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BASIC RESEARCH

Effect of herbal medicine Juzentaihoto on hepatic and intestinal heat shock gene expression requires intestinal microflora in mouse

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Abstract

AIM: To evaluate the role of intestinal microflora in the effects of multi-herbal medicine on gene expression in the gut and liver.

METHODS: The multi-herbal medicine Juzentaihoto (JTX) was administered to five germ-free mice and regular mice for 2 wk. Among the results of the comprehensive gene chip analysis of the intestine and liver, we featured heat shock proteins (HSPs) 70 and 105 because their gene expression changed only in the presence of microflora. Real-time RT-PCR was performed to confirm the expression levels of these HSP genes. To determine whether JTX acts directly on the HSP genes, sodium arsenite (SA) was used to induce the heat shock proteins directly. To examine the change of the intestinal microflora with administration of JTX, the terminal restriction fragment polymorphism (T-RFLP) method was used. To identify the changed bacteria, DNA sequencing was performed.

RESULTS: Heat shock protein gene expression, documented by gene chip and real-time RT-PCR, changed with the administration of JTX in the regular mice but not in the germ-free mice. JTX did not suppress the direct induction of the HSPs by SA. T-RFLP suggested that JTX decreased unculturable bacteria and increased *Lactobacillus johnsoni*. These data suggested that JTX changed the intestinal microflora which, in turn, changed HSP gene expression.

CONCLUSION: Intestinal microflora affects multi-herbal product JTX on the gene expression in the gut and liver.

Key words: Herbal medicine; Juzentaihoto; Intestinal microflora; Terminal restriction fragment length polymorphism; Heat shock protein

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INTRODUCTION

Increasingly, herbal products are used worldwide. Although many studies have suggested efficacy of herbal products, often the mechanism of action is not well understood. Kampo medicine is a Japanese traditional herbal medicine, and it also uses substances that are of pharmaceutical grade and integrated in the national medical system. More than 70% of Japanese physicians prescribe Kampo medicine together with western medicine[1]. Juzentaihoto (JTX; *Shi-Quan-Da-Bu-Tang* in Chinese) is one of the Kampo medicines that consists of 10 herbs. It has been traditionally used for centuries for general malaise. Currently, JTX is widely prescribed for anemia, rheumatoid arthritis, chronic fatigue syndrome and inflammatory bowel diseases. It is also widely used for the prevention of cancer metastasis and infection in immuno-compromised patients[1]. The mechanism of its action has been vigorously investigated.

For example, JTX stimulates hematopoietic stem cell growth[2], enhances T-cell-mediated suppression of melanocytic tumor cell growth[3] and inhibits tumor metastases[4]. Although one of the mechanisms of action may be alteration of intestinal microflora[5,6] and stimulation of the host immune system *via* the mucosal immune system[6], the relationship between host gene expression and intestinal microflora change has not been clarified.

We investigated the genome-wide analysis of mRNA of the intestine and liver and compared the effect of JTX on germ-free (GF) mice with no intestinal microflora and specific pathogen-free (SPF) mice that have normal intestinal bacteria, which enabled us to discriminate the intestinal microflora-dependent and -independent effects of JTX. Within the comprehensive gene expression analysis, we focused on heat shock proteins 105 (HSP105) and 70 (HSP70) because their expression changed together with cytokine-related molecules, such as interleukin-related factor 7 (IRF-7), with the administration of JTX only in SPF mice, but not in GF mice. Also, heat shock proteins in the gut are reported to be maintained and controlled by the intestinal microflora and may reflect the inflammatory state of the gut[7,8]. Our previous study showed that JTX suppressed the serum ammonia elevation after a partial hepatectomy of mouse[9]. In that study, the change of the intestinal microflora after partial liver excision was minimized with JTX administration.

In the present study, we investigated the species of intestinal bacteria which may affect the change of HSPs expression. Terminal restriction fragment length polymorphism (T-RFLP) analysis is a very useful tool for comparing microbial communities, including the unculturable bacteria[10]. The estimation of the number of bacterial species in intestinal microflora by culture-independent approaches using 16S rDNA clone library suggests that only 20% to 30% of the total intestinal bacteria are culturable[11,12]. Recent studies of murine intestinal microflora using T-RFLP analysis revealed the existence of many previously unidentified microorganisms in the intestinal tracts of mice[13,14].

As a result of these investigations, we propose a novel mechanism of action for herbal product: altered gene expression as a result of altered intestinal microflora.

MATERIALS AND METHODS

Animals

For the study of comparison of germ-free (GF) mice with specific pathogen-free (SPF) mice, male IQI mice (age 7 to 9 wk) kept under GF or SPF conditions were provided from the Central Institute of Experimental Animals (Central Institute for Experimental Animals, Kawasaki, Japan). IQI mice have been established as an inbred strain from ICR mice. Male ICR mice from CLEA Japan Inc. (age 7 to 9 wk) were used in the rest of the experiments. The GF mice were maintained in isolators which were free from bacteria throughout the experiments. The SPF mice were housed in an air-conditioned room (temperature 24℃ ± 1℃) with a controlled light/dark cycle (light on between 6:30 am and 7:00 pm), and food and water were available *ad libitum*. The mice were randomly divided into two groups, three to six in each group. One group was orally treated with JTX (Tsumura & Co. Tokyo, Japan) solution (1.0 g/kg body weight), and another was treated with an equal volume of water as the control. JTX was administered using a stainless steel gastric tube once a day for 14 d. Figure 1 shows the chemical profile of JTX. For antibiotic treatment, ciprofloxacin (CPFX) was fed in the diet (240 mg/kg body weight per day) for a week. The intake of chow was recorded daily. Sodium arsenite (SA) (Sigma Chemical, St. Louis, MO) was diluted in saline (5 mg/kg) and injected intraperitoneally. This protocol was approved by the Guidance for the Care and Use of Laboratory Animals of Keio University School of Medicine that is in accordance with the NIH Guide for Care and Use of Laboratory Animals.

RNA preparation from the mice tissues

Mice were sacrificed and the liver, small intestine and large intestine were harvested for preparation of total RNA. Each of the samples (0.1 g tissue) was homogenized in a 1 mL of TRI reagent (Sigma-Aldrich Japan, Tokyo, Japan) with POLYTRON tissue homogenizer (Kinematica, Littau-Lucerne, Switzerland) and incubated for 10 min at room temperature. Chloroform (0.2 mL/1 mL TRI reagent) was added to the samples and the suspensions were centrifuged at 13 200 × *g* for 15 min at 4℃. The water phase was transferred to a new tube and the RNA was prepared with a conventional isopropanol/ethanol precipitate technique. To check the quality and quantity of RNA, ultraviolet (UV) absorbance at 260 nm was determined and the electrophoresed RNA was visualized by ethidium bromide staining.

Real-time RT-PCR

Real-time RT-PCR was performed to confirm the expression levels of HSP105, HSP70 RNA and β2-microgloblin (as a house-keeping gene) with sequence-specific oligonucleotide primers (Invitrogen, Carlsbad, CA). RNA templates were quantified by the QuantiTect SYBR Green RT-PCR kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The sequences of the primers were as follows: HSP105 (forward: 5'-GTG TCA GGG TCC TGT GGA GT-3', reverse: 5'-CCT TGG CAA GAA TAA CTG CTT-3'); HSP70 (forward: 5'-TGC CTT CAA CAT GAA GAG CG-3', reverse: 5'-TTG TGC ACG AAC TCC TCC TT-3'); β2-microgroblin (forward: 5' -CGG CTT GTA TGC TAT CCA GA, reverse: 5'-AGA AAG ACC AGT CCT TGC TG-3'). Real-time RT-PCR reactions were carried out with 100 ng of total RNA as the templates through 30 amplification cycles of 94℃ for 15 s, 60℃ for 30 s and 72℃ for 20 s. After amplification, samples were kept at 55℃ for 1 min and the temperature was raised gradually by 0.5℃ in every 10 s to perform the melt-curve analysis. All procedures of real-time RT-PCR were performed on the iCycler iQ™ Real-Time PCR and Detection System (Bio-Rad Laboratories, Tokyo, Japan), the software for this instrument. The threshold cycles (Ct) were used to quantify the mRNA expression levels of samples with β2-microgloblin normalization.

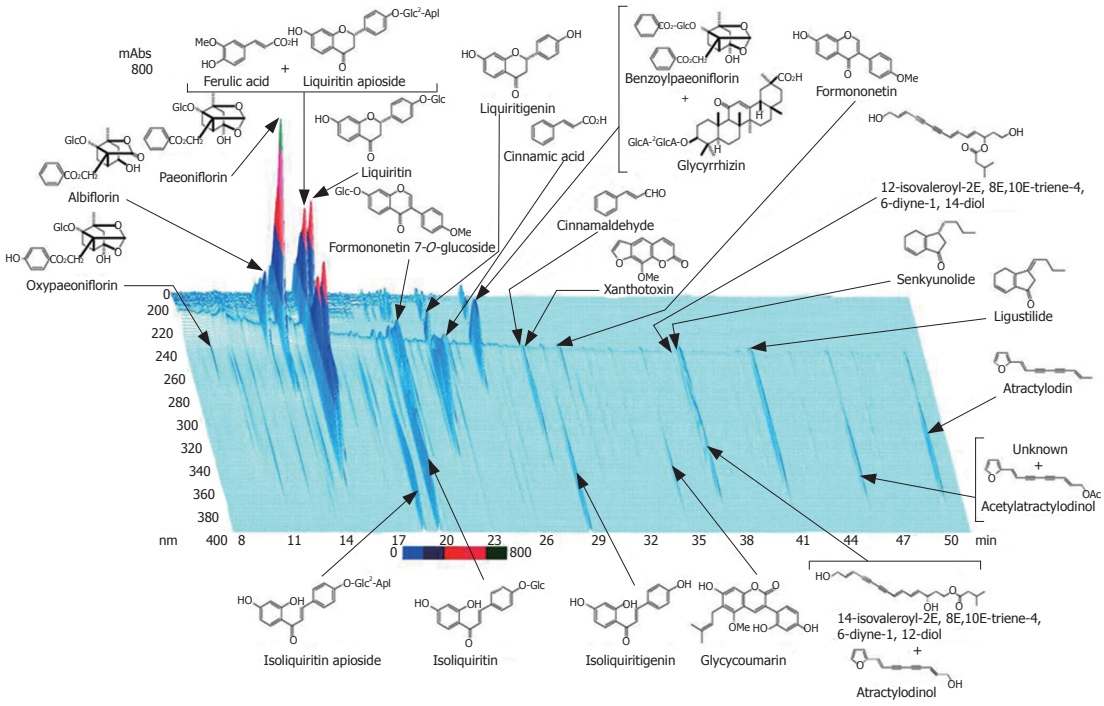

Figure 1 Chemical profile of JTX product analyzed by three-dimensional HPLC.

Sampling of feces

After 2 wk administration of JTX or water, mice were sacrificed and the feces of the small intestine and large intestine were collected. Samples were stored at -80℃ until use.

Cell lysis and DNA isolation from samples

DNA extraction and purification were based on the methods described by Clement and Kitts[15], using an Ultra Clean Soil DNA Isolation kit (Mo Bio Laboratories, Inc. Solana Beach, CA) with some modifications according to Kibe *et al*[16].

T-RFLP analysis and sequencing

The primers used for the PCR amplification of 16S rDNA sequences were 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3')[16]. 27F was labeled at the 5' end with the 6'-carboxyfluorescein (6-FAM) (Applied Bio Systems, Tokyo, Japan). PCR was performed as described by Kibe *et al*[16]. Purified PCR products were digested with 20 U of *Hha* I or *Msp* I (Takara Bio Inc.) in a total volume of 10 μL at 37℃ for 3 h. GS-500 Rox and GS-1000 Rox (Applied Biosystems) were used as internal standard markers. The length of the terminal restriction fragments (T-RFs) were analyzed by electrophoresis on a model ABI Prism 310 Genetic Analyzer (Applied Biosystems) and Genecan analysis software (Applied Biosystems). The distances between samples were represented graphically by constructing a dendrogram based on Jaccard matching coefficients of T-RFLP profiles. Unweighted pair-group method with arithmetic mean (UPGMA) was used for establishing the dendrogram type.

To identify the bacteria, T-RFs were electrophoresed, isolated, ligated with pT7Blue T vector and transformed into *E. coli*[17]. More than 100 transformants per T-RF were subjected to the direct DNA sequencing. The sequences were sent to database for BLAST[18] searches to determine identities/similarities or homologies to known sequences.

Statistical analysis

The data were expressed as mean ± SD and groups were compared using Student's *t*-test corrected for small sample size. For multiple comparisons, Dunnet's method was used. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of JTX on the expression of HSP105 and 70 mRNA

Gene chip analysis showed that JTX administration decreased the mRNA expression of HSP105 and HSP70 in the small or large intestine and the liver in SPF mice, but not in GF mice (data not shown). The real-time RT-PCR analysis confirmed this effect (Figure 2). Significantly lower levels of HSP105 mRNA were observed in the intestines and liver of SPF mice treated with JTX compared to that of SPF mice with water. JTX administration seemed to affect HSP105 mRNA expression in the opposite direction in GF mice compared to SPF mice though the changes were not significant. In SPF mice, HSP70 mRNA showed a similar reduction to HSP105 mRNA, but did not reach statistical significance. Moreover, the effect of JTX on HSP70 mRNA levels was inconsistent among tissues in GF mice.

The same experiment was performed with the widely used SPF ICR mice which were already stated. Three doses of JTX (1, 0.5, and 0.25 g/kg) were used. SPF ICR mice with JTX, at the doses of 0.5 and 1.0 g/kg, expressed significantly lower amounts of HSP105 and HSP70 mRNA as compared to the control mice in all examined tissues (Figure 3).

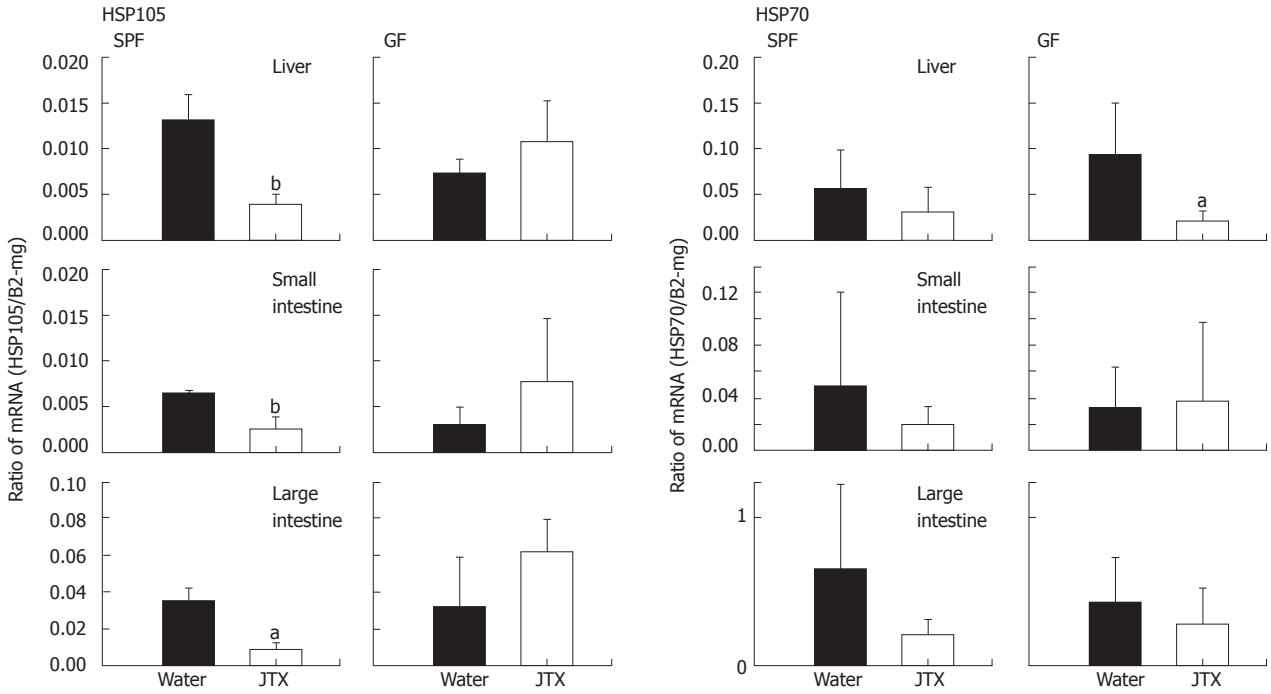

Figure 2 Effect of JTX (1 g/kg body weight for 2 wk) on the expression of HSP105 and HSP70 mRNA in the liver, small intestine and large intestine detected by real-time RT-PCR in SPF and GF IQI mice (n = 5, mean ± SD, $^aP < 0.05$, $^bP < 0.01$ *vs* water).

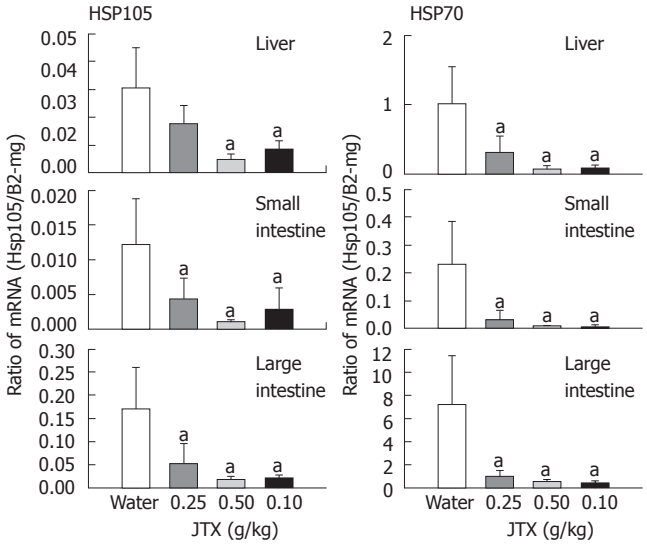

Figure 3 Effect of JTX (0.25, 0.5 and 1 g/kg body weight for 2 wk) on the expression of HSP105 and HSP70 mRNA detected by real-time RT-PCR in the liver, small intestine and large intestine in ICR mice (n = 4-5; mean ± SD, $^aP < 0.05$ *vs* water).

Change in the intestinal microflora by JTX

The differential expression of HSP105 and HSP70 mRNA by JTX between GF and SPF mice suggested strongly that the effect of JTX on HSP gene expression depended on intestinal microflora. To investigate how JTX affected intestinal microflora in the small intestine and large intestine, T-RFLP analysis was performed. Figure 4 shows one representative profile. We performed dendrogram analysis based on the Jaccard matching coefficients. UPGMA was used as the dendrogram type (Figure 5).

Table 1 Identification of decreased (98- and 115-base) and increased (1000-base) peaks in the T-RFLP chart by the administration of JTX

Base	Frequency (%)	Bacterial strain or clone	Homology (%)
98 base and	91.8	MC81C01	99-100
115 base	5.9	MC82C09	99-100
	2.4	*Bifidobacterium lactis*	97
1000 base	100.0	*Lactobacillus johnsonii*	99

Analysis of T-RFLP profiles of the small intestine showed significant differences between the JTX-treated group and the control group. Dendrogram analysis of T-RFLP patterns showed that the differences between the JTX-treated and the control group for large intestinal microflora were not statistically significant. Compared to the large intestinal microflora, the number of bacteria was much less in the small intestine. The several peaks with same sizes (some are indicated by the arrows in Figure 4) showed remarkable change by JTX treatment in all samples of the small intestine and large intestine. These disappeared (98-base T-RF produced by *Hha* I digestion and 118-base T-RF by *Msp* I , Figure 4) and appeared (1000-base T-RF by *Msp* I , data not shown) DNA fragments were cloned and 100 clones per each T-RF were subjected to DNA sequencing. Results are shown in Table 1. A 1000-base T-RF was supposed to be derived from *Lactobacillus johnsoni*. Over 90% of the 98- and 115-base T-RF clones showed a high homology with MC81C01, bacteria which were unknown except for the DNA sequence of 16S rDNA and was unculturable. The results clearly indicated that JTX administration brought about some change to the intestinal microflora.

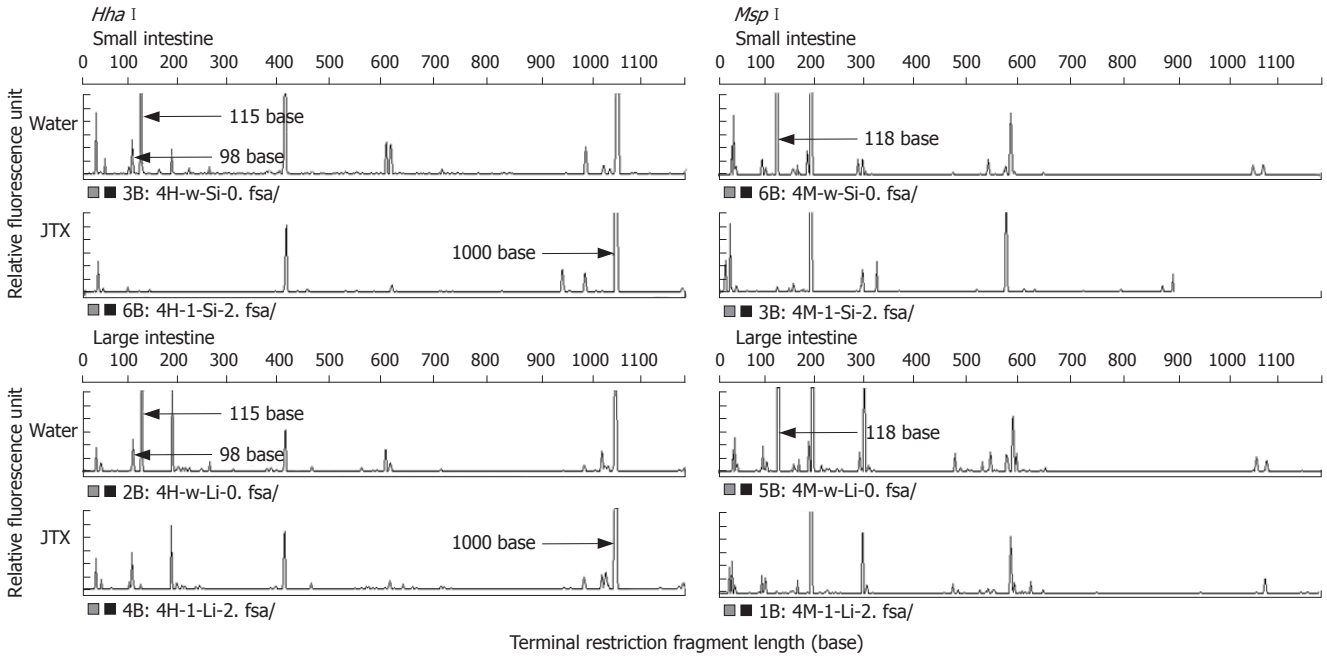

Figure 4 Change of the intestinal microflora in JTX-treated SPF ICR mice assessed by T-RFLP analysis digested with *Hha* I and *Msp* I (n = 5). The arrows indicate the remarkably changed peaks. Even though it is not clear that 1000-base peak was increased in the *Hha* I restricted fragments in this scale, it is obviously increased when the scale is changed.

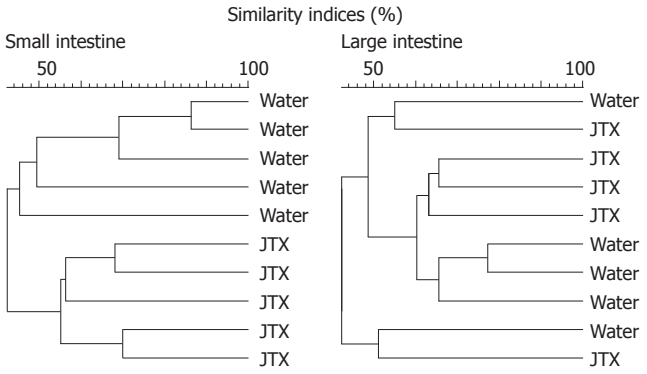

Figure 5 Dendrogram analysis of the intestinal microflora based on T-RFLP. Jaccard matching coefficients was performed and UPGMA were used as the dendrogram type.

Change in the expression of HSP105 and HSP70 mRNA by ciprofloxacin

The results described above addressed the possibility that JTX decreased HSPs *via* altered intestinal microflora. To investigate whether changes of intestinal microflora affect the expression of HSPs, mice were continuously provided the antibiotic ciprofloxacin (CPFX) in the chow for a week. CPFX is a fluoroquinoline antibiotic which inhibits DNA synthesis in bacteria *via* affecting bactrerial DNA gyrase. This antibiotic has an effect on both Gram-positive and -negative bacteria[19]. The apparent decrease in HSPs was observed in all examined cases, although the significant changes were shown only in the expression of HSP105 and HSP70 mRNA in the liver and HSP105 in the large intestine (Figure 6).

SA induced HSP105 and HSP70 mRNA without relation of the JTX administration

To clarify whether a direct interaction with intestinal

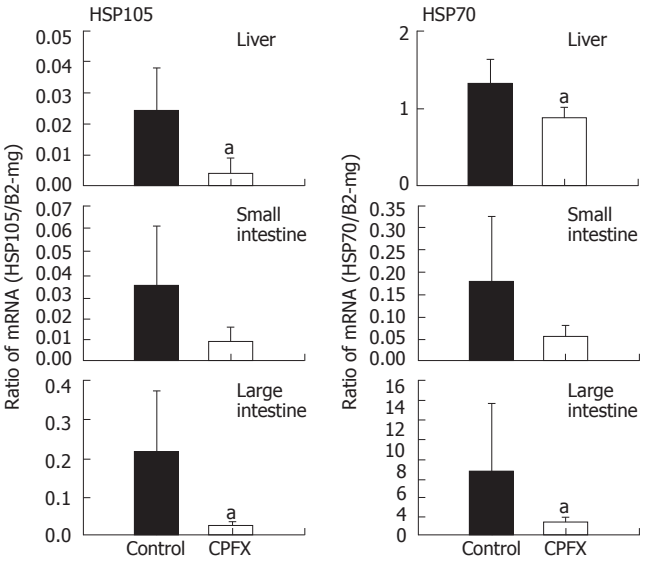

Figure 6 Real-time RT-PCR showing the change of expression of HSP105 and HSP70 mRNA in the liver, small intestine and large intestine of mice by the administration of CPFX in SPF ICR mice (n = 5, mean ± SD, $^aP < 0.05$, $^bP < 0.01$ *vs* control).

epithelial cells is involved in JTX's suppressive effect on HSPs expression, we examined the effect of JTX on the direct induction of the heat shock response. Sodium arsenite (SA) has been used to directly induce heat shock proteins in animals and various cell lines even though the mechanism is not known[20-22]. Intraperitoneal injection of SA induced HSP105 and HSP70 mRNA in the liver, small intestine and large intestine of ICR mice (Figure 7). The induction of these mRNA reached a peak within 1 h in these three organs. The levels of HSP70 and HSP105 expression turned back to a steady state within 4 h and 8 h, respectively. Mice with JTX administration for 2 wk were

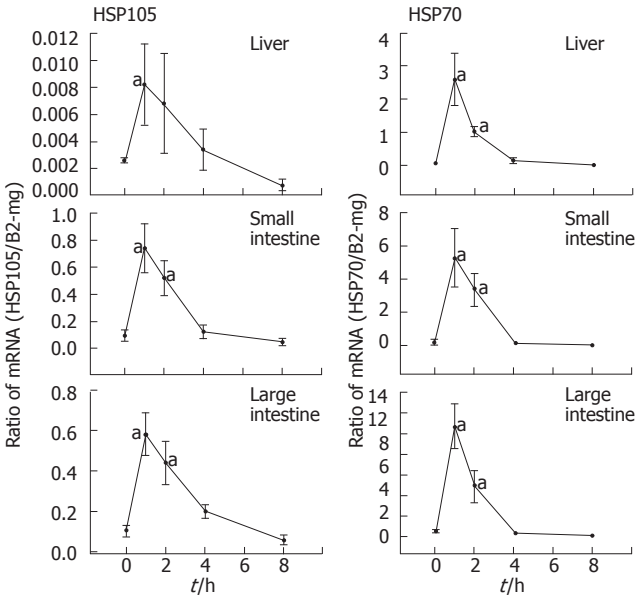

Figure 7 Induction of HSP105 and HSP70 mRNA by sodium arsenite detected by real-time RT-PCR in the liver, small intestine and large intestine of mice (n = 3, mean ± SD, $^aP < 0.05$ *vs* 0-h value).

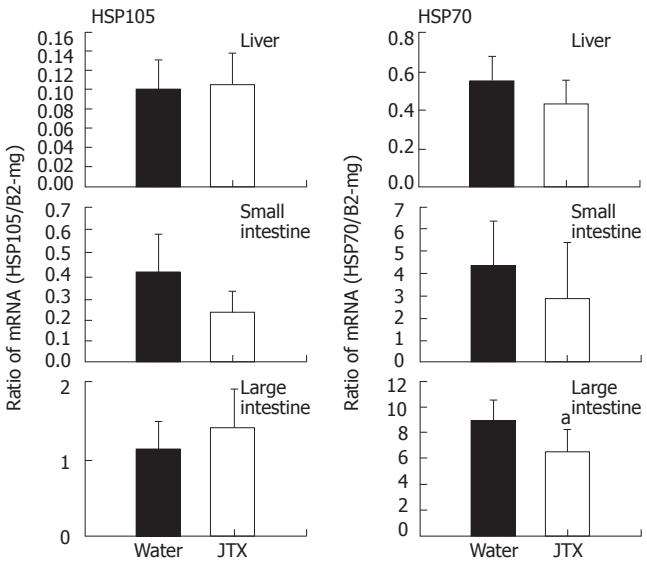

Figure 8 Effect of JTX on HSP105 and HSP70 induction by sodium arsenite detected by real-time RT-PCR in the liver, small intestine and large intestine of SPF ICR mice (n = 6, mean ± SD, $^aP < 0.05$, $^bP < 0.01$ *vs* water).

injected SA and sacrificed after 1 h. Though the modest decrease in HSP70 in the large intestine (significant) and in HSP105/70 in the small intestine (not significant) were observed, the induction ability of heat shock response was essentially retained in JTX-treated mice (Figure 8).

DISCUSSION

Kampo medicines have been suggested to have antibiotic or prebiotic effects[23,24]. Some constituent herbs of JTX and its ingredients have also been reported to have antimicrobial effects[25,26] and a putative stimulating effect on the growth of certain bacterial species[5]. Kampo medicines have been considered to modulate intestinal microflora and exert their beneficial effects in part by this modulation. However, to our knowledge, changes in the intestinal microflora by the administration of Kampo medicines have not been detected despite great effort till now. Two possible reasons for this are considered. First, recent studies using DNA-based estimation have suggested that 70%-80% of the total intestinal bacteria are unculturable, so that the conventional detection methods based on the bacterial cultures may miss significant changes in intestinal microflora[11,12]. Second, Kampo medicines contain many species of prebiotic compounds in relatively small quantities. The changes induced by Kampo drug administration are assumed to be subtle and complex compared to those induced by a simple prebiotic compound, such as oligosaccharide or starch.

In this study, we used T-RFLP analysis, which allowed us to detect the change of intestinal microflora including currently unculturable bacteria as a pattern of Figure 4 chart. Though the changes of peaks by JTX administration were small and complex, the dendrogram analysis based on Jaccard matching showed a significant change of gut bacteria in the small intestine (Figure 5). On the other hand, because the number of the bacteria is much larger in the large intestine than in the small intestine, the change of the microflora is rather difficult to visualize. Several peaks in T-RFLP chart that were changed with JTX were derived from the unculturable bacteria like MC81C01 or MC82C09 shown by DNA sequencing (Table 1). The present study indicates that T-RFLP analysis is a useful tool for detecting the effect of Kampo medicines on the intestinal bacterial population.

The result of T-RFLP also suggested that *Lactobacillus johnsoni* increased in JTX-treated mice. *Lactobacillus johnsoni* are being increasingly used as probiotics in functional foods and as vaccine carriers. This bacteria has various activities such as a preventive effect against gastrointestinal infection by several pathogens including *H pylori*[27] and *Salmonella enterica*[28]. It is also known to have functions, such as immunomodulation[29,30], anti-oxidative stress[31] and a therapeutic effect for chronic fatigue syndrome[32]. These are also expected in JTX[3], so it is possible that the pharmacological effect of JTX is, at least partly, due to the increase of *Lactobacillus johnsoni*. The function of *Lactobacillus johnsoni* is mediated by the modulation of pH and by production of lactic acid, bacteriocins, and other antibacterial molecules[28,33]. It may be possible through this function, MC81C01 or MC82C09 (98 and 115 base peaks) are suppressed as a result.

In this study, the effect of JTX on the expression of HSPs was observed only in the mice which had intestinal microflora (Figure 2). HSPs are classified into several families: HSP27, HSP60, HSP70, HSP90 and HSP105/110. The 70-ku heat shock protein (HSP70) family is one of the most highly conserved members of the HSP family[34]. HSP70 expresses ubiquitously and protects cells against lethal heat shock by denaturing proteins in an ATP-dependent manner[34]. HSP105/110 protein can be a substitute for HSP70 family proteins to modulate the aggregation of denatured protein when the

cellular ATP level decreases[35].

The role of HSPs in the gut is not well understood. The presence of HSP110 protein in the feces and in the large intestine has been detected[36]. Intestinal bacteria, such as *E. coli*[7] and *Salmonella enteritidis*[8], have been reported to induce certain HSPs in intestinal epithelial cells. Similar effects of intestinal bacteria-derived substances, such as LPS[37-40], *Staphylococcus* aureus enterotoxin B[41] and butyric acid[42], have also been known. Thus HSPs in the gut might be controlled by a certain intestinal bacteria and may reflect the status of intestinal microflora in the gut.

As a fact, the administration of the antibiotic CPFX reduced the expression of HSPs (Figure 6). Another report showed that metronidazole decreased HSP72 and HSP25 in the colonic mucosa[37]. Furthermore, comparing the expression of HSPs in GF mice and SPF mice, GF mice appeared to express less HSP105 and HSP70 mRNA though the significant differences were shown only in those of HSP105 in the liver and small intestine (data not shown). These results suggested that the intestinal microflora induces the expression of HSP105 and 70 mRNA in the gut and liver.

Though many studies have not examined the HSPs in the liver, bacteria-derived substances including LPS[43] are transported to the liver by the portal vein, and therefore, affect the expression of liver HSPs. It may be possible that this change of expression in the liver is caused by the LPS from the bacteria in the intestine. Reduced expression of HSPs in GF or antibiotic-treated mice reflects decreased inflammation in the intestinal tract burdened by intestinal bacteria. The incidence of liver tumors has been reported to be higher in conventional mice than in GF mice presumably because bacterial metabolism resulted in the production of various mutagens[44]. Furthermore, GF mice are generally expected to live longer than SPF mice[35]. The expression levels of HSPs in the intestines and/or liver, therefore, may correspond to the level of inflammation produced in the intestinal tract. Certainly, we observed that HSP105 and 70 gene expression changed in the gene chip analysis together with cytokine-related molecules, especially interferon alpha-related molecules.

The expression of HSPs changed by JTX depended clearly on the intestinal microflora because the change was not observed in GF mice. Also, because the induction of HSPs by SA was not affected by JTX administration, JTX did not decrease directly the expression of HSPs in intestinal epithelial cells. Therefore, this study addresses the possibility that a change of intestinal bacteria by JTX caused the change of HSPs expression in the intestine and liver. Likewise, JTX may change other gene expression *via* intestinal microflora leading to a change of the host immune state. A recent study has suggested that only single bacteria can direct the maturation of the host immune system[45].

The meaning of the change of the intestinal microflora is not clear in this study because these were the observations in normal mice. Our previous study showed that the change of the intestinal flora is very important in the perturbation model[9]. In that, we partially excised the mouse liver and induced elevations of serum ammonia. This elevation was suppressed by JTX. Because the liver function was not improved with JTX, we evaluated the intestinal microflora. The change of the microflora after partial hepatectomy was minimized with JTX and this change was speculated to be the main reason of the suppressed serum ammonia elevation. Follow-up human studies are desirable to confirm this phenomenon with herbal products.

This study did not identify the active compound(s) responsible for the observed modulation of intestinal microflora. Various major ingredients, many of which are saccharide-containing compounds, isolated from herbs have been known to be usable (hydrolyzed) for very limited bacterial species and allow such bacteria to grow selectively[46-48]. Therefore, JTX may contain such ingredients responsible for the selective growth of specific bacterial strains. Further, several polysaccharides have been identified from JTX as immuno-modulating compounds mainly functioning in the digestive tracts though the mechanism of action has not been elucidated[49,50].

To investigate whether polysaccharides in the JTX are responsible to regulate HSPs gene expression, we fractionated JTX into polysaccharide-enriched and polysaccharide-poor fractions by ethanol precipitation. IQI mice were administered with each fraction of JTX orally for 2 wk and the change of expression of HSPs was investigated by gene chip analysis. Unexpectedly, both fractions did not decrease HSP105 or HSP70 mRNA levels (data not shown). Though the reason for this is not clear, two possibilities are considerable. Firstly, active compound(s) may have been lost or inactivated during the fractionation of JTX. For example, because polysaccharide-poor fractions contained ethanol, volatile ingredients may be lost through freeze-dry process to obtain powder extracts. Secondly, collaboration of multiple actions by different compounds may be necessary to exert the observed effect[51]. Further and extensive investigation, in any case, is necessary for the clarification of the active component(s) and the mechanism(s) of action of JTX.

In conclusion, this study indicates that the administration of JTX changes the intestinal microflora and, as a result, changes expression levels of HSP105 and HSP70 in the liver and intestine. The expression levels of HSPs in these organs may correspond to the level of inflammation produced in the intestinal tract and JTX plays a role to reduce the inflammation. We propose the novel mechanism of multi-herbal products, i.e. altered gene expression as result of altered intestinal microflora.

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