

Prevention of Pancreatic Cancer

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1. Introduction

It is an intriguing problem whether human cells and/or bodies have the physiological function which supervises and selects survival and death fate in response to various kinds of stressors such as radiation. For example, what are the physiological effects of X-ray irradiation for examination of breast conditions including the risk of genetic mutation and cancer occurrence (Fig. 1A).

Based on a protease-activation signal in *E.Coli* SOS response we have attempted to search for human SOS response, particularly signals which protect stability of genetic information (Fig.1B) by estimation of plasminogen activator-like protease (PA) activity in peripheral blood-derived lymphocytes and cultured human RSa cells with hyper-mutability and their variant hypo-mutable cells (Fig.1C) (Suzuki *et al.*, 2005). Levels of PA activity in lymphocytes are changed after X-ray irradiation for breast examination and after exposure *in vitro* to chemical such as bisphenol A (Takahashi *et al.*, 2000). The activity in variant cells was found to be associated with heat shock protein (HSP) 27 expression, resulting in enhancement of error-free DNA repair function (Wano *et al.*, 2004). A HSP27-bound protein, annexin II, was also suggested to play some roles on the error-free function in human cell nuclei (Tong *et al.*, 2008 and Jin *et al.*, 2009). We recently developed a method to analyze base substitution mutation of the *K-ras* codon 12 and found that a decreased mutation frequency accompanied increased GRP78 expression in human RSa cells (Hirano *et al.*, 2008 and Zhai *et al.*, 2005). The repair function seems to suppress base substitution mutation of *K-ras* codon 12. The base substitution mutation was also found to be regulated by extracellular factors, human interferon and serum factors from cancer patients and stressors-exposed persons (Suzuki *et al.*, 2005, Hirano *et al.*, 2008 and Chi *et al.*, 2007). This regulation seems to be mediated by PA activation and the following chaperones expression (Suzuki *et al.*, 2005, Isogai *et al.*, 1994, Takahashi *et al.*, 2003 and Kita *et al.*, 2009). Thus, studies on molecular mechanisms to supervise cellular mutability, including frequency of Ras gene mutation, are important for discussion about relationships with a network of proteases and chaperones and/or cytokines.

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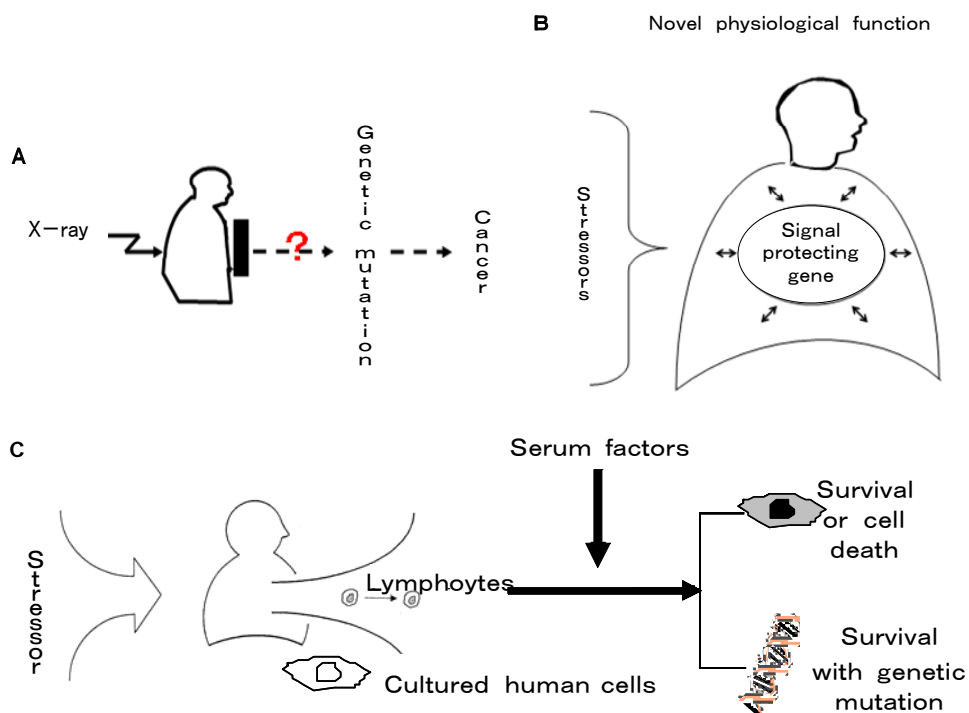


Fig. 1. Strategy of the method to search for human SOS response.

(A) A question concerning X-ray effects on genetic mutation and the following cancer. (B) A physiological function which supervises protection mechanisms for genetic information stability. (C) Modulation of cellular fate by serum factors and plasminogen activator-like protease (PA) activation, elucidated by the observation of peripheral blood lymphocytes and cultured human cells after stressors exposure.

Incidence of K-ras gene mutation is high in pancreatic carcinomas, and therefore suppression of the mutation could be beneficial for inhibiting pancreatic cancer occurrence (Hirano *et al.*, 2008). We were intrigued by the possibility that this carcinogen-induced mutation could be suppressed, via modulation of GRP78 expression, by agents such as foods.

In the present chapter, cellular levels of GRP78 in R5a cells that had been cultured with aqueous extracts of Japanese miso and the unfermented ingredients of miso are shown using immunoblotting analysis. The mutability of the treated cells are also evaluated after ultraviolet light C (UVC)(principally 254 nm in wave length) irradiation using the differential dot-blot hybridization test for *K-ras* codon 12 mutation. In previous reports, dietary supplementation with long-term fermented miso has been shown to act as a chemopreventive agent against gastric and colon carcinogenesis in rats (Ohara *et al.*, 2002a, 2002b, and Ohuchi *et al.*, 2005). Miso is a fermented food that has formed an important part of the Japanese diet for over 1300 years (Yoshikawa *et al.*, 1998). It is prepared by the microbial fermentation of a mixture of raw materials (soybean, wheat, barley and rice) over

a long period until the ripe miso is obtained (Hesseltine & Shibasaki, 1961). Little is known about the ability of miso to modulate the mutability of human cells.

2. Human SOS response and suppression of *K-ras* mutation by Japanese miso possibly via GRP78 expression in human RSa cells

2.1 Effect of miso samples on RSa cell viability

2.1.1 Preparation of miso samples

The Japanese Enbunhikae miso (EM) was purchased from Marui Co., Ltd. (Chino, Japan). Rice-koji, a raw material used in the preparation of miso, was obtained from Hanamaruki Co., Ltd (Nagano, Japan). Aqueous extracts of miso and rice-koji were prepared as follows: each (10 g) was suspended in 20 ml of MilliQ water, and the suspension was heated at 90 °C for 5 minutes and then at 70 °C for 10 minutes. The suspension was centrifuged at 1,780 × g and 4 °C for 10 minutes and the supernatant was then filtered through a 0.22 µm membrane. The dose of sample used in each treatment is shown as a percentage of volume per volume (v/v); 1% is equivalent to 5 mg of miso or rice-koji per ml.

2.1.2 Culture conditions and UVC irradiation

RSa cells were established from human embryo-derived fibroblast cells by double infection with the Simian virus 40 and the Rous sarcoma virus. The cells were confirmed to have high UV sensitivity and low DNA-repair activity (Kuwata *et al.*, 1976, Suzuki & Fuse, 1981, Suzuki, 1984). Cells were cultured in Eagle's minimal essential medium (EMEM) (Nissui, Tokyo, Japan) containing 10% calf serum (CS) (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere containing 5% CO₂. UVC was generated by a 6 W National germicidal lamp (Panasonic, Osaka, Japan). The intensity of UVC was 1 J/m²/s, as measured by a UV radiometer, UVR-254 (Tokyo Kogaku Kikai, Tokyo, Japan). Irradiation of the cells with UVC was performed as described previously (Suzuki & Fuse, 1981) and mock irradiation of cells was carried out in the same manner but without UVC irradiation.

2.1.3 Colony survival assay

To determine the optimal concentration of miso sample for use in the culture medium in cell mutability tests, the colony survival capacity of RSa cells cultured with or without EM extracts was examined (Fig. 2). The survival capacity of cells treated with or without miso extracts was measured using a colony survival assay as reported previously (Suzuki *et al.*, 1984). Logarithmically growing cells were seeded in 100 mm dishes (800 cells/dish), incubated for 20 h to allow the cells to attach, and then treated with or without miso extracts. One h after treatment, the cells were grown in fresh medium containing 5% CS in 100 mm dishes for about 14 d and were then stained with 0.2% methylene blue in 30% methanol. The results of the colony survival assay are expressed as percentages of the colony numbers observed for miso extracts-treated cells relative to those of untreated cells. Colony survival rates were over 85% when a miso extract concentration of 1% was used, but at concentrations higher than 1% a decrease of more than 10% in survival rate was observed (Fig. 2). An MTT assay showed that miso extract concentrations of less than 1% were not cytotoxic after 48 h of culture (data not shown).

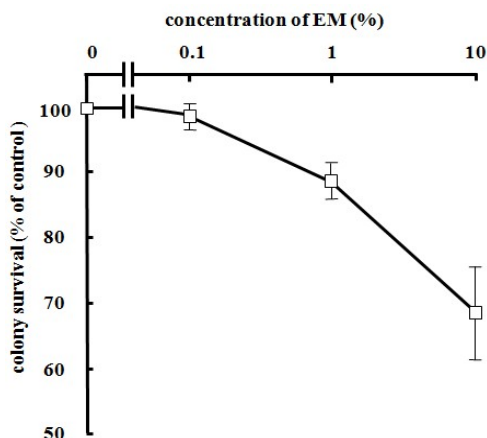


Fig. 2. Effect of EM extracts on cell survival.

RSa cells were treated with or without the indicated concentrations of EM extracts for 1 h. After treatment, the cells were cultured with fresh medium containing 5% CS in 100 mm dishes for 14 d and were then stained with 0.2% methylene blue in 30% methanol. Data are the means \pm SD for three experiments.

2.2 Effect of miso extracts on GRP78 expression and the mutability of RSa cells

2.2.1 GRP78 expression

GRP78 expression was analyzed by immunoblotting as described previously (Wano *et al.*, 2004). Cells were lysed with a lysis buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.5% NP-40 and protease inhibitors, 1 mM phenylmethylsulfonyl fluoride, 0.05 mM leupeptin, 0.05 mM antipain and 0.05 mM pepstatin A. The cell lysates were centrifuged at $15,000 \times g$ and $4^\circ C$ for 20 min, and the supernatant was then treated with SDS sample buffer. Detection of the GRP78 protein was performed using mouse monoclonal anti-GRP78 (1:2000 dilution; SPA-827; StressGen, Victoria, Canada) antibodies. β -Actin was also analyzed using mouse monoclonal anti- β -actin antibodies (1:30000 dilution; ab40864; Abcam, Cambridge, UK) as a loading control. The antigen-antibody complexes were detected by horseradish peroxidase (HRP) conjugated anti-mouse IgG (Amersham Biosciences, Buckinghamshire, UK) following the ECL system (GE Healthcare, Buckinghamshire, UK). The GRP78 protein was quantified using Multi Gauge Ver2.2 image analysis software (Fuji Foto film, Tokyo, Japan) and expressed relative to the quantity of β -actin measured. When RSa cells were cultured with EM extracts at a concentration of 1%, expression of GRP78 was enhanced by over 1.5 fold compared with the expression observed in mock-treated cells (Fig 3A).

2.2.2 Mutability

Mutations in *K-ras* codon 12 were detected according to a method described previously (Suzuki N & Suzuki H., 1993). Logarithmically growing cells were inoculated at near confluency (5×10^5 cells/100 mm dish) to avoid cell selection by the lethal effects of UVC irradiation, as described elsewhere (Suzuki N & Suzuki H., 1995). Six d after UVC

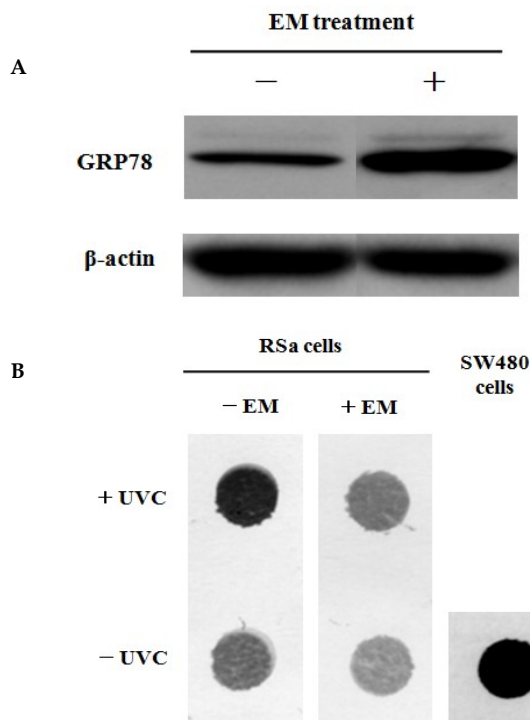


Fig. 3. Effect of EM extracts on GRP78 expression (A) and the mutability (B) of RSa cells. Cells were cultured with or without EM extracts at a concentration of 1% for 24 h. (A) Cell lysates were prepared after miso treatment and subjected to immunoblotting analysis of GRP78 and β -actin proteins. (B) After miso treatment, cells were irradiated with UVC or left unirradiated as controls (6 J/m^2). Six d after UVC irradiation, genomic DNA was extracted, and the *K-ras* codon 12 mutation was detected by PCR and differential dot-blot hybridization, as described in Materials and Methods.

or mock irradiation, genomic DNA was extracted using a standard proteinase K/SDS/phenol/chloroform procedure. DNA of SW480 cells carrying the *K-ras* mutation at codon 12 was used as a positive control for genomic DNA. Target sequences of sample DNA were amplified *in vitro* by PCR using the primers 5'-GACTGAATATAAACTGTGG-3' and 5'-CTATTGTTGGATCATATTCG-3', and the amplified DNA ($0.25 \mu\text{g}$) was dot-blotted onto nylon membranes. After hybridization with digoxigenin-11-dUTP-3' end-labeled *K-ras* codon 12 probes, the membranes were reacted with alkaline phosphatase conjugated polyclonal sheep anti-Dig Fab (Boehringer Ingelheim, Mannheim, Germany) and colored with the nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate solution (Boehringer Ingelheim, Mannheim, Germany). As a control probe, the oligonucleotide 5'-GTTGGAGCTGGTGGCGTAGG-3' was used, and a mixed mutant probe, containing the following oligonucleotides mixed at the same concentration ratios, was used: 5'-GTTGGAGCTAGTGGCGTAGG-3', 5'-GTTGGAGCTCGTGGCGTAGG-3', 5'-GTTGGAGCTGTGGCGTAGG-3', 5'-GTTGGAGCTGATGGCGTAGG-3', 5'-

GTTGGAGCTGCTGGCGTAGG-3', 5'-GTTGGAGCTGTTGGCGTAGG-3'. Photographs were taken as permanent records of the results. To determine whether miso extracts suppress the mutability of RSa cells, the *K-ras* codon 12 mutation assay was performed. A black dot, indicating base substitution mutation, was detected after hybridization of the PCR products from genomic DNA of SW480 cells containing the *K-ras* codon 12 mutation, and this was used as a positive control (Fig. 3B). Under the assay conditions, the intensity of the black dot was clearly enhanced after UVC irradiation in mock-treated RSa cells (Fig. 3B). However, EM extracts-treated RSa cells did not show black dot signals either after UVC or mock irradiation (Fig. 3B).

2.3 Effect of GRP78 siRNA on the modulation of UVC cell mutability by miso extracts

To further examine whether GRP78 expression levels are causally related to the miso treatment modulation of RSa cells mutability, its expression was inhibited by GRP78 siRNA transfection. Duplex small interfering RNA (siRNA) with Stealth modification against human GRP78 (GRP78 siRNA) was synthesized based on the protein's nucleotide sequence (Invitrogen), as described previously (Suzuki T *et al.*, 2007). The sequence of the duplex was

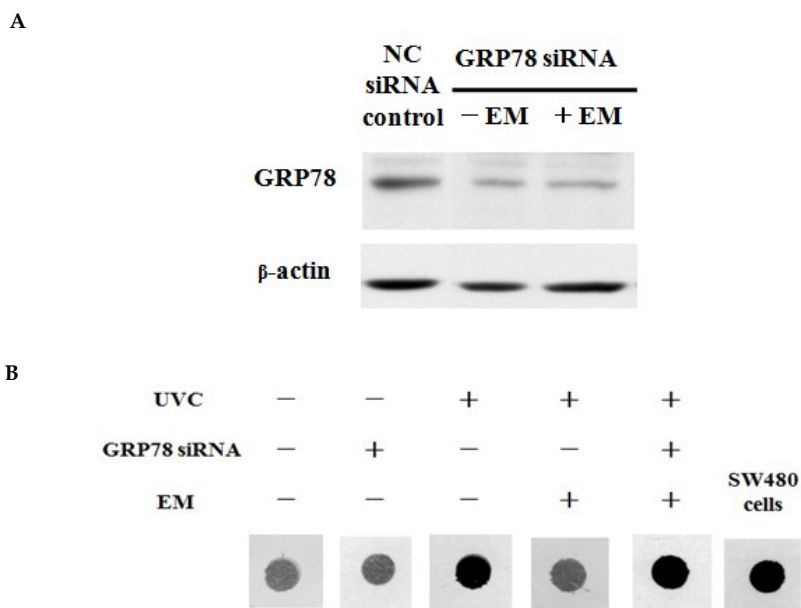


Fig. 4. Effect of GRP78 siRNA transfection on UVC-induced mutagenicity.

(A) After GRP78 or NC siRNA transfection, cell lysates were separated by SDS-PAGE and analyzed by immunoblotting analysis using anti-GRP78 and anti- β -actin (loading control) antibodies. (B) After siRNA transfection, cells were treated with or without EM extracts for 24 h and then irradiated with UVC (6 J/m^2). Mutability of RSa cells was determined by the *K-ras* codon 12 mutation assay using PCR and differential dot-blot hybridization, as described in Materials and Methods.

as follows: 5'-UAC CCU UGU CUU CAG CUG UCA CUC G-3' / 3'-AUG GGA ACA GAA GUC GAC AGU GAG C-5'. Stealth RNAi negative control duplex (NC siRNA), with a GC content similar to that of the above Stealth RNAi, was used as a negative control. The NC siRNA was designed to minimize sequence homology to any known vertebrate transcript and for use in RNA interference (RNAi) experiments as a control for sequence independent effects following Stealth RNAi delivery in any vertebrate cell line. Treatment of cells with siRNA was carried out as described previously (Harborth *et al.*, 2001), with minor modifications. The siRNAs (128 nM) were transfected for 5 h into RSa cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were detached from the test dish 48 h after transfection and used for each experiment.

In GRP78 siRNA-transfected cells, cellular levels of GRP78 protein were decreased in EM extracts-treated cells as well as in mock-treated cells, while no decrease was observed in NC siRNA-transfected cells (Fig. 4A). The decrease observed was up to 50% of the NC siRNA control (Fig. 4A). In RSa cells with GRP78 siRNA transfection that had been treated with miso extracts, the dot signal of *K-ras* codon 12 mutation was enhanced after UVC irradiation (Fig. 4B).

2.4 Effect of miso components on cell mutability

The effect of rice-koji, a component of miso, on the mutability of RSa cells was examined. The survival rate, measured by a colony survival assay (Fig. 5) and an MTT assay (data not shown), of cells treated with rice-koji at concentrations of less than 10% were more than 80%. On the basis of these results, we used 1% rice-koji in subsequent experiments to allow direct comparison with experiments using EM extracts.

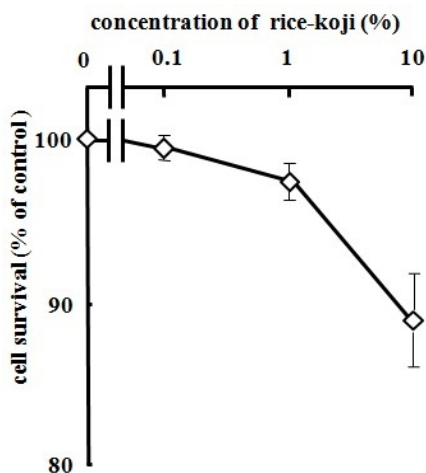


Fig. 5. Effect of rice-koji on cell survival.

RSa cells were treated at the indicated concentrations with rice-koji. After rice-koji treatment (1h) cells were grown in fresh medium containing 5% CS in 100 mm dishes for about 14 d, and then stained with 0.2% methylene blue in 30% methanol. Data are the means \pm SD for three experiments.

Expression levels of GRP78 in rice-koji-treated RSa cells were slightly increased, at about 1.5 fold of the untreated cells (Fig. 6A). Rice-koji-treated cells showed no detectable black dot signal in the *K-ras* codon 12 mutation test with or without UVC irradiation treatment (Fig. 6B).

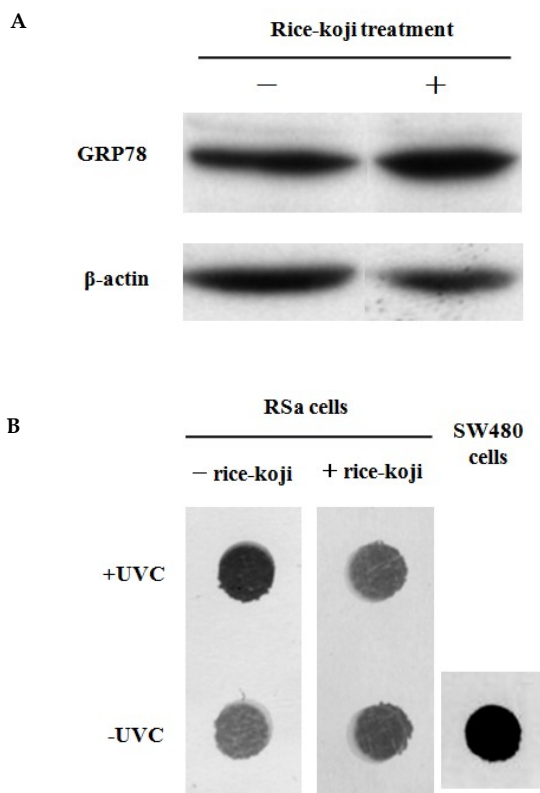


Fig. 6. Effect of rice-koji on GRP78 expression and mutability.

(A) Cells were treated with rice-koji (1%) for 24 h, and the cell lysates were subjected to immunoblotting analysis of GRP78 and β -actin proteins. (B) After rice-koji treatment, cells were irradiated with UVC (6 J/m²). Six d after UVC irradiation genomic DNA was extracted, and the *K-ras* codon 12 mutation was detected using PCR and differential dot-blot hybridization.

We also examined whether GRP78 siRNA transfection affected UVC mutagenicity in rice-koji-treated RSa cells. In GRP78 siRNA-transfected cells, GRP78 expression levels decreased to about 40% of those in NC siRNA-transfected cells (Fig. 7A). Cells transfected with GRP78 siRNA and then treated with rice-koji, showed black dot signals indicating UVC-induced *K-ras* codon 12 mutation, similar to the signal observed in cells treated with UVC irradiation alone, and in SW480 cells (Fig. 7B).

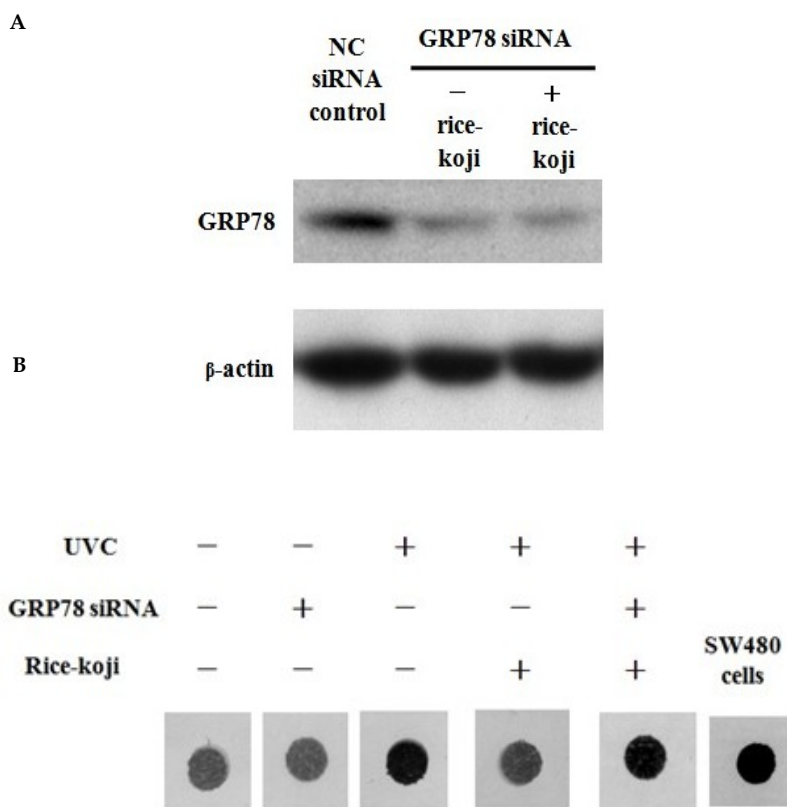


Fig. 7. Effect of GRP78 siRNA transfection on UVC-induced mutagenicity in rice-koji treated cells.

(A) After GRP78 or NC siRNA transfection, cell lysates were separated by SDS-PAGE and analyzed by immunoblotting analysis using anti-GRP78 and anti- β -actin (loading control) antibodies. (B) After siRNA transfection, cells were treated with or without rice-koji extracts for 24 h and then irradiated with UVC (6 J/m^2). Mutability of R5a cells was determined by the *K-ras* codon 12 mutation assay using PCR and differential dot-blot hybridization.

3. Conclusion

The *K-ras* base substitution mutation is thought to be a cancer-causing DNA sequence change in human cells, and its incidence is particularly high in pancreatic carcinomas. We previously found that proteases are activated in stress-exposed cells and/or human bodies, leading to modulation of chaperone expression and cellular mutability (so-called SOS response). Increased levels of glucose-regulated protein 78 (GRP78) expression are associated with the suppression of the *K-ras* mutation in human R5a cells irradiated with ultraviolet C UVC. R5a cells are hyper-mutable and are used to examine the modulation

of cell mutability using various agents. Here, we describe investigations into the effect of R_{Sa} cell treatment with Japanese miso on GRP78 expression and the suppression of UVC mutagenicity. Aqueous extracts of miso and its components were tested. Miso treatment was found to increase GRP78 levels, as estimated by immunoblotting analysis, and to decrease UVC-induced *K-ras* codon 12 base substitution mutation frequency. Increases in GRP78 expression and decreases in mutation frequency were not observed in cells whose GRP78 levels had been down-regulated using GRP78 siRNA transfection. This suggests that miso extracts suppress UVC mutagenicity by increasing GRP78 expression in human cells.

In the present study Japanese miso was tested concerning the modulation activity of genetic mutation, *K-ras* codon 12 base substitution mutation, in addition of human cytokines and serum factors. Several dietary factors have been postulated to act as risk factors for human carcinogenesis (Sugimura, 2000 and Mirvish, 1983). In Japan, intensive studies of the causal relationship between diet and cancer incidence have focused on stomach, lung and colorectal cancers (Masaki *et al.*, 2003, Ngoan *et al.*, 2002, Takezaki *et al.*, 2001, Ozasa *et al.*, 2001, Tajima & Tominaga, 1985). The association of dietary factors with pancreatic cancer has been significantly less well studied. In this study, the modulation of cell mutability via the GRP78 chaperone was examined by measuring GRP78 expression in Japanese miso-treated human R_{Sa} cells. It was found that levels of GRP78 expression increased upon treatment of R_{Sa} cells with EM and rice-koji extracts (Figs. 3A and 6A). The pretreatment of cells with these extracts was also found to suppress UVC mutagenicity (Figs. 3B and 6B). An intimate relationship between GRP78 up-regulation and hypo-mutability was also suggested by the results of experiments using GRP78 silencing (Figs. 4 and 7).

We reported that the down-regulation of GRP78 in R_{Sa} cells reduces DNA repair capacity in the nucleotide excision repair pathway. Nucleotide excision repair, a highly conserved DNA repair system in human cells, is essential for protection against UVC-induced DNA damage leading to, for example, (6-4)-photoproducts and cyclobutane thymine dimers (Batty & Wood, 2000, de Laat *et al.*, 1999). Thus, one plausible mechanism for the observed hypo-mutable change in R_{Sa} cells pre-cultured with miso and rice-koji extracts may be the enhancement of cellular DNA repair function by the up-regulation of GRP78 expression.

The *K-ras* point mutation-enhancing activity of conditioned medium is detected from culture of human pancreatic cancer cells (Hirano *et al.*, 2008), suggesting involvement of extracellular factors from pancreatic cancer cells in tumor-worsening process. Extracellular materials released from cancer cells play crucial roles in development of cancers and resistance to anticancer treatment (Hidalgo *et al.*, 2010). Pancreas carcinoma shows resistance to chemotherapeutic agents (Gong *et al.*, 2010). Thus, we tried to search for the above materials in conditioned medium from pancreatic cancer cells, and identified one of HSP27-bound proteins, annexin II, by molecular mass analysis. Although the functions of extracellular annexin II are not fully understood, annexin II is known to act as a cell surface receptor for extracellular ligands and is suggested to play roles in regulation of proteolytic cascades including PA activities (Hajjar *et al.*, 1994), signal transduction (Singh, 2007), and tumor invasion and metastasis (Chung *et al.*, 1996, Esposito *et al.*, 2006, Singh *et al.*, 2007, Mai

et al., 2000). Studies on the mechanisms of PA-involved SOS functions are required for prevention of pancreatic cancer.

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