Diet-induced macrophage inhibitory cytokine 1 promotes prostate cancer progression

Mingguo Huang^{1,2,4}, Shintaro Narita^{1,4}, Takamitsu Inoue¹, Norihiko Tsuchiya^{1,4}, Shigeru Satoh¹, Hiroshi Nanjo³, Takehiko Sasaki^{2,4} and Tomonori Habuchi^{1,4}

¹Department of Urology ²Research Center for Biosignal ³Department of Clinical Pathology, Akita University Graduate School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan

⁴CREST, Japan Science and Technology Agency (JST), Tokyo 102-0076, Japan

Correspondence should be addressed to M Huang
Email
huangmg@gipc.akita-u.ac.jp

Abstract

Recent studies have indicated that a high-fat diet (HFD) plays an important role in prostate cancer (PCa) progression. Palmitic acid (PA) is one of the most abundant saturated free fatty acids (FAs) and is associated with carcinogenesis. In this study, we investigated the mechanism underlying the association of dietary fat, including PA, with PCa progression. In four PCa cell lines, *in vitro* PA administration stimulated the expression of macrophage inhibitory cytokine 1 (MIC1), which is a divergent member of the transforming growth factor- β family. *In vivo*, LNCaP xenograft tumor growth, serum MIC1 levels, and FA levels in xenograft tumors were significantly higher in mice receiving an HFD containing high amounts of PA than in those receiving a low-fat diet (LFD). In addition, tumor cells with high MIC1 expression invaded to venules and lymph vessels in the LNCaP xenograft. *In vitro* studies showed that proliferation and invasive capacity were significantly higher in PCa cells cultured with serum from HFD-fed mice than in those cultured with the serum from LFD-fed mice. This effect was attenuated by the addition of neutralizing antibodies against MIC1, but not by isotype control antibodies. Clinically, serum MIC1 levels were significantly higher in PCa patients than in healthy controls, and higher levels were associated with higher pathological grade and obesity. In conclusion, our results indicate that an HFD containing PA may promote growth and invasiveness of PCa cells through the upregulation of MIC1 expression.

Key Words

- ▶ prostate cancer
- ▶ high-fat diet
- ▶ palmitic acid
- ▶ macrophage inhibitory cytokine 1

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Introduction

Prostate cancer (PCa) is the second leading cause of cancer-related death in the USA (Hsing & Devesa 2001), and epidemiological studies suggest that obesity and/or a high-fat diet (HFD) can increase the incidence of PCa (Blair & Fraumeni 1978, Andersson *et al.* 1997). The incidence of PCa is increasing rapidly in Japan, presumably because of increasingly westernized lifestyles, although it is still much lower than that in western nations (Tominaga 1985, Namiki *et al.* 2010). Furthermore, Japanese migrants in the USA

have a markedly higher risk for PCa, suggesting that the development of PCa may be influenced by environmental factors, including diet (Shimizu *et al.* 1991). An HFD is believed to affect gene expression, cellular activity, and other important changes in circulating biological factors related to prostate carcinogenesis and progression (Narita *et al.* 2008, Huang *et al.* 2011, 2012). Recent large population-based studies have indicated that saturated fatty acids (SFAs), especially palmitic acid (PA), are

significantly associated with higher incidence of PCA (Giovannucci *et al.* 1993, Kurahashi *et al.* 2008). In addition, high SFA consumption has been associated with biochemical recurrence as defined by increasing prostate-specific antigen (PSA) levels in patients treated with prostatectomy for PCA (Strom *et al.* 2008).

PA, one of the most abundant SFAs, is a major source of energy and is an important component of the lipids that comprise the cellular membrane. PA can be obtained from dietary fat or synthesized *de novo* by FA synthase (Flavin *et al.* 2011). It has also been reported that PA activates inflammatory pathways and induces the production of many cancer-related cytokines, such as interleukin 1 (IL1), IL6, and tumor necrosis factor- α (TNF- α) in both humans and in mice (Haversen *et al.* 2009, Wen *et al.* 2011). In addition, a previous study suggested that PA was associated with the progression of breast cancer cells through the increased expression of macrophage inhibitory cytokine 1 (MIC1; Kim *et al.* 2008a). However, there is little evidence for the mechanism by which an HFD and/or PA induce cancer progression, especially that of PCA.

MIC1 is a divergent member of the transforming growth factor- β (TGFB) family and is also known as placental bone morphogenetic protein (PLAB), prostate-derived factor, and growth/differentiation factor 15 (Bootcov *et al.* 1997, Paralkar *et al.* 1998). MIC1 was first identified in monocytes and is also expressed in lung, liver, pancreas, kidney, prostate glands, and adipose tissue (Hromas *et al.* 1997). MIC1 is synthesized by proteolytic cleavage of an N-terminal propeptide, generating a dimeric or monomeric bioactive molecule that is secreted into the extracellular medium (Bootcov *et al.* 1997, Bauskin *et al.* 2005). Serum MIC1 levels are elevated in many patients with metastatic cancer, including that of the breast, colon, pancreas, and prostate (Brown *et al.* 2003, 2006, Koopmann *et al.* 2004, Wollmann *et al.* 2005).

In this study, we first examined the effects of PA on PCA cells. Because MIC1 was found to be elevated by PA administration, we then analyzed the role of MIC1 in HFD-associated PCA progression using *in vitro* and *in vivo* models. In humans, we also assessed the relationship among cancer aggressiveness, obesity, and serum MIC1 levels.

Subjects and methods

Cells, culture conditions, and reagents

Human PCA LNCaP, PC3, and DU145 cells were purchased from the American Type Culture Collection (Manassas,

VA, USA). Nonmalignant prostate PNT1B cells (Degeorges *et al.* 1995) and PCA C42 cells (Wu *et al.* 1994) were kindly provided by Dr Leland W K Chung (Emory University). The cells were maintained in RPMI 1640 media (Invitrogen) containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. FA-free (2%) BSA (fraction V; Sigma) was used as a vehicle for PA (Sigma) treatment. PA was dissolved in water with 2% (w/v) BSA and filtered through a 0.22- μ m filter (SLGV004SL; Millipore, Billerica, MA, USA) before use. For PA treatment, 1×10^6 cells in a 35-mm dish were serum starved for 4 h and treated with 0.125 or 0.25 mmol/l PA in serum-free DMEM (Invitrogen) for 24 h. Recombinant human MIC1 (rMIC1) was purchased from R&D Systems (Minneapolis, MN, USA) and used at a concentration of 20 ng/ml. A capture antibody specific for MIC1, MAB957 (R&D Systems), and the isotype control antibody, MAB004 (R&D Systems), was used at a concentration of 10 μ g/ml.

Animal study

The Institutional Review Board of the Akita University School of Medicine approved all animal experiments. Six-week-old BALB/c-nu/nu mice ($n=24$) were obtained from Japan SLC (Shizuoka, Japan) and fed an autoclaved CE-2 diet (Japan SLC). For obtaining xenografts, 1×10^6 LNCaP cells were s.c. inoculated with 0.25 ml of ice-cold BD Matrigel (BD Bioscience, Bedford, MA, USA) and 0.25 ml RPMI medium. Four weeks after injection, mice with a palpable tumor were randomly assigned to two different dietary groups ($n=12$ for each group): HFD and low-fat diet (LFD). The average tumor size was 91.2 ± 13.9 and 84.9 ± 12.1 mm³ for the HFD and LFD groups respectively. The energy composition of the HFD was 59.9% from fats, 21.4% from carbohydrates, and 18.6% from proteins, whereas that of the LFD included 9.5% from fats, 67.7% from carbohydrates, and 22.8% from proteins. These diets were prepared by Test Diet (Purina Mills Test Diets, Richmond, IN, USA). The FA content in the diets was measured, and it was found that the HFD and LFD included 6.6% (w/w) and 0.3% (w/w) PA respectively (Japan Food Research Laboratories, Tokyo, Japan). Body weight and tumor volume were measured weekly throughout the experiment. Tumor volume was calculated using the following formula: length (cm) $\times$ width (cm) $\times$ height (cm) $\times$ 0.5236 (Narita *et al.* 2008). Mice were killed at week 14, and xenograft tumors were excised for histopathological examination and FA quantification. Mouse serum was separated, filtered, and stored at -80°C until use.

siRNA constructs

siRNA was purchased from Ambion (Carlsbad, CA, USA). The sequences of siRNAs used in this study are as follows: *MIC1* siRNA; *siMIC1-1*, 5'-GGCAGAAUCUUCGUCCGCAtt-3'; *siMIC1-2*, 5'-CCCGAUUCCUGCCCAACAtt-3'; *siMIC1-3*, 5'-GAGUUGCACUCCGAAGACUtt-3'; and *GL2 Luciferase* siRNA (control; Qiagen), 5'-AACGTACGCGGAATACTTCGA-3'. Transfection of siRNAs was carried out using Lipofectamine 2000 (Invitrogen). Cells were treated with 50 nmol/l siRNA in reduced-serum DMEM. *MIC1* knockdown was verified using quantitative RT-PCR (qRT-PCR) and western blotting.

Quantitative RT-PCR

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen), and cDNA was synthesized using Superscript 2 reagent (Invitrogen), according to the manufacturer's instructions. PCR conditions were 90 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s. The following primers were used: *MIC1*, forward 5'-CGCGCAACGGG-GACCACT-3', reverse 5'-TGAGCACCATTGGGATTGTAGC-3'; *TGF- β* , forward 5'-GACATCAACGGGTTCAC-3', reverse 5'-GAAGTTGGCATGGTAGC-3'; and β -*actin*, forward 5'-ATCTGGCACCACACCTTCTA-3', reverse 5'-CGTCA-TACTCCTGCTTGCTGATCCACATCTGC-3'. All experiments were performed in triplicate.

Cell proliferation assay

A total of 1×10^4 cells were seeded in a 96-well plates and cultured in DMEM containing 5% FBS and 5% mouse serum for 24 h with or without the *MIC1*-specific neutralizing antibody MAB957, the isotype control antibody MAB004, or r*MIC1*. Cell proliferation was assessed using a non-radioactive 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT)-based cell proliferation assay kit (Roche), according to the manufacturer's instructions. Absorbance was measured at 570 nm using an ELISA reader (Bio-Rad). All experiments were performed in triplicate.

Western blot analysis

Proteins were isolated from the cultured cells and supernatants. Equal amounts of protein or culture medium were separated by SDS-PAGE and transferred to PVDF membranes (ATTO Instruments, Tokyo, Japan). The membranes were blocked for 1 h at room temperature using 1% nonfat dry milk in PBS containing 0.1% Tween

20 and incubated for 1 h at room temperature in anti-*MIC1*, anti-ERK1/2, anti-phospho-ERK1/2 (p-ERK1/2, Thr202/Tyr204), or anti- β -actin (Cell Signaling Technology, Beverly, MA, USA) antibodies, diluted 1:1000 in blocking buffer. Membranes were washed and incubated with goat anti-rabbit IgG coupled to horseradish peroxidase (1:3000, Santa Cruz Biotechnologies). Proteins were visualized using an ECL system (Amersham Biosciences) and quantified by densitometry.

Matrigel invasion assay

The *in vitro* invasion assay was performed in triplicate using Growth Factor Reduced BD BioCoat Matrigel Invasion Chambers (BD Biosciences), according to the manufacturer's instructions. Briefly, 5×10^4 cells were seeded in the upper chamber with a medium containing 5% FBS or 5% mouse serum with or without 10 μ g/ml MAB957 or MAB004, or 20 ng/ml r*MIC1*. Subsequently, 20% FBS DMEM was placed in the lower chamber, followed by incubation for 24 h. In siRNA experiments, cells had been previously treated with 50 nmol/l *MIC1* siRNA for 24 h. Subsequently, the noninvading cells were removed and the membranes were stained with a Diff-Quik cell-staining kit (Sysmex, Kobe, Japan) to count the invading cells.

FFA quantification

Quantification of FFA storage in xenograft tumors was performed in triplicate using a FFA quantification kit (Biovision, Mountain View, CA, USA), according to the manufacturer's instructions. Briefly, 10 mg tumor tissue was extracted by homogenization with 200 μ l chloroform-Triton X-100 (1% Triton X-100 in pure chloroform). The extract was centrifuged, the organic lower phase was collected, and samples were air dried at 50 °C. The dried lipids were dissolved in 200 μ l FA assay buffer and converted to acyl-CoA. The oxidized reaction and generated color were measured at 570 nm, and the concentration was calculated using a PA standard curve.

Histopathological analysis

The sections of formalin-fixed paraffin-embedded xenograft tumors were stained with rabbit anti-human *MIC1* polyclonal antibody (Cell Signaling) used at a 1:100 dilution. After overnight incubation, the tissue sections were incubated with HRP-labeled anti-rabbit antibody at a 1:5000 dilution. To assess the invasion of venules and lymph vessels, Elastica-Masson and D2-40

(Nichirei Biosciences, Inc., Tokyo, Japan) staining were performed. The intensity of MIC1 staining intensity in venules and lymph vessels of the xenograft tumors was scored semi-quantitatively on a three-point scale as follows: 1, low; 2, moderate, and 3, high.

Determination of serum MIC1 levels

Human serum samples were obtained from 168 PCa patients and 54 healthy controls (male kidney donors for transplantation). The mean age, BMI, and PSA levels of PCa patients were 67.9 ± 5.5 years, 24.1 ± 2.2 kg/m², and 46.1 ± 64.2 ng/ml respectively. The mean age, BMI, and serum creatinine levels of the healthy kidney donors were 60.9 ± 10.9 years, 22.9 ± 2.4 kg/m², and 0.7 ± 0.1 mg/dl respectively (Supplementary Table 1, see section on supplementary data given at the end of this article). The Institutional Review Board of the Akita University School of Medicine approved all experiments, and samples were obtained after written informed consent was provided by all patients. From each patient, 4 ml of blood was drawn and the serum was separated by centrifugation, and then aliquoted and stored at -80°C until assay. The subjects were enrolled between 2006 and 2010, after being referred to the Department of Urology at Akita University. The serum samples from the patients were used as a pretreatment before anticancer therapy. Serum MIC1 levels were measured using a sandwich ELISA Kit (Oxford Biomedical Research, Inc., Oxford, MI, USA), according to the manufacturer's instructions. We also measured the concentration of human MIC1 both in the serum of the xenograft mice and in the conditioned medium of the PCa cells treated with PA using human-specific ELISA that does not cross-react with mouse MIC1.

Statistical analysis

Statistical analyses were performed using Microsoft Excel (Microsoft Corporation) and SPSS version 12 (IBM) Software. The statistical significance of the differences between two means in each experiment was evaluated using the unpaired Student's *t*-test. Differences among three or more groups were tested using one-way ANOVA for continuous variables or the Kruskal–Wallis test. The correlation coefficient was calculated to investigate the relationship between serum MIC1 levels and volume of xenograft tumors. A positive correlation was considered when an *r* value of >0.5 was obtained. Differences were considered significant if the *P* value was <0.05 .

Results

Upregulation of MIC1 by PA in PCa cells

Taking into account the recent evidence suggesting that PA stimulates MIC1 expression in breast cancer cells (Kim et al. 2008a), we first examined whether PA stimulated MIC1 expression in PCa and benign prostate cells. MIC1 mRNA expression was increased in PCa LNCaP, PC3, and DU145 cells after treatment with 0.25 mmol/l PA. Furthermore, expression was significantly higher in PCa cells treated with 0.25 mmol/l PA for 24 h than in benign PNT1B cells (Fig. 1A). In contrast, *TGFB* mRNA expression was not significantly changed in PCa cells after treatment with 0.25 mmol/l PA (Supplementary Fig. 1, see section on supplementary data given at the end of this article). We also examined MIC1 protein expression after PA treatment in LNCaP and C4-2 cells. MIC1 protein expression was increased after treatment with 0.25 mmol/l PA in a time-dependent manner (Fig. 1B). Next, we assessed soluble MIC1 (sMIC1) expression in the conditioned medium of LNCaP and C42 cells with or without PA treatment. Dimeric sMIC1 (molecular weight, ~ 28 kDa) expression was significantly higher in PCa cells treated with PA. However, there was no difference in protein expression for the two different concentrations of PA (0.25 and 0.125 mmol/l; Fig. 1C). The MIC1 concentration in the conditioned medium from the above experiments was measured and found to be significantly higher in a medium containing cells treated with PA (Fig. 1D).

Increased tumor growth, FA storage, and MIC1 expression in LNCaP xenograft tumors in HFD mice

After 4 weeks, mice with palpable tumors were randomly assigned to two different diet groups (HFD and LFD, 12 mice in each group). There was no significant difference in body weight or caloric intake between the HFD and LFD groups (data not shown). At the end of the experiment (week 14), the mean tumor volume was significantly higher in the HFD group than in the LFD group (2569.9 ± 151.1 and 2010.3 ± 120.7 mm³, respectively, $P=0.025$; Fig. 2A), which was in accordance with the results of previous studies (Huang et al. 2012). The amount of FA storage in tumors was found to be significantly higher in the HFD group than in the LFD group (172.2 ± 43.9 and 119.6 ± 17.8 nmol/mg, respectively, $P<0.001$; Fig. 2B). Furthermore, serum MIC1 levels were significantly higher in the HFD group than in the LFD group

Figure 1

PA stimulates MIC1 expression in PCa cells. (A) PCa cell lines, LNCaP, PC3, and DU145 cells, and benign prostate PNT1B cells were treated with 0.25 mmol/l palmitic acid (PA) or 2% BSA for 24 h. Cells were harvested, total RNA was extracted and quantitative RT-PCR was performed to detect *MIC1*. Mean $\pm$ s.d., $^{**}P < 0.01$, $^{***}P < 0.001$. (B) LNCaP and C42 cells were cultured with 0.25 mmol/l PA or 2% BSA for the indicated time periods. Western blot analysis was performed with anti-human MIC1 and anti- β -actin antibodies. (C) LNCaP and C42 cells were cultured in serum-free

DMEM with 2% BSA or 0.125 or 0.25 mmol/l PA for 24 h. Proteins from equal volumes of culture medium were subjected to western blot analysis using anti-human MIC1 antibody. (D) The MIC1 concentration of the conditioned medium derived from (C) was measured using a human MIC1 ELISA Kit. Mean $\pm$ s.d., $^{*}P < 0.05$, $^{**}P < 0.01$. (D) LNCaP and C42 cells were cultured in serum-free DMEM with 2% BSA or 0.125 or 0.25 mmol/l PA for 24 h. Proteins from equal volumes of culture medium were subjected to western blot analysis using anti-human MIC1 antibody.

(24 155.2 ± 4281.6 and 18 343.8 ± 7019.9 pg/ml, respectively, $P = 0.012$; Fig. 2C). There was a significant correlation between tumor volume and serum MIC1 levels (total, $r = 0.642$, $P = 0.022$; HFD group, $r = 0.710$, $P = 0.016$; and LFD group, $r = 0.629$, $P = 0.028$; Fig. 2D). We confirmed MIC1 protein expression in the xenograft tumors using immunohistochemistry. MIC1 was mainly expressed in the cytoplasm of the PCa cells in the xenograft tumors, but it was also detected in the tumor stroma. The intensity of MIC1 immunostaining was higher in the HFD group (Fig. 2E, left) than in the LFD group (Fig. 2E, right). These findings indicated that the HFD upregulated FA storage and MIC1 overexpression in the LNCaP xenograft tumors as well as accelerating tumor growth.

Upregulation of MIC1 modulates PCa cell proliferation and ERK activation

To address the direct effects of the HFD and MIC1 overexpression on PCa cell proliferation, we performed an MTT-based cell proliferation assay. The proliferation rate was significantly higher in LNCaP and C42 cells

cultured in a medium containing 5% HFD mouse serum than in those cultured in a medium containing 5% FBS. These effects were attenuated in the presence of an MIC1-specific capture antibody MAB957, but not in the presence of control antibody MAB004 (Fig. 3A and Supplementary Fig. 3, see section on supplementary data given at the end of this article). Furthermore, the cell proliferation rate observed in the medium containing 5% FBS in the presence of MAB957 or MAB004 was similar to the rate in the medium without antibodies (Supplementary Fig. 3). The proliferation rate of LNCaP and C42 cells cultured in a medium containing 5% HFD mouse serum was 1.2 and 1.3 times higher, respectively, than that of the cells cultured in a medium containing 5% LFD mouse serum ($n = 4$ per group, $P < 0.05$; Fig. 3A). The cell proliferation rate in both LNCaP and C42 cells increased after treatment with rMIC1 in a dose-dependent manner, and it was significantly higher than that after treatment with FBS alone ($P < 0.05$; Fig. 3A). Because MIC1 activates the ERK signaling pathway in breast cancer (Kim et al. 2008b), we next examined ERK1/2 expression in the PCa cells treated with xenograft mouse serum or rMIC1.

Figure 2

A high-fat diet (HFD) increases tumor growth, fatty acid (FA) storage, and MIC1 expression and secretion in LNCaP xenograft-recipient mice. Twenty-four mice with xenografted tumors were randomly assigned to two groups and fed an HFD or an LFD. (A) Tumor volume was measured at 14 weeks. Mean $\pm$ s.d., **P* < 0.05. (B) FA storage in xenograft tumor tissue was measured using a free fatty acid quantification kit. Mean $\pm$ s.d., **P* < 0.05. (C) Serum MIC1 levels in xenograft-recipient mice were measured using a human MIC1

ELISA Kit. Mean $\pm$ s.d., **P* < 0.05. (D) Serum MIC1 levels and tumor volumes from xenograft-recipient mice were plotted for each individual animal, and the correlation coefficient (*r*) was calculated using SPSS version 12 Software (total, *r* = 0.642, *P* = 0.022; HFD group, open squares, *r* = 0.710, *P* = 0.016; LFD group, open triangles, *r* = 0.629, *P* = 0.028). (E) Xenograft tumor sections from mice fed the HFD (left) or the LFD (right) were examined using immunostaining for anti-human MIC1 antibody (bar, 100 μm).

pERK1/2 expression was significantly higher in the LNCaP cells cultured with 5% HFD mouse serum, 5% LFD mouse serum, or 20 ng/ml rMIC1 than in those cultured with 5% FBS. The effects were significantly inhibited by MAB957 (Fig. 3B and Supplementary Fig. 5, see section on supplementary data given at the end of this article). These findings indicated that MIC1 is one of the important factors enhancing PCa cell proliferation in the presence of HFD mouse serum and that this proliferation involves ERK1/2 activation.

Upregulation of MIC1 modulates invasive capacity in PCa cells

Next, we investigated the effects of diet-induced upregulation of MIC1 on PCa invasiveness using the Matrigel invasion assay. The invasive capacity was significantly higher in PC3 and DU145 cells cultured in a medium containing xenograft mouse serum than in those cultured in FBS media, and the effects were attenuated in the presence of MAB957, but not in the presence of control antibody MAB004 (*n* = 4 per group, **P* < 0.05; Fig. 4A, B and

Figure 3

Administration of MIC1 stimulates cell proliferation and ERK1/2 activation in PCa cells. (A) LNCaP and C4-2 cells were cultured with DMEM containing 5% FBS or 5% mouse serum in the presence or absence of the anti-MIC1 neutralizing antibody MAB957 or recombinant MIC1 at the indicated concentrations for 24 h. MTT assay was performed and cell viability was compared with that of cells cultured with FBS. Mean $\pm$ s.d., * P < 0.05. (B) LNCaP cells were cultured with DMEM containing 5% FBS or 5% HFD mouse serum in the presence or absence of MAB957 or MAB004 or 20 ng/ml rMIC1 for 15 min. In the same experiments, the DMEM containing 5% HFD mouse serum had been previously incubated with 10 μ g/ml MAB97 or MAB004 at 4 °C overnight. Western blot analysis was performed using anti-ERK1/2, anti-phospho-ERK1/2 (p-ERK1/2), and anti- β actin antibodies.

Supplementary Fig. 4, see section on [supplementary data](#) given at the end of this article). In addition, PCa cell invasiveness in the FBS medium in the presence of MAB957 or MAB004 was similar to that observed in the medium without antibodies (**Supplementary Fig. 4**). The invasive capacity of PC3 and DU145 cells was significantly higher after treatment with 20 ng/ml rMIC1 than after treatment with 5% FBS (* P < 0.01; **Fig. 4A and B**). Furthermore, siRNA-mediated knockdown of MIC1 decreased invasive capacity of both PC-3 and DU145 cells by 0.6 times (P < 0.05;

Fig. 4A and B and Supplementary Fig. 2A and B, see section on [supplementary data](#) at the end of this article).

Invading cancer cells exhibit high MIC1 expression in LNCaP xenograft tumors

We next assessed MIC1 expression in tumor and peritumoral vessels of the LNCaP xenograft tumors using MIC1 immunohistochemistry and evaluated the relationship between MIC1 expression and invasive capacity *in vivo*. To delineate the specific location, Elastica–Masson staining and D2-40 immunostaining were performed to detect venules and lymph vessels respectively. Several sections of the LNCaP xenograft tumors showed clusters of tumor cells invading venules (**Fig. 4C**, left) and lymph vessels (**Fig. 4C**, right). MIC1 expression in vessels around the LNCaP xenograft tumors was higher in the HFD group than in the LFD group (venules, 2.7 ± 0.4 and 2.0 ± 0 , $P = 0.116$; lymph vessels, 2.5 ± 0.5 and 2.0 ± 1.0 , $P = 0.453$ respectively). Although not statistically significant, the LNCaP xenograft tumors exhibited greater invasion of peritumoral venules and lymph vessels in the HFD group than in the LFD group (33.3 and 16.7%, $P = 0.346$; 50.0 and 16.7%, $P = 0.083$ respectively; **Fig. 4C**). These findings indicate that the HFD increased the capacity of tumor cells to invade peritumoral vessels by enhancing MIC1 expression in LNCaP cells in tumors originating from xenografts.

Increased serum MIC1 levels in PCa patients correlate with disease aggressiveness and BMI

To investigate the role of MIC1 expression in cancer aggressiveness and obesity in PCa patients, we measured serum MIC1 levels in 54 healthy controls and 168 patients with localized PCa treated with radical prostatectomy. Serum MIC1 levels were significantly higher in the PCa patients than in the controls (1154.2 ± 889.9 and 784.0 ± 299.6 pg/ml, respectively, $P = 0.002$; **Fig. 5A**). When the PCa patients were divided into three groups according to Gleason score (GS), serum MIC1 levels were significantly higher in patients with high-grade tumors than in patients with low-grade and moderate-grade tumors (ANOVA, $P = 0.013$; high grade vs moderate, $P = 0.008$; high grade vs low grade, $P = 0.003$; high grade vs control, $P = 0.001$; moderate vs low grade, $P = 0.271$; moderate vs control, $P = 0.017$; and low grade vs control, $P = 0.036$; **Fig. 5B**). As shown in **Supplementary Table 2**, see section on [supplementary data](#) given at the end of this article, serum MIC1 levels in the PCa patients were significantly associated with clinical stage (P < 0.01), GS (P < 0.02), and serum PSA levels (P < 0.01).

Figure 4

Increased MIC1 enhances the invasiveness of Pca cells. Representative images (A) and a histogram (B) showing the invasive capacity of PC3 and DU145 cells cultured for 24 h with DMEM containing 5% FBS in the presence or absence of *MIC1* siRNA and 5% xenograft mouse serum, in the presence or absence of the anti-MIC1 neutralizing antibody MAB957 or 20 ng/ml rMIC1. All invaded cells were counted and their numbers were

compared with those of cells cultured with FBS. Mean $\pm$ s.d., * $P < 0.05$.

(C) Immunohistochemistry with anti-human MIC1 antibody was performed for xenograft tumor sections, and the number of cancer cells invading venules (black arrow, left) and lymph vessels (white arrow, right) was determined using a microscope. Incidence and intensity of invasion were evaluated (below).

When the PCa patients were divided into two groups according to median BMI, mean serum MIC1 levels were higher in patients with a higher BMI than in those with a lower BMI (1411.3 ± 1353.3 and 1137.5 ± 1076.4 pg/ml, respectively, $P = 0.087$; Fig. 5C). These findings indicated that increased serum MIC1 levels may be associated with cancer aggressiveness and obesity in PCa patients.

Discussion

PA is one of most abundant SFA, and epidemiological studies have suggested that higher PA intake is a risk factor for the incidence and progression of PCa (Giovannucci et al. 1993, Kurahashi et al. 2008). Unlike unsaturated FA, SFA activates inflammasomes and induces many inflammatory cytokines in several target tissues

Figure 5

Serum MIC1 levels were significantly higher in patients with localized PCa than in controls and were associated with a high Gleason score (GS). (A) Serum MIC1 levels from 54 controls and 168 patients with PCa were measured using a human MIC1 ELISA Kit. Mean $\pm$ s.d., ** $P < 0.01$. (B) Patients with PCa were categorized into three groups (GS < 7 , GS = 7–8, and GS = 9–10) and the serum MIC1 levels in each group were compared with those in the healthy controls. Mean $\pm$ s.d., * $P < 0.05$, ** $P < 0.01$. (C) Patients with clinically localized PCa were divided into two groups according to BMI (< 24 or > 24) and serum MIC1 levels were compared between the two groups (1411.3 ± 1353.3 and 1137.5 ± 1076.4 pg/ml, respectively, $P = 0.087$).

(Oberbach *et al.* 2010, Wen *et al.* 2011). Increased PA levels regulate DNA-damaged cells and promote the transformation of primary cells, including precancerous cells (Zeng *et al.* 2008). Although the precise function of PA in PCa cell viability and progression remains unclear, recent evidence has suggested that PA induces *MIC1*

expression in breast cancer MDA-MB-231 cells (Kim *et al.* 2008a). Secreted factors from adipocytes were shown to stimulate MIC1 expression in MDA-MB-231 cells, and PA was shown to be one of the strongest factors inducing MIC1 (Kim *et al.* 2008a). In this study, we demonstrated that PA stimulated MIC1 expression and secretion in four PCa cell lines, i.e. LNCaP, C42, Du145, and PC3. Furthermore, this study shows that serum MIC1 levels in mice were significantly increased in the HFD group compared with the LFD group, and these levels were significantly correlated with tumor growth in the LNCaP xenograft mouse. These findings indicate that high MIC1 levels induced by PA may be involved in HFD-mediated PCa progression.

The role of MIC1 in PCa remains unclear and controversial. One recent study has demonstrated that cell viability was significantly increased in *MIC1* cDNA-transfected LNCaP cells compared with normal LNCaP cells through the activation of the ERK1/2 signal pathway (Chen *et al.* 2007). In contrast, *MIC1* siRNA-expressing LNCaP cells exhibited resistance to protein kinase C-mediated apoptosis, indicating that MIC1 negatively affects LNCaP cell survival (Shim & Eling 2005). Our *in vitro* study showed that rMIC1 stimulated the proliferation of LNCaP and C42 cells in a dose-dependent manner. Furthermore, LNCaP and C42 cell viability was significantly increased in the medium containing HFD mouse serum, and this effect was diminished by treatment with MAB957, indicating that extracellular MIC1 may be a growth enhancer in some types of PCa cells. Recent studies have shown that MIC1-overexpressing TRAMP mice exhibited significantly increased metastasis to the liver, kidney, lung, and rectum compared with normal TRAMP mice (Husaini *et al.* 2012). In addition, intracellular and extracellular MIC1 levels were significantly higher in the metastatic PCa cells (Senapati *et al.* 2010). In this study, PCa cell-invasive capacity was significantly enhanced in media containing xenograft recipient mouse serum compared with FBS media, and this effect was attenuated in the presence of MAB957. In addition, the invasive capacity of PCa cells was increased by rMIC1 treatment and decreased by treatment with *MIC1* siRNA. Furthermore, serum MIC1 levels and MIC1 expression in the xenograft tumors were significantly higher in the HFD group than in the LFD group. MIC1-overexpressing tumor cells efficiently invaded peritumoral venules and lymph vessels in the LNCaP xenograft mouse. ERK is an essential proliferative and metastatic factor that is rapidly activated by MIC1 in breast and gastric cancer cells (Kim *et al.* 2008b). In our study, ERK activation was significantly

increased in the LNCaP cells by treatment with HFD mouse serum, and the effect was diminished by the addition of MAB957. These findings indicate that upregulation of ERK signaling by diet-induced increases in MIC1 may be associated with HFD-induced PCa progression.

Similar to results described in a recent report, the increased serum MIC1 levels observed in the present study were associated with the clinical stage, pathological grade, and PSA levels in PCa (Brown *et al.* 2009). Serum MIC1 levels were also elevated in obese patients and further increased by the presence of type 2 diabetes mellitus (T2DM; Dostalova *et al.* 2009). In addition, a recent report has shown that obesity-mediated hyperinsulinemia also induced an increase in serum MIC1 levels (Karczewska-Kupczewska *et al.* 2012). In this study, PA and an HFD including PA stimulated the production and secretion of MIC1 in PCa cells *in vitro* and *in vivo*. These findings indicate that the dietary component or HFD-mediated biological alterations, such as obesity or hyperinsulinemia, may stimulate MIC1 production. In contrast, MIC1 induced anorexia and weight loss in late-stage advanced PCa (Johnen *et al.* 2007). Moreover, overexpression of MIC1 may lead to decreases in food intake, body weight, and adiposity, both under normal and obesogenic dietary conditions (Macia *et al.* 2012). Therefore, the balance between increased caloric intake from an HFD and HFD-associated hyper-production of MIC1 may be important for body weight regulation. In this study, serum MIC1 levels significantly increased in Japanese patients with localized early-stage PCa and correlated with the pathological Gleason grade. In addition, serum MIC1 levels tended to be higher in patients with higher BMI (BMI >24) than in those with a lower BMI (BMI <24). Although these findings are intriguing, it remains unknown whether high MIC1 levels are associated with higher grade PCa or whether obesity plays a causative role in PCa progression.

Our previous investigation indicated that an HFD affects many important cytokine signaling pathways, including IGF, TWEAK, and MCP1, and promotes proliferation and invasiveness in PCa (Narita *et al.* 2008, Huang *et al.* 2011, 2012). A recent study has demonstrated that an HFD stimulates the production of inflammatory cytokines, including IL6, IL1, and TNF- α (Cortez *et al.* 2013). In this study, an HFD containing high amounts of PA influenced MIC1 expression and promoted PCa progression. These findings support the view that altered cytokine levels and signaling influenced by FA dietary content may play a significant role in PCa progression (Tete *et al.* 2012).

In conclusion, we found that PA stimulated MIC1 expression in PCa cells and that an HFD containing PA accelerated PCa tumor growth *in vivo* and enhanced MIC1 expression. In addition, MIC1 modulated the proliferation and invasive capacity of PCa cells *in vitro*. In humans, serum MIC1 levels may be associated with cancer aggressiveness and obesity. Therefore, an HFD containing PA may promote growth and invasiveness of PCa cells through the upregulation of MIC1 expression.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-13-0227>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

M Huang, S Narita, and T Habuchi designed the experiments; M Huang, S Narita, and H Nanjo performed the experiments; M Huang, S Narita, T Inoue, N Tsuchiya, S Satoh, H Nanjo, T Sasaki, and T Habuchi analyzed the data; and M Huang, S Narita, and T Habuchi wrote the paper.

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