

Niclosamide suppresses migration of hepatocellular carcinoma cells and downregulates matrix metalloproteinase-9 expression

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Abstract. Metastasis negatively affects the prognosis of hepatocellular carcinoma (HCC). In the present study, niclosamide, which is known to suppress the proliferation of HCC cells, was investigated for possible suppressant effects on the migration of HCC cells. HLF and PLC/PRF/5 HCC cells were cultured in the presence of niclosamide. Cell proliferation was analyzed using the MTS assay. Cell migration was measured by performing a scratch assay. Expression levels of cyclin D1 and matrix metalloproteinase 9 (MMP9) were analyzed by performing reverse transcription-quantitative polymerase chain reaction. Compared with the control treatment, treatment with 10 μ M niclosamide suppressed the proliferation of the HLF and PRL/PRF/5 cells to 49.9 ± 3.7 and $17.9 \pm 11.5\%$ ($P < 0.05$), respectively. Furthermore, compared with the control treatment, treatment with 1.0 μ M niclosamide downregulated the expression of cyclin D1 to 52.4 ± 4.4 and $23.9 \pm 5.4\%$ ($P < 0.05$) in the HLF and PRL/PRF/5 cells, respectively. In the scratch assay, treatment of the HLF cells with niclosamide (1.0 μ M) decreased the distance of the scratched line from the growing edge to 4.6 ± 1.0 mm compared with the 9.2 ± 1.4 mm observed with the control treatment ($P < 0.05$). Similarly, treatment of the PRL/PRF/5 cells with niclosamide (1.0 μ M) also decreased the distance of the scratched line from the growing edge to 3.0 ± 0.8 mm compared with the 5.5 ± 0.9 mm observed with the control treatment ($P < 0.05$). Further, MMP9 expression levels in the HLF cells treated with 1.0 μ M niclosamide decreased to $22.4 \pm 1.76\%$ ($P < 0.05$) compared with those in the untreated control HLF cells. Similarly, expression level of MMP9 in the PRL/PRF/5 cells treated with 1.0 μ M niclosamide decreased to $18.7 \pm 10.7\%$ ($P < 0.05$) compared with those in the untreated control PRL/

PRF/5 cells. Overall, niclosamide downregulated the expression of MMP9 in and suppressed the migration of HCC cells.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies and its prognosis is poor with metastasis (1,2). HCC cells migrate at an early stage (3). One of the problems associated with HCC is that its cells migrate into the surrounding fibrous tissues and proliferate (4). *In vitro*, HCC cells can proliferate without nutrients or growth factors such as fetal bovine serum (FBS) (5). It is important to detect molecular events at an early stage of HCC cell migration to improve HCC treatment. Matrix metalloproteinase 9 (MMP9) expression level is upregulated 20-fold after co-culture of HCC cells and parenchymal cells (6).

MMP9 is a gelatinase involved in cancer metastasis (7). The overexpression of MMP9 is associated with the poor prognosis of cancer (8). Research focused on elucidating the mechanisms involved in the upregulation of MMP9 expression is therefore important. Polymorphism of the promoter region of MMP9 (-1562)C/T increases the risk of metastasis (9). MMP9 is upregulated in the presence of inflammatory cytokines such as interleukins 8 and 17 (10). However, details of the mechanisms involved in the upregulation of MMP9 expression in HCC are not clear.

The Wnt signaling pathway, which is activated when Wnt ligands bind their Frizzled (Fz) receptors, has been implicated in HCC (11). The small interfering (si)RNA of Fz9 suppresses the proliferation and migration of HCC cells (12). Studies suggest that the Wnt pathway is specifically involved in the migration of HCC cells, and it is expected that inhibition of this pathway may improve the survival rate of HCC patients by suppressing the proliferation and migration of HCC cells. One limitation of siRNA, however, is that its antitumor effect depends on its transfection efficiency (13).

Niclosamide was originally developed for the treatment of tapeworm infection and is clinically used worldwide (14,15). Sack *et al* first suggested that niclosamide may be a promising candidate for the treatment of colorectal cancer through the inhibition of HCC cell proliferation (16) via the modulation of the Wnt pathway (17). The mechanisms proposed for the Wnt pathway-mediated inhibitory effects of

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niclosamide include internalization of Fz1 (18) and inhibition of the canonical Wnt signaling pathway (19). Niclosamide also downregulates the expression of cyclin D1, one of the target molecules of the Wnt pathway, via glycogen synthase kinase-3 β (17,20). Thus far, no study has reported the effects of niclosamide on the migration of HCC cells or on its regulation of MMP9 expression.

The present study was therefore conducted to investigate the effects of niclosamide on the migration of HCC cells and the expression of MMP9.

Materials and methods

Cell culture. HCC HLF and PLC/PRL/5 cell lines were purchased from Riken Cell Bank (Tsukuba, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (Life Technologies, Grand Island, NY, USA) and incubated in a humidified chamber at 37°C in 5% carbon dioxide.

Scratch assay. The cells were plated on 4-well chamber slides (BD Biosciences, Franklin Lakes, NJ, USA) and allowed to yield a confluent monolayer sheet of cells, which were then scratched with 200- μ l pipettes, incubated for 48 h and stained with hematoxylin and eosin. The stained slides were observed under an AX80 microscope (Olympus, Tokyo, Japan). The distance from the location of the original scratched line to the new growing edge of the cells was measured at five different points. The migration distance for each plate was calculated as the mean average of these five measurements.

Cell proliferation analysis. The HLF and PRL/PRF/5 cells were trypsinized, harvested, seeded onto 96-well flat-bottomed plates (Asahi Techno Glass, Tokyo, Japan) at a density of 1,000 cells per well and then incubated for 24 h in DMEM supplemented with 10% FBS. Subsequent to culturing, the cells were treated with niclosamide (Sigma-Aldrich) at various concentrations (0, 0.01, 0.03, 0.1, 0.3, 1, 3 and 10 μ M) for 72 h and subjected to MTS assay (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. MTS is bio-reduced by cells into a colored formazan product that can be detected at a specific wavelength. The absorbance of each reaction plate was measured at a wavelength of 490 nm using an iMark Microplate Absorbance Reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The cells were cultured in 6-well plates (Asahi Glass Co., Ltd., Tokyo, Japan) and treated with niclosamide for 48 h, after which total RNA (5 μ g) was isolated using Isogen (Nippon Gene Co., Ltd., Tokyo, Japan). The RNA was converted reverse transcribed into cDNA using the Super Script III and oligo(dT) primers (Life Technologies Ltd., Carlsbad, CA, USA) according to the manufacturer's instructions. The PCR primers were as follows: Ribosomal protein L19 (RPL19; BC095445), forward 5'-CGAATGCCAGAGAAGGTCAC-3' and reverse 5'-CCATGAGAATCCGCTTGTTT-3' (157 bp); cyclin D1 (NM_053056), forward 5'-AGAGCGGAGGAGACA AACAG-3' and reverse 5'-AGGCGGTAGTAGGACAG

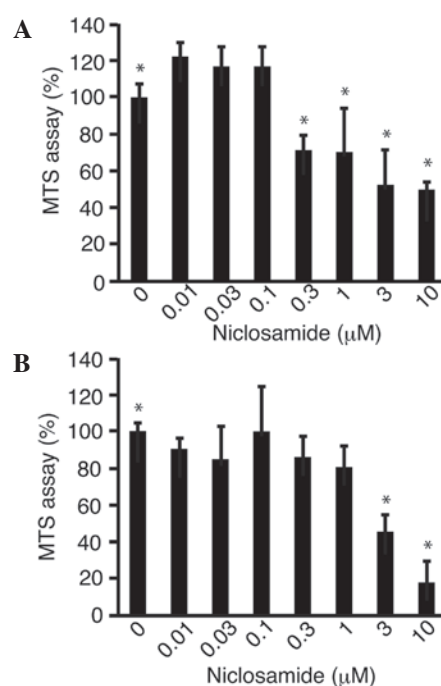


Figure 1. Cell proliferation assay. (A) HLF cells or (B) PLC/PRL/5 cells were cultured with niclosamide and subjected to MTS assays. The error bars represent the standard deviation (n=3). *P<0.05 vs. 0 μ M.

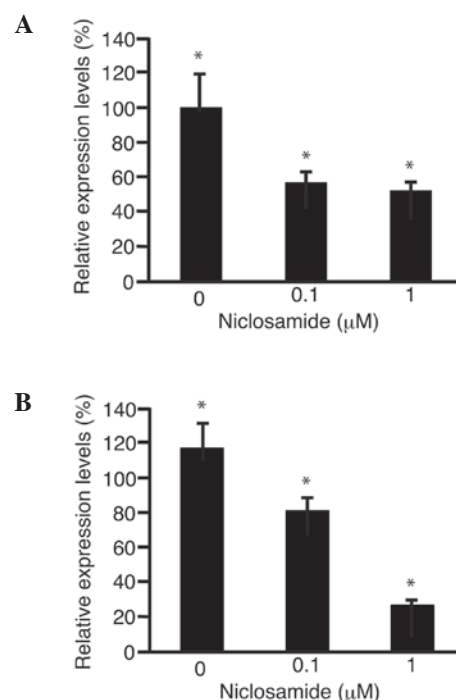


Figure 2. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) of cyclin D1. (A) HLF cells or (B) PLC/PRL/5 cells were cultured with niclosamide, and subjected to qRT-PCR for detection of cyclin D1 expression. The error bars represent the standard deviation (n=3). *P<0.05 vs. 0 μ M.

GAAGTTG-3' (180 bp); and MMP-9 (NM_004994) forward 5'-CCTGGGCAGATTCCAAACCT-3' and reverse 5'-GCAAGTCTTCCGAGTAGTTTGGAT-3' (89 bp). RT-qPCR was performed using Fast SYBR Green Master Mix (Life Technologies Ltd.) for 40 cycles, using 5 sec for denaturation at 95°C and 5 sec for annealing-extension at 60°C with

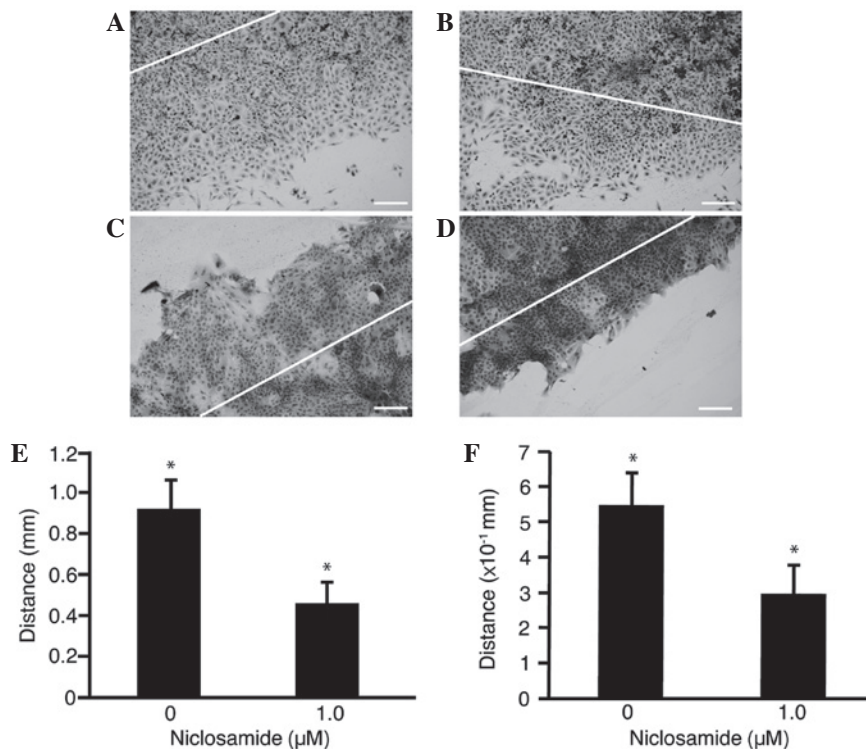


Figure 3. Scratch assay. (A and B) HLF or (C and D) PLC/PRL/5 cells were cultured on chamber slides. Upon attaining confluence, the cells were scratched with the tip of a 200-μl pipette tip and treated (B and D) with 1.0 μM niclosamide, or (A and C) without niclosamide. The white line represents the location of the original leading edge of the scratch. The distance from the line to the new growing edge of the cells was measured in the (E) HLF and (F) PLC/PRL/5 cells. Original magnification, x100; scale bar, 200 μm; error bar, standard deviation; n=3; *P<0.05 vs. 0 μM.

the Mini Opticon System (Bio Rad, Hercules, CA). RPL19 was used as an internal control.

Statistical analysis. One-factor analysis of variance was performed on the data from the cell proliferation studies, RT-qPCR and scratch assays using JMP10.0.2 software (SAS Institute, Cary, NC, USA). P<0.05 was used to indicate a statistically significant difference.

Results

The effects of niclosamide on the proliferation of the HLF (Fig. 1A) and PRL/PRF/5 (Fig. 1B) cells were examined using the MTS assay. The proliferation of the HLF and PRL/PRF/5 cells treated with niclosamide (10 μm) was significantly (P<0.05) suppressed to 49.9±3.7 and 17.9±11.5%, respectively, compared with that of the untreated control cells.

Cyclin D1 is a downstream molecule associated with the Wnt pathway and involved in cell proliferation (21). The HLF and PRL/PRF/5 cells were cultured with niclosamide. The expression levels of cyclin D1 in the HLF (Fig. 2A) and PRL/PRF/5 (Fig. 2B) cells cultured in the presence of niclosamide were analyzed using RT-qPCR. The results showed that cyclin D1 expression was significantly (P<0.05) downregulated to 52.4±4.4 and 23.9±5.4% in the HLF and PRL/PRF/5 cells, respectively, compared with the levels in the untreated control cells.

Change in cell motility was also investigated in the HLF (Fig. 3A and B) and PRL/PRF/5 (Fig. 3C and D) cells treated with niclosamide (1.0 μm). A reduction in the distance of the

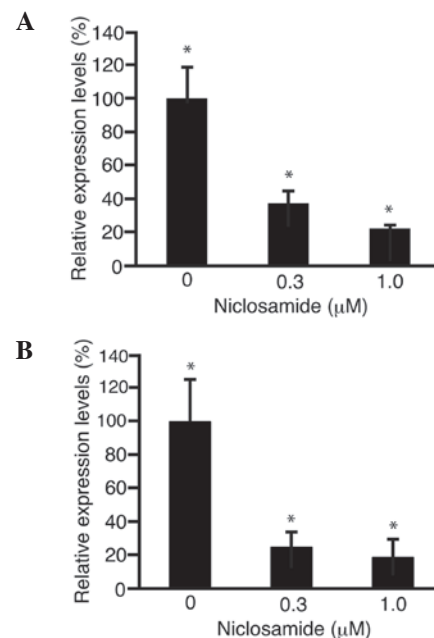


Figure 4. Real-time quantitative polymerase chain reaction (qRT-PCR) of matrix metalloproteinase 9. HLF cells (A) and PLC/PRL/5 cells (B) were cultured with niclosamide and subjected to qRT-PCR with matrix metalloproteinase 9. Error bar: standard deviation, *P<0.05 vs. 0 μM, n=3.

scratched line from the growing edge was observed in the treated cells (Fig. 3B and D) compared with the untreated control cells (Fig. 3A and C). The HLF (Fig. 3E) and PRL/PRF/5 (Fig. 3F) cells treated with niclosamide showed significant (P<0.05) decreases in cell migration (4.6±1.0 and

3.0±0.8 mm, respectively) compared with that of the untreated control HLF and PRL/PRF/5 cells (9.2±1.4 and 5.5±0.9 mm, respectively).

MMP9 is involved in cell motility, and as expected, its expression level in the HLF (Fig. 4A) and PRL/PRF/5 (Fig. 4B) cells treated with niclosamide (1.0 µm) was significantly ($P<0.05$) decreased to 22.4±1.76% and 18.7±10.7% compared with that in the untreated cells.

Discussion

Niclosamide is currently used to treat tapeworm infection (15). Application of niclosamide in the treatment of cancer was first reported for colorectal cancer (16). Niclosamide suppresses the proliferation of HCC cells by inhibiting the Wnt signaling pathway (17).

Niclosamide has been previously shown to suppress the migration of breast cancer cells and colon cancer cells (16,22). The present results revealed a similar suppression of HCC cell migration by niclosamide, suggesting that it may suppress the migration of cancer cells. These results are promising and may be clinically significant, as niclosamide could improve the prognosis of cancer patients by suppressing metastasis. While the mechanism by which niclosamide suppresses the migration of cancer cells was not completely clear, the present study clearly showed that MMP9 expression was downregulated by niclosamide, thereby strongly suggesting its involvement in the suppression of cancer cell migration. Previous studies and the present study have indicated that niclosamide is promising for the treatment of HCC (23).

The present data clearly showed that niclosamide suppressed the migration of HCC cells, and that MMP9 decreased with niclosamide treatment. This suggests that niclosamide suppresses migration via inhibiting the expression of MMP9 (7). However, the mechanism underlying decreased MMP9 expression remains unclear. Our previous study revealed that niclosamide suppresses the Wnt pathway (17). Therefore, it is suggested that the expression of MMP9 decreases with suppression of the Wnt pathway, and that MMP9 may be a downstream molecule of the Wnt pathway.

In conclusion, niclosamide significantly suppressed the migration of the HCC cells, probably by mechanisms involving the downregulation of MMP9 expression. Future studies in line with these results could involve investigation of the promoter activity of MMP9, which would further elucidate the effects of niclosamide at a transcriptional level.

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