Differences in human gene expression induced by
tokishakuyakusan containing different grades of
Angelica radix

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Abstract
Pharmacological effects of two grades of Japanese Angelica radices were compared by global transcriptional analysis in humans, and the differences were revealed by alterations in gene expression. An excellent-grade radix of Angelica acutiloba Kitagawa (Yamato-toki), produced in Nara, Japan, and a low-grade radix produced in China were dispensed to tokishakuyakusan formulation. These formulations were administered to separate groups of female participants for 4 weeks. Excellent-grade and low-grade Angelica radix were administered to 24 and 18 subjects, respectively. A global transcriptional dataset was obtained by cDNA microarray using RNA from peripheral blood nuclear cells of subjects. Partial least squares discriminant analysis and Student’s t-test were combined to extract the transit data: (1) genes altered by the administration of both types of tokishakuyakusan and (2) genes altered by either type. Transients in (1) were categorized to have protein transport and transcription functions, with significance determined by the z-test for comparing two proportions. Transients in (2) were categorized to have translation, metabolic process, transcription, and ion transport functions. Results of (2) suggest that the difference exist in the protein level, metabolism, and cell regulation. Estrogen-related genes are included in the transcription category of (1), and neuronal-related genes are included in the metabolic process and ion transport categories of (2). These transitions may be related to the positive effect of each preparation in alleviating complaints.

Key words cDNA microarray, Kampo medicine, Toki.
Abbreviations FDR, false discovery ratio; PCA, principal component analysis; PLS-DA, partial least squares-discriminate analysis; VIP, variable importance value.

Introduction
Angelica acutiloba radix is one of the most popular herbal medicines used by the Japanese people; it is traditionally used to treat gynecological disorders such as irregularity of menstruation, menstrual pain and anemia.1) Two Angelica radix varieties, A. acutiloba Kitagawa (Yamato-toki) and A. acutiloba Kitagawa var. sugiyama Hikino (Hokkai-toki), are used medically in Japan in accordance with the Japanese Pharmacopoeia. Yamato-toki is known to be of higher grade than Hokkai-toki. Yamato-toki produced in Nara prefecture, Japan, is considered to be the highest-grade radix. The radix grade is usually based on appearance, aroma, color, and taste, as determined by highly trained
specialists. On the basis of clinical experiences, herbal
doctors and Kampo pharmacists have realized that grading
of radixes correspond to their pharmacological
effects. However, this realization has not been docu-
mented with supporting scientific evidence in medical or
pharmaceutical literature.

The pharmacological effects of tokishakuyakusan
were reported by cDNA microarray analysis.21 Gene ex-
pression induced by the administration of tokishaku-
yakusan on peripheral blood nucleated cells was
discussed. In this study, tokishakuyakusan was dis-
pensed with an excellent-grade radix produced in Nara
and with a low-grade radix produced in China; the gene
expression profiles of the two formulations were com-
pared using the partial least squares discriminate anal-
ysis (PLS-DA; a regression analysis) and Student’s t-test
(a statistical analysis). Extracted transients were func-
tionally categorized with the z-test to compare the two
formulations.

Materials and Methods

Galenicals: The Japanese Angelica radix price is usu-
ally classified into one of three grades: excellent-, mid-
dle- and low-grade in the market. Two Angelica radices,
an excellent-grade radix produced in Nara, Japan, and a
low-grade radix produced in China, were purchased
from Fukuda-shoten (Nara, Japan). They were sepa-
rately dispensed to tokishakuyakusan. Other galenicals
dispensed to tokishakuyakusan were described in a pre-
vious report.21 The same lot of each galenical was dis-
pensed to two formulation types, except for Angelica
radix.

Three-dimensional high-pressure liquid chromato-
ography analysis and principal component analysis
based on the data from liquid chromatography cou-
pled with tandem mass spectrometry: Three-
dimensional high-pressure liquid chromatography (3D-
HPLC) analysis was carried out using a Waters Alliance
system (Waters, Milford, MA) equipped with a binary
solvent delivery system and an autosampler. Chromato-
graphy was performed on a 4.6 \times 250 \text{mm} Cosmosil
5C18-MS-II column (Waters). The mobile phase con-
sisted of (A) 0.05% trifluoroacetic acid (TFA) in
acetonitrile (ACN) and (B) 0.05% TFA in water. The
flow rate was 1.0 mL/min. The column and autosampler
were maintained at 40°C and 10°C, respectively. The elution
conditions were as follows: a linear gradient
from 5% to 57% B (0.0-30.0 min), isocratic conditions
with 57% B (30.1-50.0 min), and a linear gradient up to
100% B (50.1-55.0 min). Absorption spectrometry was
performed using a Waters 2996 photodiode array.
Absorbance from 210 nm to 410 nm was measured. Angelica radix was chopped and mashed to a powder.
After freeze-drying, 10 mg of the powder was extracted
with 1.0 mL water at 100°C for 60 min. Peaks were
identified by comparison with standard compounds;
gallic acid and paoniflorin standards purchased from
Wako Co. (Osaka, Japan). Other peaks were identified
on the basis of Katoh’s report.32

Liquid chromatography coupled with tandem mass
spectrometry (LC/MS) was carried out in an ACQUITY
UPLC system (Waters) equipped with a binary solvent
delivery system and an autosampler. Chromatography
was performed with an ACQUITY UPLC BEH C18
column (100 \times 2.1 \text{mm}, i.d.; Waters) with a 1.7-\mu m
particle size. The mobile phase consisted of 0.1% for-
ic acid in water (C) and 0.1% formic acid in ACN (D)
using a gradient program of 5-100% (D) in 0.0-10.0
min. The flow rate was 0.25 mL/min and the injection
volume was 10 \mu L. The column and autosampler were
maintained at 40°C and 10°C, respectively. The LC was
coupled to a Micromass LCT Premier, an orthogonal
acceleration time-of-flight mass spectrometer (TOFMS)
with an electrospray ionization device (Waters, Manches-
ter, UK). The system was tuned for optimum sensitiv-
ity and resolution using leucine-enkephalin (50 pg/\mu L
and infused via a syringe pump at 3.0 \mu L/min) in posi-
tive electrospray ionization mode. The TOF mass spec-
trometer was operated in the “W” mode and the
optimized conditions were as follows: capillary voltage,
2500 V; sample cone voltage, 50 V; desolvation tem-
perature, 350°C; source temperature, 120°C; cone gas
flow, 50 L/h; desolvation gas flow, 600 L/h; TOF flight
tube voltage, 7200 V; reflectron voltage, 1800 V; and
MCP detector voltage, 2500 V. The TOF mass spectrom-
trometer was calibrated routinely using a sodium
formate solution infused at a flow rate of 5 \mu L/min.
Sodium formate solution was prepared by mixing 0.05
mmol NaOH solution with 0.05% formic acid in a 9:1
proportion of ACN and water. Samples were diluted 5 fold, passed through a 0.45-μm pore filter (Advantec, Chiba, Japan) and analyzed by LC/MS. The creation of matrix data from LC/MS data was performed with MZmine in 5 steps.\(^5\) First, the “Centroid peak detector” was used for peak detection. Then, the “Fast aligner” was used for peak alignment. “Linear normalization” was then used with the total raw signal for normalization. Thereafter, “Filter out rare peaks” was used to delete peaks that were present in only 1 or 2 samples. Finally, “Fill in empty gaps” was used to fill the empty columns. Principal component analysis (PCA) using mean-center preprocessing was performed with Pirouette ver. 3.11 software (Infometrix, Inc., WA, USA).

**Participants:** Forty-two females, aged 21-47 years (mean ± S.D., 34.8 ± 8.8 years), gave written consent to participate in this study. Subjects were excluded if they were currently receiving any treatment, including herbal medicine; if they had received medical treatment within a month prior to the study; if they had a past history of a significant disease; if they smoked; or if they were or might have potentially been pregnant. This study was approved by the Medical Ethics Committee of Oriental Medicine Research Center, Kitasato University, and the Ethical Guidelines for Human Genome/Gene Research (Ministry of Education, Culture, Sports, Science and Technology/Ministry of Health, Labour and Welfare/Ministry of Economy, Trade and Industry). Subjects were given tokishakuyakusan for 4 weeks. Examination periods were November to December 2007 for 20 subjects who received tokishakuyakusan dispensed with the excellent-grade radix; November to December 2008 for 18 subjects who received low-grade radix; and 4 who received excellent-grade radix. Subject’s age was randomly distributed in the experiment years and the administrated radix grades. Patients were blinded to the administered medicine.

**Data processing:** Analysis of cDNA microarray based on the one-color hybridizing method and questionnaire data were described in a previous report.\(^2\) The fluorescence intensities of probes were quality-controlled using Feature Extraction and GeneSpring GX software (Agilent Technologies, Santa Clara, CA). The quantile method was used for fluorescence intensity correction in the preadministration test.\(^5\) PCA using mean-center preprocessing was carried out on the basis of the gene expression data obtained at preadministration. The intensity correction on pre- and post-administration test was performed on MA plots using the TREBAX program (http://kanaya.naist.jp/~skanaya/Web/software/trebax/trebax2.html),\(^6\)\(^8\) and the false discovery ratio (FDR) for each transient was estimated by using Storey and Tibshirani’s method.\(^9\) FDR is an index indicating the proportion of false positives (meaning that the null hypothesis is true) among selected positives (meaning that the null hypothesis is rejected). For example, when 500 genes are selected as differentially expressed with an FDR of 0.01, 5 genes (500 × 0.01) are expected to be false positives, whereas an FDR of 0.05 means that 25 genes (500 × 0.05) are expected to be false positives. PLS-DA was carried out by SIMCA-P + ver. 12 software (Umetrics, Umeå, Sweden). This regression analysis is a suitable method for group discrimination modeling of complex data.\(^10\)\(^11\) Model accuracy is assessed by the Factor, R2, and Q2 values in the analysis.\(^12\) Factor is the number of latent factors in the regression, for which a smaller number means a higher accuracy. R2 is the coefficient value of correlation by the least square method of regression. Q2 is the value of cross validation by the leave-one-out method, in which 1 means 100% matching. Q2 is calculated as follows:

\[
Q^2 = 1 - \frac{\sum_{i=1}^{N} (y_i - \hat{y}_i)^2}{\sum_{i=1}^{N} (y_i - \bar{y})^2}
\]

where \(N\) indicates sample number, \(\hat{y}_i\) is the \(i\)th dataset left out, and \(\bar{y}\) is the mean of the dataset. Functional categorization of genes were performed using a human Gene Ontology database and a software tool called “KNApSAcK human gene classifier,” which allows comparison of the ratio of genes corresponding to the functional categories in an arbitrary gene set with that in the entire human genome gene set (http://kanaya.naist.jp/GeneClassifier/top.jsp?fn=human).\(^2\)

**Galenical and questionnaire administration:** Hot-water extracts of the tokishakuyakusan formulations were administered to the participants for a 4-week period. A questionnaire based on the 3- or 5-level rating system was provided to the participants. The content of the questionnaire have been described in a previous report.\(^2\)
Results

3D-HPLC profiles of Angelica radixes and tokishakuyakusan formulations, and PCA based on LC/MS data of the formulations: 3D-HPLC profiles and peak identification with hot-water extracts of excellent- and low-grade Angelica radixes and the tokishakuyakusan formulation used in this administration experiment are shown in Fig. 1. Although the differences among the radix grades in terms of their ligustilide content and on component fingerprintings have been reported, no remarkable differences were observed in the HPLC profiles of the 2 grades of Angelica radixes. Furthermore, no remarkable difference was observed between the tokishakuyakusan formulations with the excellent-grade radix and low-grade radix, respectively (data not shown).

![Fig. 1 Comparison of the 3D-HPLC profiles of the hot-water extract of (A) an excellent-grade Angelica acutiloba Kitagawa radix, (B) low-grade Angelica acutiloba Kitagawa radix, and (C) tokishakuyakusan formulation containing excellent-grade Angelica radix. 1: gallic acid, 2: paconiflorin, 3: psoralen, 4: xanthotoxin, 5: bergapten and isopimpinellin, 6: (Z)-ligustilide, and 7: (Z)-butyldeneptinalide.](image)

Fig. 2 Score plotting of PCA based on the LC/MS data of the hot-water extracts of the tokishakuyakusan formulation. The cumulative contribution ratio of PC1 is presented on the x-axis, and that of PC2, on the y-axis. The formulations contained either excellent-grade Angelica radix (solid-red), excellent-grade radix using in administration of this experiment (dark red), low-grade radix (green), and low-grade radix using in administration of this experiment (dark-green argyle). The dark-red and dark-green circles demarcate the excellent-grade class and low-grade class, respectively.

Analysis of gene expression alteration dataset resulting from administration of two types of tokishakuyakusan: Transcriptome analysis of 41,000 probes in a cDNA microarray set was performed on the mRNA of subjects who were given tokishakuyakusan dispensed with the excellent-grade or low-grade radix. The GeneSpring GX software was used to extract 13,455 reliable probes. They contained 8,596 genes which were probes attached official full name provided by HUGO Gene Nomenclature Committee (http://www.genenames.org/aboutHGNC.html).

Two types of tokishakuyakusan formulations were administered to 2 separate groups. The PCA output for the gene expression dataset at preadministration is shown in Fig. 3. The cumulative contribution ratio was more than 60% at PC2 in the analysis. The output of
PC1 and PC2 indicates that the 2 separate groups are randomly distributed in independent classes (light red: participants in 2007 and dark red and green: participants in 2008). These results suggested that the difference in the 2 participant groups of 2007 and 2008 was not significant in terms of the background gene expression.

No unusual results were attained in the biochemical tests, hematological tests, and tests for sex hormone levels in blood (follicle stimulation hormone, luteinizing hormone, prolactin, estradiol, and progesterone); furthermore, no significant difference was noted in the test results of the 2 groups on pre- and postadministration with Student’s t-test (data not shown). These results suggested that the difference between the 2 groups on a physiological background was not significant.

PLS-DA was performed on the two datasets corrected using the TREBAX program to estimate the contribution of transients in discriminating between the administration results of the two formulations. A plot depicting predicted and actual values of the subjects is shown in Fig. 4 (Factor: 3, R2: 0.938, Q2: 0.406). The actual value for patients who were given the formulation containing the excellent-grade radix was set at 0.0, whereas that for patients given the low-grade radix was set at 1.0. Predicted values were distributed from -0.3 to 1.1. The excellent-grade radix subjects were 53% (from -0.3 to 0.5), and the low-grade radix subjects were 25% (0.8 to 1.1), of all subjects in the prediction. This prediction gave a 100% correct classification, which indicates successful PLS-DA regression modeling. The degree of contribution of each factor on discrimination between the two groups is expressed by variable importance values (VIP) in PLS-DA. High VIP values, i.e., ≥1.0, are relevant for explaining the classification; 5,171 probes showed a VIP of ≥1.0.

**Functional categorization of genes commonly regulated by two types of tokishakuyakusan:** Student’s t-test was performed on the datasets to compare the administration results of two formulations; 8,472 probes showed a difference in p-value of ≥0.05, and 5,188 probes showed a VIP of <1.0. They were recognized to be commonly regulated genes with the same tendency —upregulated or downregulated— toward each formulation; that is, the grade of radix has no effect on gene regulation induced by tokishakuyakusan. The 5,188 probes contained 514 significantly upregulated and 154 significantly downregulated probes (p <0.05 with an FDR of <0.05 on comparison between pre and post administration). The 514 upregulated probes contained 372 genes, whereas the 154 downregulated probes contained 110 genes.

The functional category especially affected by administration shows a higher value of enrichment than
the other category. The gene enrichment value was calculated by the number of genes matched with their corresponding functions in input data divided by the gene frequency. Statistically significant categories by the z-test for comparing two proportions \( p < 0.01 \), numbers matched with the corresponding function in input data \( \geq 10 \), gene enrichment value \( >1.5 \) are shown in Table 1. Protein transport and transcription are in the upregulated categories, and none in the downregulated category (C3).

**Functional categorization of genes regulated by the excellent-grade radix:** By using a \( t \)-test comparison between the two administration groups, 4,983 probes showed a \( p \)-value of \( <0.05 \), 1,887 of which have a VIP of \( \geq 1.0 \). These 1,887 probes contained 564 significantly expressed probes resulting from the administration of the formulation dispersed with the excellent-grade radix \( p <0.05 \), FDR <0.05). Two hundred twenty-one upregulated probes contained 113 genes, whereas 343 downregulated probes contained 268 genes. Significant categories by the z-test are shown in Table 2. Protein synthesis-protein metabolic process-translation is in the upregulated category, and metabolism-metabolic process and transcription-metabolic process-transcription are in the downregulated category.

**Functional categorization of genes regulated by the low-grade radix:** The 1,887 probes mentioned above contained 491 significantly expressed probes resulting from administration of the formulation dispersed with the low-grade radix \( p <0.05 \), FDR <0.05). Two hundred sixty-four upregulated probes contained 219 genes, whereas 227 downregulated probes contained 118 genes. Significant categories by the z-test for comparing two proportions are shown in Table 3. Metabolism-metabolic process and transcription-metabolic process-transcription are in the upregulated category, whereas transcription-metabolic process-ion transport is in the downregulated category.

**Comparison of patient improvement resulting from administration of two tokishakuyakusan formulations:** The questionnaire analysis was performed on the datasets before and after the administration of the two formulations. The percentages of subjects who showed improvement are shown in Table 4. On administration of the excellent-grade radix, the categories feeling cold, edema, and gynecological symptoms have a \( >75\% \) value, with a significant difference between pre and post administration \( p <0.01 \). On administration of the low-grade radix, the categories feeling cold, edema, gynecological symptoms, psychoneurotic symptoms, and

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**Table 1** Functional categories of genes significantly upregulated or downregulated by both tokishakuyakusan formulations (excellent- and low-grade Angelica radices; significance by the z-test for comparing two proportions, \( p <0.01 \))

<table>
<thead>
<tr>
<th>Regulation</th>
<th>Functional category</th>
<th>Count</th>
<th>Total</th>
<th>Enrich.</th>
<th>Symbol (Gene ID)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up</td>
<td>1. Transport</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>facilitation</td>
<td>10</td>
<td>309</td>
<td>3.29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protein transport</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protein transport</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Up</td>
<td>2. Transcription</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Metabolic process</td>
<td>25</td>
<td>1130</td>
<td>2.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transcription</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Down</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#1. Functional categorization by the KNAPSAK human gene classifier. #2. Number of genes matched with their corresponding functions in input data \( n \geq 10 \), gene enrichment value \( >1.5 \). #3. Number of genomic genes. #4. Gene frequency enrichment value calculated by the following.

\[
\text{Enrich.} = \frac{\text{number of B/1,067 of input data}}{\text{number of C/23,540 of all genomic genes}}
\]

#5. Genes showing unidentifiable differences in expression between both administrations of tokishakuyakusan, showing a VIP of \( <1.0 \) by PLS-DA and a \( p \)-value of \( \geq 0.05 \) by Student’s \( t \)-test.
Table 2  Functional categories for genes significantly upregulated or downregulated by tokishakuyakusan dispensed with excellent-grade *Angelica* radix (significance by the z-test for comparing two proportions, $p < 0.01$)

<table>
<thead>
<tr>
<th>Regulation</th>
<th>Functional category</th>
<th>Count</th>
<th>Total</th>
<th>Enrich.</th>
<th>Symbol (Gene ID)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up</td>
<td>3. Protein synthesis</td>
<td>Metabolic process</td>
<td>Translation</td>
<td>11</td>
<td>238</td>
</tr>
<tr>
<td>Up</td>
<td>4. Metabolism</td>
<td>Metabolic process</td>
<td>Translation</td>
<td>11</td>
<td>430</td>
</tr>
<tr>
<td>Up</td>
<td>5. Transcription</td>
<td>Metabolic process</td>
<td>Transcription</td>
<td>22</td>
<td>1130</td>
</tr>
</tbody>
</table>

#1. Functional categorization by the KNAPsAcK human gene classifier. #2. Number of genes matched with their corresponding functions in input data ($n \geq 10$, gene enrichment value $>1.5$). #3. Number of genomic genes. #4. Gene frequency enrichment value calculated by the following.

(number of B/1,067 of input data) $\div$ (number of C/23,540 of all genomic genes)

#5. Genes showing identifiable differences in expression between two administrations of tokishakuyakusan, showing a VIP of $\geq 1.0$ and a $p$-value of $<0.05$ (Student’s $t$-test).

Table 3  Functional categories for genes significantly upregulated or downregulated by tokishakuyakusan dispensed with the low-grade *Angelica* radix (significance by the z-test for comparing two proportions, $p < 0.01$)

<table>
<thead>
<tr>
<th>Regulation</th>
<th>Functional category</th>
<th>Count</th>
<th>Total</th>
<th>Enrich.</th>
<th>Symbol (Gene ID)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up</td>
<td>6. Metabolism</td>
<td>Metabolic process</td>
<td>Translation</td>
<td>11</td>
<td>430</td>
</tr>
<tr>
<td>Up</td>
<td>7. Transcription</td>
<td>Metabolic process</td>
<td>Transcription</td>
<td>24</td>
<td>1130</td>
</tr>
<tr>
<td>Down</td>
<td>8. Transport facilitation</td>
<td>Transport</td>
<td>Ion transport</td>
<td>10</td>
<td>555</td>
</tr>
</tbody>
</table>

#1. Functional categorization by the KNAPsAcK human gene classifier. #2. Number of genes matched with their corresponding functions in input data ($n \geq 10$, gene enrichment value $>1.5$). #3. Number of genomic genes. #4. Gene frequency enrichment value calculated by the following.

(number of B/1,067 of input data) $\div$ (number of C/23,540 of all genomic genes)

#5. Genes showing identifiable differences in expression between two administrations of tokishakuyakusan, showing a VIP of $\geq 1.0$ and a $p$-value of $<0.05$ (Student’s $t$-test).
Table 4  Percentage of subjects with improved conditions resulting from administration of tokishakuyukan dispensed with excellent- or low-grade Angelica radix

<table>
<thead>
<tr>
<th>Complaint category</th>
<th>Percentage of improved subjects</th>
<th>Excellent-grade radix</th>
<th>Low-grade radix</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Feeling cold</td>
<td>88**</td>
<td>100**</td>
<td></td>
</tr>
<tr>
<td>(2) Edema</td>
<td>83**</td>
<td>100**</td>
<td></td>
</tr>
<tr>
<td>(3) Gynecological symptoms</td>
<td>75**</td>
<td>83**</td>
<td></td>
</tr>
<tr>
<td>(4) Psychoneurotic symptoms</td>
<td>67</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>(5) Constitutional symptoms</td>
<td>54</td>
<td>94**</td>
<td></td>
</tr>
<tr>
<td>(6) Mouth, nose, eyes and throat</td>
<td>63</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>(7) Cardiovascular and respiratory symptoms</td>
<td>42</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>(8) Digestive symptoms</td>
<td>46</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>(9) Urological symptoms</td>
<td>63</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>(10) Skin symptoms</td>
<td>46</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>(11) Muscle, bone and joints</td>
<td>54</td>
<td>67</td>
<td></td>
</tr>
</tbody>
</table>

#1: Data for excellent-grade radix was shown in a previous report.25 **: p < 0.01, Student’s t-test; +: p < 0.05, comparison of gene expression between pre and post administration.

Table 5  Notable alterations in gene expression

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene ID</th>
<th>Official full name</th>
<th>VIP#1</th>
<th>p-value#2</th>
<th>Ratio#3</th>
<th>Significant category#4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Excellent</td>
<td>Low</td>
</tr>
<tr>
<td>Estrogen-related genes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCOA7</td>
<td>135112</td>
<td>Nuclear receptor coactivator 7</td>
<td>0.76</td>
<td>3.9×10^-1</td>
<td>1.28**</td>
<td>1.20+</td>
</tr>
<tr>
<td>NR2C2</td>
<td>7182</td>
<td>Nuclear receptor subfamily 2, group C, member 2</td>
<td>0.10</td>
<td>7.8×10^-1</td>
<td>1.11**</td>
<td>1.09++</td>
</tr>
<tr>
<td>Nitric oxide (NO)-related gene</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCH1</td>
<td>2643</td>
<td>GTP cyclohydrolase 1</td>
<td>0.93</td>
<td>1.5×10^-1</td>
<td>1.11**</td>
<td>1.23++</td>
</tr>
<tr>
<td>Angiotensin-related genes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCBT1</td>
<td>55213</td>
<td>Regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing protein 1</td>
<td>0.12</td>
<td>5.7×10^-1</td>
<td>1.14+</td>
<td>1.21+</td>
</tr>
<tr>
<td>AGTR1</td>
<td>185</td>
<td>Angiotensin II receptor</td>
<td>0.83</td>
<td>1.1×10^-1</td>
<td>0.51**</td>
<td>0.65++</td>
</tr>
<tr>
<td>Vasopressin/oxytocin-related gene</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LNPEP</td>
<td>4012</td>
<td>Lecylcystinyl aminopeptidase</td>
<td>0.21</td>
<td>2.6×10^-3**</td>
<td>1.14+</td>
<td>1.38+</td>
</tr>
<tr>
<td>Neuron-related genes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHRNB2</td>
<td>1141</td>
<td>Cholinergic receptor, nicotinic, beta 2</td>
<td>1.68</td>
<td>1.4×10^-3**</td>
<td>0.99</td>
<td>0.49++</td>
</tr>
<tr>
<td>ATP1A3</td>
<td>478</td>
<td>ATPase, Na+/K+ transporting, alpha 3 polypeptide</td>
<td>1.01</td>
<td>1.7×10^-4**</td>
<td>0.97</td>
<td>1.36++</td>
</tr>
<tr>
<td>ATP1A1</td>
<td>476</td>
<td>ATPase, Na+/K+ transporting, alpha 1 polypeptide</td>
<td>1.59</td>
<td>1.7×10^-3**</td>
<td>0.92</td>
<td>1.22++</td>
</tr>
<tr>
<td>CACNA1H</td>
<td>8912</td>
<td>Calcium channel, voltage X 1D-dependent, T type, alpha 1H subunit</td>
<td>1.00</td>
<td>1.3×10^-2**</td>
<td>0.86</td>
<td>0.62++</td>
</tr>
<tr>
<td>ACCN1</td>
<td>40</td>
<td>Amiloride-sensitive cation channel 1</td>
<td>1.31</td>
<td>1.2×10^-3**</td>
<td>1.07</td>
<td>0.60++</td>
</tr>
<tr>
<td>Tumor-related genes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFRSF21</td>
<td>27242</td>
<td>Tumor necrosis factor receptor superfamily, member 21</td>
<td>0.09</td>
<td>4.5×10^-3**</td>
<td>1.26++</td>
<td>0.95</td>
</tr>
<tr>
<td>TNFSF10</td>
<td>8743</td>
<td>Tumor necrosis factor superfamily, member 10</td>
<td>1.15</td>
<td>4.6×10^-1</td>
<td>1.22+</td>
<td>1.12</td>
</tr>
<tr>
<td>TP53BP2</td>
<td>7159</td>
<td>Tumor protein p53 binding protein, 2</td>
<td>0.97</td>
<td>2.3×10^-2*</td>
<td>1.10**</td>
<td>1.24++</td>
</tr>
<tr>
<td>TNFRSF8</td>
<td>943</td>
<td>Tumor necrosis factor receptor superfamily, member 8</td>
<td>0.35</td>
<td>4.3×10^-3**</td>
<td>0.89++</td>
<td>1.1</td>
</tr>
<tr>
<td>TNFRSF6B</td>
<td>8771</td>
<td>Tumor necrosis factor receptor superfamily, member 6b</td>
<td>0.51</td>
<td>1.3×10^-2**</td>
<td>0.84+</td>
<td>1.11</td>
</tr>
<tr>
<td>TP73</td>
<td>7161</td>
<td>Tumor protein p73</td>
<td>0.15</td>
<td>2.1×10^-1</td>
<td>0.78+</td>
<td>0.67++</td>
</tr>
<tr>
<td>TNFRSF1B</td>
<td>7133</td>
<td>Tumor necrosis factor receptor superfamily, member 1B</td>
<td>1.69</td>
<td>4.6×10^-6**</td>
<td>0.66++</td>
<td>1.21+</td>
</tr>
</tbody>
</table>

#1: VIP by PLS-DA. #2: p-value by Student’s t-test between two datasets resulting from both administrations of tokishakuyukan. #3: Exponential values of mean of log(ratio) of gene expression of subjects on each administration. #4: Corresponding numbers for functional categories in Tables 1-3. Excellent: administration of tokishakuyukan dispensed with the excellent-grade radix. Low: administration of tokishakuyukan dispensed with the low-grade radix. **: p < 0.01; +: p < 0.05 by Kruskal-Wallis analysis. ++: FDR <0.01; +: FDR <0.05, comparison of gene expression between pre and post administration.
constitutional symptom have a >80% value, with a significant difference (p < 0.05).

Feeling cold, edema, and gynecological symptoms were commonly improved categories resulting from administration of the two formulations. Psychoneurotic symptoms and constitutional symptoms were improved only by the low-grade radix.

**Notable transients in significantly express transients:** Feeling cold, edema, and gynecological symptoms were commonly improved categories resulting from administration of both formulations. Therefore, notable transients, which could be related to patient improvement and which were commonly regulated by both formulations, were searched in the significant gene expression dataset. Estrogen-related genes, a nitric oxide (NO)-related gene, angiotensin-related genes, and a vasopressin/oxytocin-related gene were extracted (Table 5). NCOA7 and NR2C2 encode receptor-related proteins to hormones, including estrogen. The GCH1 protein is related to NO synthesis. PCBTBT1 encodes the angiotensin II receptor interact protein. AGTR1 encodes the angiotensin II receptor. The LNPEP protein cleaves vasopressin and oxytocin. Estrogen-related genes, NCOA7 and NR2C2, are contained in the transcription-metabolic process-transcription of the significant functional category in Table 1.

Psychoneurotic symptoms were improved only by the administration of the low-grade radix. Notable transients, which could be related to improvement of psychoneurotic symptoms and which were regulated only by the low-grade radix, were searched among the significantly expressed transients. CHRNA2 encodes the neuronal acetylcholine receptor. ATP1A1 and ATP1A3 encode the Na⁺/K⁺ transporting ATPases, which are related to membrane polarization. CACNA1H encodes the calcium channel subunit, which regulates membrane polarization. The ACCN1 protein may play a role in neurotransmission. All genes, except for ACCN1, were contained in the metabolism-metabolic process or transport facilitation-transport-ion transport significant functional categories in Table 3.

Tumor-related genes were also identified and shown in Table 5. TNFRSF21 induces cell apoptosis. TNFSF10

![Fig. 5](image_url) Plot of logarithmic values of altered gene expression induced by excellent grade- and/or low grade-Angelica radix tokishakuyakusan formulations. **: p < 0.01, Student’s t-test; *: p < 0.05, comparison of gene expression induced by administration of excellent- and low-grade Angelica radixes. Subjects administered the excellent grade-radix formulations in 2007 (open) and 2008 (filled square) and those administered low-grade radix formulations in 2008 (open triangle).
encodes a cytokine that belongs to the tumor necrosis factor ligand family. \textit{TP53BP2} regulates apoptosis and cell growth. \textit{TNFRSF8} is a positive regulator of apoptosis. The TNFRSF6B protein belongs to the tumor necrosis factor receptor superfamily. \textit{TP73} encodes the tumor protein p73, which is a member of the p53 family of transcription factors involved in cellular responses to stress and development. \textit{TNFRSF1B} regulates apoptosis. They are not included in the significant functional category.

The formulations containing excellent-grade radix were administered to subjects in 2007 and 2008 to assess the reproducibility of this study. The distribution of logarithmic values of altered expression of notable genes in the case of the participants administered excellent-grade radices in 2007, 2008 and also those in the case of participants administered low-grade radices in 2008 are shown in Fig. 5. Although 1 or 2 aberrant values were noted between the 2007 and 2008 participants who were administered excellent-grade radices, no notable bias was observed in the distribution of any of the values, except in the case of genes showing significantly different expression between participants administered the excellent-grade and low-grade radices, respectively (**: \( p < 0.01 \) and *: \( p < 0.05 \)), as determined by the \( t \)-test. This distribution trend confirms the experimental repeatability in the case of administration of excellent-grade radix.

**Discussion**

\textit{Angelica} radix grade difference is shown by human transcriptome analysis, and genes that seem to have functions related to the pharmacological effects of each medicine are reported in this study.

Tianniam \textit{et al.} reported a method of discriminat

ing the quality of \textit{Angelica} radix using the metabolite fingerprinting technique based on LC/MS data.\textsuperscript{13} In this report, the PCA output based on the LC/MS data showed independent 2 classes of tokishakuyakusan formulations containing excellent- and low-grade \textit{Angelica} radices although no remarkable difference was observed between the 2 grades in their 3D-HPLC profiles. Our PCA result is consistent with Tianniams' report. The 2 formulation types used in this study were located in each class. This suggested that the 2 formulations used in this experiment were typical excellent grade- and low grade-radix formulations, respectively, which were representative of each respective formulation group.

PLS-DA was performed to estimate transient contribution resulting from the administration of two formulations. Accurate prediction of subjects indicates successful PLS-DA modeling. The parent population is not accounted for in the multivariate analyses including PLS-DA. This leads to a trend of overestimation. To avoid this problem, a statistical analysis, Student’s \( t \)-test, was performed on the dataset. PLS-DA and \( t \)-test were applied in combination to extract genes that are essential to the classification of the results of two administrations. The combined tests can remove the transients, which are not essential to classification, showing a VIP of \( \geq 1.0 \) by PLS-DA, \( p \geq 0.05 \) by \( t \)-test, or a VIP of \(< 1.0 \) and \( p < 0.05 \) from the dataset.

The pharmacological effects of two radices were compared using the functional categories of transients. The categories corresponding to genes commonly regulated by both formulations, with significance determined by the \( z \)-test for comparing two proportions, are shown in Table 1. Protein transport and transcription categories indicate that administration changed the protein status in the tissue. The radix grade has an influence on this status. The significant functional categories corresponding to genes regulated by the excellent- and low-grade radices are shown in Tables 2 and 3, respectively. The two formulations showed different effects on gene expression. The genes upregulated by the excellent-grade radix were categorized to protein synthesis-protein metabolic process-translation, and the downregulated genes to metabolism-metabolic process and transcription-metabolic process-translation. The genes upregulated by the low-grade radix were categorized to metabolism-metabolic process and transcription-metabolic process-translation, and those downregulated to transcription-metabolic process-ion transport. Thus, the difference was found in protein status and metabolism, such as translation and ion transport. Each grade of radix affected the same functions, whereas the corresponding transitions did not overlap. The pharmacological differences of the radix grades are exhibited in the gene expression alterations.

The a number of items on the list of patient
complaints were improved with both tokishakuyakusan formulations as shown in Table 4. An image was created with these results; it showed that the excellent-grade radix was responsible for the intrinsic effects of the formulation, whereas the low-grade radix produced broad effects, which included intrinsic effects.

Estrogen-related genes NR2C2 and NCOA7 were commonly regulated by both formulations. They are included in a significant functional category, transcription, in Table 1. Estrogen is a hypothalamic thermoregulator\textsuperscript{14,15} and it is essential to the maintenance of the menstrual cycle. These indicate that estrogen may be related to the improvement of patient complaints, such as feeling cold and gynecological symptoms. These complaints were improved by both formulation types. The transition of NR2C2 and NCOA7 may be also related to these symptom improvements.

Improvement of the “feeling cold” complaint could be also related to vasodilation. Edema complaint may be related to the regulation of the amount of water in the body. NO and angiotensin are vasodilation control factors.\textsuperscript{16,17} Vasopressin/oxytocin and angiotensin could also be related to the regulation of body water.\textsuperscript{18,19} Genes related to these factors were extracted from the common transitions altered by both formulation types with the same tendency, upregulation or downregulation, even if they were not included in a significant functional category. GCH1, a NO-related gene, PCBTTB1 and AGTR1, angiotensin-related genes, LNPEP, a vasopressin/oxytocin-related gene, are shown in Table 5. Common improvement of feeling cold and edema complaints could be related to common transitions of GCH1, PCBTTB1, and AGTR1.

Psychoneurotic symptoms were improved by administration of the low-grade radix. Neuron-related genes, namely, CHRNBB2, ATP1A1, ATP1A3, CACNA1H, and ACCN1 were regulated only by the low-grade radix. Tokishakuyakusan has therapeutic effects on cerebral disorders and physiological effects on the transmitter status of neuronal cells,\textsuperscript{20} suggesting that the transition of neuron-related genes regulated by the low-grade radix could be related to improvement of psychoneurotic symptoms. Analysis of the questionnaire datasets showed that psychoneurotic symptoms were improved only by administration of the low-grade radix. Transition of CHRNBB2, ATP1A1, ATP1A3, CACNA1H and ACCN1 may be related to improvement of psychoneurotic symptoms.

Seven tumor-related genes, TNFRSF21, TNFSF10, TP53BP2, TNFRSF8, TNFRSF6B, TP73, and TNFRSF1B, were identified as notable transients on a previous report.\textsuperscript{21} The regulatory effect of tokishakuyakusan administration on tumor-related gene expression was a novel finding. They cannot be functionally categorized with statistical significance. TP73 was commonly regulated by both formulation types. TP53BP2 also shows a trend to be commonly altered, even if the significant difference by t-test was shown. Although there has been no clinical report to show that tokishakuyakusan is effective for patients with cancer, these results might imply that tokishakuyakusan affects the proliferation or development of tumor cell. Physiological analysis of formulation affection in cancer patients is a profitable future study.

The differences in component contents in the hot extract of galenicals can be identified by extensive chemical analysis with an independent extraction procedure even if the extraction and analysis are performed in parallel. Therefore, differences in the formulation quality were assessed in terms of the alteration in gene expression of the subjects. Two subject groups were predicted accurately (100%) on PLS-DA analysis on the basis of altered gene expression datasets (Fig. 4), even though the excellent-grade radix group comprised subjects who were administered excellent-grade radix in 2007 and 2008. Furthermore, no notable bias was observed in the excellent-grade subjects of 2008 to the excellent-grade of 2008’s in terms of the distribution of logarithmic values of express-alteration on the notable transients (Fig. 5). These results suggest that there was not significant difference between the formulation quality in 2007’s and 2008’s formulation quality.

Herbal doctors and Kampo pharmacists have realized that the radix grades correspond to pharmacological responses. However, psychoneurotic symptoms were improved by the low-grade radix but not by the excellent-grade radix. Our results suggest that overall realization based on clinical experiences is not assessed simply by one observation of the different effects of radix grades. Intrinsic effects of the excellent-grade radix may avert the side effects of tokishakuyakusan. The differences in organic components of the two radix grades
have been reported.\textsuperscript{21-25} Unfortunately, component differences cannot be directly linked to the complaint improvements based on the current scientific knowledge. Further studies are required to completely assess the radix grade in clinical experiments and to link their pharmacological differences with the difference in formulation—for example, molecular, biological, or biochemical analysis using mice dosing tests, or tests using a large population of human samples accompanied by accurate and large-scale chemical analysis of the radix and its formulation.

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