser (488 nm). The percentages of cells in different phases of the cell cycle were analyzed by using FlowJo software (Tree Star, Ashland, OR). All experiments were performed in triplicate to assess for consistency of response.

**Xenograft model analysis.** Animal experiments were performed according to the guidelines of the Committee on Experimental Animals of Kagawa University and use of animals by National Cancer Research Institute (NCRI) (21). We purchased 30 male athymic mice (BALB/c-nu/nu; 8 weeks old; 20-25 g) from Japan SLC (Hamamatsu, Japan). The animals were maintained under specific pathogen-free conditions using a laminar airflow rack and had continuous free access to sterilized food (gamma-ray-irradiated food, CL-2; Clea Japan, Tokyo, Japan) and autoclaved water. Each mouse was subcutaneously inoculated with Huh7 cells (5x10^6 cells/animal) in the flank region. After the xenografts were identifiable as a mass of more than 6 mm in maximal diameter in all recipients, the animals were randomly assigned to three groups. The metformin-treated group was injected 5 times a week intraperitoneally (i.p.) at 1 mg/body or 2 mg/body per day for 2 weeks. The control group was administered only PBS (n=10) for 2 weeks. After the initiation of the metformin administration, the tumor growth was monitored by the same investigators (H.M. and T.M.), and the tumorigenesis of HCC was monitored every
day. Tumor size was measured weekly by measuring the two greatest perpendicular tumor dimensions. To examine the significance of the differences between growth curves in this study, all the measurements of tumor volume for each growth curve from the start of the treatment to the end, typically about 30 observations, were analyzed by one-way analysis of variance (ANOVA). The tumor volume was calculated as follows: tumor volume (mm$^3$) = [tumor length (mm) x tumor width (mm)$^2$]/2 (22). All animals were sacrificed on day 14 after treatment. All animals were alive during the observation.

**Antibody arrays of phosphorylated receptor tyrosine kinase (p-RTK).** The RayBio™ Human Phospho Array Kit (cat. no. ARY 001) was purchased from RayBiotech Inc. (Norcross, GA). The assay for the p-RTK array was performed according to the manufacturer's instructions. Briefly, p-RTK array membranes were blocked with 5% BSA/TBS (0.01 M Tris-HCl, pH 7.6) for 1 h. The membranes were then incubated with 2 ml of lysate prepared from cell lines or tumorous tissues after normalization with equal amounts of protein. After extensive washing with TBS including 0.1% v/v Tween-20 (3 washings of 10 min each), and TBS alone (2 washings of 10 min each) to remove unbound materials, the membranes were incubated with anti-phospho-tyrosine-HRP antibody for 2 h at room temperature. The unbound HRP antibody was washed out with TBS including 0.1% Tween-20. Finally, each array membrane was exposed to X-ray film using a chemiluminescence detection system (Perkin-Elmer Co.).

**Antibody array for angiogenesis.** To assess whether molecules important in angiogenesis are regulated by the antitumor effect of metformin, the RayBio Human Angiogenesis Antibody Array (RayBiotech Inc.) was used according to the protocol. This method is a dot-blot-based assay which enables detection and comparison of 19 different angiogenesis-specific cytokines. Briefly, angiogenesis protein array membranes were blocked with 5% BSA/TBS (0.01 M Tris-HCl, pH 7.6) for 1 h. The membranes were then incubated with 2 ml of lysate prepared from tumor tissues after normalization with equal amounts of protein. After extensive washing with TBS including 0.1% v/v Tween-20 (3 washings of 5 min each) to remove unbound materials, the membranes were incubated with anti-phospho-tyrosine-HRP antibody for 2 h at room temperature. Unbound HRP antibody was washed out with TBS including 0.1% Tween-20. Finally, each array membrane was exposed to X-ray film using a chemiluminescence detection system (Perkin-Elmer Co.).

**Densitometric analysis.** The densitometric densities of p-EGFR and p-IGF-1R in the cell lines and p-EGFR, Mer and angiogenin in tumorous tissue were visualized in black and white, respectively. The densities of p-EGFR, p-IGF-1R and Mer obtained from the membrane array were analyzed by means of a Kodak Image Station (Kodak, Tokyo, Japan).

**Analysis of miRNA microarray.** The samples of cancer cell lines were processed for total RNA extraction with an miRNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA samples typically showed A260/280 ratios of between 1.9 and 2.1, as determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). After RNA measurement with an RNA 6000 Nano kit (Agilent Technologies), the samples were labeled using a miCURY Hy3/Hy5 Power labeling kit and were hybridized on a human miRNA Oligo chip (v.14.0; Toray Industries, Tokyo, Japan). Scanning was performed with the 3D-Gene Scanner 3000 (Toray Industries). 3D-Gene extraction version 1.2 software (Toray Industries) was used to read the raw intensity of the image. To determine the change in miRNA expression between metformin-treated and control samples, the raw data were analyzed via GeneSpringGX v 10.0 (Agilent Technologies). Samples were first normalized relative to 28sRNA and baseline corrected to the median of all samples. Replicate data were consolidated into two groups: those from metformin-treated animals and those from control animals, and were organized by using the hierarchical clustering and analysis of variance (ANOVA) functions in the GeneSpring software. Hierarchical clustering was done by using the clustering function (condition tree) and Euclidean correlation as a distance metric. Two-way ANOVA analysis and asymptotic p-value computation without any error correction on the samples were performed to search for the miRNAs that varied most prominently across the different groups. The p-value cutoff was set to 0.05. Only changes >50% on at least one of the time points for each sample were considered significant. All the analyzed data were scaled by global normalization. The statistical significance of differentially expressed miRNAs was analyzed by Student's t-test. All our microrarray data in this study were submitted as a complete data set to the NCBI Gene Expression Omnibus (GEO), no.44204 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=lgzlzioeioqijc&acc=GSE44204).

**Results**

**Metformin inhibits the proliferation of human HCC cell lines.** To evaluate the effect of the cell growth of metformin on human HCC cells in vitro, we examined metformin's effect on proliferation in three HCC cell lines, namely, Alex, HLE and Huh7. Cells were grown in 10% fetal bovine serum (FBS) and treated with 1 or 5 mM metformin, or without metformin as a control. To discern the direct relationship between the decrease in cell viability and the inhibition of cell proliferation, we followed the course of proliferation over 3 days after the addition of metformin. Metformin (0, 1 and 5 mM) led to a decrease in cell proliferation in a dose- and time-dependent manner in all three cell lines (Fig. 1), with the reduction being especially prominent in Huh7. On the other hand, metformin did not suppress the proliferation of normal hepatocytes. Thus the results showed that metformin inhibited the proliferation of HCC cells, but not the proliferation of normal hepatocytes (Hc cells).

**Effects of metformin on cell cycle regulatory proteins in Huh7.** To study whether or not metformin affects the cell cycle in Huh7 cells, we examined the expression of various cell cycle-related molecules in Huh7 cells with and without metformin treatment using western blot analysis. The samples
were cells treated with 10 mM metformin or left untreated for 24-72 h. The most remarkable changes were the loss of cyclin D1, Cdk4 and cyclin E, a key protein implicated in the transition of the G0/G1 phase. In short, the cyclin D1, Cdk4 and cyclin E levels had already declined rapidly at 24 h after the addition of metformin, and were no longer detectable at 24 h (Fig. 2A).

As shown in Fig. 2A, we also studied the expression of two other cell cycle-related proteins, Cdk6 and Cdk2, implicated in the G0/G1 transition. The results showed that the catalytic subunit of cyclin E, Cdk2 and the catalytic subunit of cyclin D1, Cdk6 were almost unchanged at all time points after the addition of metformin (Fig. 2A). Similar things occurred in other cancer cell lines, such as Alex and HLE (data not shown). As an internal control, the amount of β-actin was also measured and was almost the same in each sample (Fig. 2A).

Next, to further investigate the inhibition of Huh7 cell proliferation in the presence of metformin, the cell cycle progression was examined using flow cytometry. We treated proliferating Huh7 cells with 10 mM metformin for different durations. After the addition of 10 mM metformin, an increasing number of cells started to accumulate in G0/G1, 60.7% after 24 h, 59.7% after 48 h and 67.7% by 72 h (Fig. 2B). In parallel, after the addition of 10 mM metformin, we observed reductions in the percentage of cells in the
S phase and G2/M phase. These data suggest that metformin inhibits the cell cycle progression from G0/G1 into S phase, resulting in G1 cell cycle arrest.

Metformin inhibits tumor proliferation in vivo. In order to determine whether metformin could affect tumor growth in vivo, metformin was injected daily intraperitoneally (i.p.) at 1 or 2 mg per day in xenografted mice. Animals in the control group received i.p. administration of the vehicle (PBS). As shown in Fig. 3A and B, animals in the control group developed rapidly-growing subcutaneous HCC. On the basis of the integrated values of the tumor growth curves, i.p. administration of metformin led to a substantial inhibition of tumor growth, by 25% (1 mg/day) and 52% (2 mg/day) (Fig. 3A and B). These growth rates were significantly above those of the control (both p<0.01, respectively). In addition, 2 mg metformin treatment significantly inhibited tumor growth compared with that in 1 mg metformin-treated mice (p<0.05). All animals remained alive throughout the experiment, and metformin exhibited no apparent changes in mice and did not affect their weight (data not shown).

To determine whether metformin also affected cell cycle-regulatory protein levels in vivo, we performed a western blot analysis in tumorous tissues obtained from the xenograft experiments. Metformin (1-2 mg dose) reduced the levels of cell cycle-related molecules (cyclin D1, Cdk4, cyclin E) in treated tumors compared with controls (Fig. 3C). However, the expressions of Cdk2 and Cdk6 in tumorous tissue remained unchanged. These data were similar to the results of the in vitro observation (Fig. 2A). In summary,
in the in vivo study, as in the in vitro study, metformin decreased tumor growth by reducing cell cycle-regulatory protein levels, especially in cyclin D1, Cdk4 and cyclin E, resulting in G1 cell cycle arrest.

Differences in phosphorylated-receptor tyrosine kinases (p-RTKs) between metformin-treated and control samples in vitro and in vivo. We used a phosphorylated-RTK array system to identify the key RTKs associated with the antitumor effect of metformin. By using an antibody array (Fig. 4A), we simultaneously screened the expressions of 42 different activated RTKs in Huh7 cells and tumorous tissues with or without metformin. In the in vitro study, the expressions of phosphorylated-epidermal growth factor (p-EGFR) and phosphorylated-insulin-like growth factor-1 receptor (p-IGF-1R) in Huh7 cells were reduced by treatment with metformin (Fig. 4B), while in the in vivo study, the levels of p-EGFR and Mer in tumorous tissues (Fig. 4C) were reduced by the treatment with metformin. The densitometric ratios of the p-EGFR and p-IGF-1R spots of the metformin-treated cell lines to the non-treated cell lines were 51 and 12%, respectively (Fig. 4D). In addition, the ratios of p-EGFR and Mer of the metformin-treated tumorous tissue to the non-treated tissue were 67 and 55%, respectively (Fig. 4E).

Differences in angiogenesis in vitro and in vivo with and without metformin. We used an angiogenesis array system to identify the key angiogenesis-related molecules associated with the antitumor effect of metformin. The antibody array (Fig. 5A) allowed us to simultaneously screen the expressions of 20 different angiogenesis molecules in Huh7 cells (Fig. 5B) and tumor tissues (Fig. 5C) with or without metformin.
Among the 20 angiogenesis molecules screened, angiogenin was reduced in in vitro and in vivo study with metformin treatment. The densitometric densities on angiogenin in cell line and tumorous tissue were visualized in black and white, respectively. The densitometric ratios of the angiogenin spots from metformin-treated cell line and tumorous tissues to those of cell line and tumorous tissues not treated with metformin were 51% (Fig. 5D) and 54% (Fig. 5E).

Differences in miRNA expression between the metformin-treated and control cell lines. Using a custom microarray platform, we analyzed the expression levels of 985 human miRNA probes in the cell lines treated with metformin and the control cell line. As shown in Table I, when the expression of miRNAs was studied in Huh7 cells treated with 10 mM metformin and without metformin in vitro, 33 of 985 miRNAs were significantly upregulated in Huh7 cells.
at 72 h after metformin treatment (Table I), while 18 of 985 miRNAs downregulated (Table II).

Unsupervised hierarchical clustering analysis, using Pearson's correlation, showed that cell lines treated with metformin clustered together and separately from the untreated cell lines (Fig. 6). These subsets of 119 microRNAs in cell lines exhibited >1.5-fold alterations in expression levels between the metformin-treated and control groups.

**Discussion**

HCC is the sixth most common malignancy and the third most common cause of cancer-related death worldwide (1). Conventional chemotherapy does not provide significant clinical benefits or prolonged survival for patients with advanced HCC (23). Therefore, it is necessary to identify new therapies for the treatment of HCC.

The role of the anti-diabetic drug metformin in glucose and fatty acid metabolism is very well known (20,22). Recent data suggest that metformin was shown to protect from cancer and inhibit the proliferation of various human cancer cell types, such as those of the prostate (6), breast (7), colon (8,9), stomach (10), and esophagus (11) and HCC (12). However, the antitumor mechanism of metformin in various cancers, including hepatocellular carcinoma (HCC), has not been fully clarified. Here we show that metformin inhibits the growth of HCC cells and tumorigenesis in vitro and in vivo. In addition, we have identified miRNAs, receptor-type tyrosine kinase...
and angiogenesis molecules associated with the antitumor effect of metformin in HCC.

In the present study, metformin led to a strong, dose-dependent inhibition of cell proliferation in all HCC cell lines. However, to our surprise, metformin did not inhibit the proliferation of normal hepatocytes. These data suggest that metformin exhibits an anti-proliferation effect on HCC cells, but does not have an anti-proliferation effect on normal hepatocytes. These results suggest that metformin would be very effective for HCC treatment, since it specifically suppressed the proliferation in cancer cells, but not in normal cells.

Table I. Statistical results and chromosomal of miRNAs upregulated in Huh7 cells treated with metformin.

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Fold (treated/non-treated) mean ± SD</th>
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<th>Chromosomal localization</th>
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and angiogenesis molecules associated with the antitumor effect of metformin in HCC.

In previous reports, downregulation of cyclin D1 in response to metformin has been demonstrated in various cancer cell lines (8,9). We have also reported that cyclin D1 was reduced in gastric cancer cells treated with metformin in vitro. In the present study, we have also shown that the major cell cycle regulators (cyclin D1, Cdk4 and cyclin E) could be intracellular targets of the metformin-mediated anti-proliferative effect in HCC. To be sure, flow cytometry also revealed that metformin arrested HCC cells at the G0/G1 phase in vitro.

An in vivo experiment using subcutaneous HCC-bearing athymic nude mice also demonstrated that metformin markedly suppressed the growth of HCC, and the expression levels of numerous cell cycle-related molecules (cyclin D1, Cdk4, cyclin E) were found to be reduced by treatment with metformin, indicating that metformin may inhibit the expression of cell cycle-related molecules. These data suggest that the antitumor effect of metformin may be related to the role of G1 arrest of HCC cells.

Our in vitro study was performed using a higher dose of metformin than the human therapeutic concentration (6 to 30 µM). This has been a previously raised criticism of similar work in other cancer cell types, such as the prostate (6), breast (7), colon (8,9), stomach (10), and esophagus (11) and HCC (12). However, it is important to consider that cells in culture are grown under hyperglycemic conditions (24). Tissue culture media alone contains high concentrations of glucose, and 5-10% fetal bovine serum is typically added, resulting in excessive growth stimulation. This may explain why the doses of metformin required to realize the antitumor effect of metformin in cancer cells.
In regard to p-EGFR and p-IGF-1R, some upregulated by many proteins involved in cell cycle progression. EGFR and IGF-R1 are expressed in HCC. In addition, the pathway of EGFR and IGF-1R plays an important role in controlling cell cycle events. EGFR and IGF-1R are involved in the development of various cancers, including HCC. The let-7 family acts as a tumor suppressor by binding its target oncogenes, including Ras (38), HMGA2 (39) and c-Myc (40). Therefore, the downregulated let-7 may reflect the general cellular responses to death-inducing stimuli. However, additional detailed studies will be needed to assess whether or not each miRNA reflects a cause or a consequence of exposure to metformin treatment.

In conclusion, our results revealed that metformin inhibits human HCC cell proliferation and tumor growth, possibly by suppressing the cell cycle-related molecules via alteration of miRNAs. Metformin is a drug widely used for the treatment of type 2 diabetes with limited side-effects. Therefore, metformin may become a novel and effective therapy for the treatment and long-term management of HCC, providing additional benefits at low cost.
The antidiabetic drug Let-7 regulates self renewal and Reduced Antitumor effect of Increased expression of 19.