Abstract. Numerous effective anticancer drugs have been developed from botanical sources, and there remains a significant untapped resource in herbal medicines. In this study, we evaluated the chemical composition of extracts from American ginseng after steaming, the antiproliferative effects of the ginsenosides in the extracts on SW-480 human colorectal cancer cells, and their apoptotic mechanisms. American ginseng roots were steamed at 120°C for 2 or 4 h. Representative ginsenosides in the unsteamed and steamed extracts were determined using HPLC. The antiproliferative effects of the ginsenosides Rb1, Rg3 and Rh2 on SW-480 cells were determined by the MTS method. The effect of extract steamed for 4 h on apoptosis of SW-480 cell was assayed by flow cytometry after staining with annexin V/PI. The expression of 84 apoptotic-related genes, including TNF, mitochondria and p53 pathways, was determined using real-time quantitative PCR array analysis. The mitochondrial membrane potential ($\Delta \psi_m$) was analyzed after staining with FC-1. Steaming of American ginseng increased Rg3 and Rh2 content and antiproliferative activity significantly. The quantitative PCR array data demonstrated that multiple genes in mitochondrial pathway are involved in American ginseng-induced apoptosis of SW-480 cells and the expression profiling was validated by the cellular functional assay. The mitochondrial pathway may play a key role in American ginseng-mediated cancer cell apoptosis.

Introduction

Human colorectal cancer is a leading cause of cancer-related death in nearly all developed countries and the second most prevalent cancer worldwide. In the USA, the expected number of new colon cancer diagnoses in 2008 is 148,810 with 49,960 deaths (1). Half of all patients diagnosed with colorectal cancer eventually die from the disease. Less than 10% of patients with metastatic colorectal cancer will survive more than five years after diagnosis. Current treatment of colon cancer is surgical resection combined with chemotherapy using cytotoxic drugs and radiation therapy (2,3). Since this therapy is moderately successful for late stage cancers, preventive strategies and novel approaches for treatment of colorectal cancer are required.

Numerous effective anticancer drugs have been developed from botanical sources, and there remains a significant untapped resource in herbal medicines. Botanicals could potentially contain effective anticancer compounds for use alone or as adjuncts to existing chemotherapy to improve efficacy and reduce drug-induced toxicity (4-6).

American ginseng is an obligate shade perennial native to eastern North America. The commonly used part of the plant is the root, which is harvested in late summer to fall after several years of cultivation (7). American ginseng extract inhibited the growth of breast cancer cells (8) and enhanced the antiproliferative effect of cisplatin on human breast cancer cells, suggesting that it possesses anticancer activity (9). In separate studies, we have shown that steam-processing significantly improved the anticancer activity of ginseng on human colorectal cancer cells. The extract from roots steamed for 2 h had a greater effect than that of roots steamed for 1 h (10,11). In our studies, however, only the ginsenoside content of the crude plant material was determined, although the extract of these botanicals is often taken as a dietary supplement. Yet the mechanism whereby American ginseng root extract prevent cancer cell proliferation has not been studied.

In the present study, the content of representative ginsenosides in unsteamed and steamed extracts of American ginseng root was determined. Processing time extended up to 4 h before analysis of ginsenoside content with high
performance liquid chromatography (HPLC). Subsequently, the effects of the steamed extract on apoptosis of SW-480 colorectal cancer cells were assayed by flow cytometry, and the expression level of key genes involved in apoptotic pathways was determined using real-time quantitative PCR array.

Materials and methods

Reagents. Ginsenoside standards Rb1, Re, Rd, Re, Rg2, Rg3 and Rh2, purchased from Delta Information Center for Natural Organic Compounds (Xuancheng, AH, P.R. China), were of biochemical-reactent grade and at least 95% pure as confirmed by HPLC. HPLC grade ethanol, n-butanol, acetonitrile and absolute ethanol were obtained from Fisher Scientific (Pittsburgh, PA, USA). Trypsin, Leibovitz’s L-15 medium, fetal bovine serum (FBS), and penicillin/streptomycin solution (x200) were obtained from Mediatech, Inc. (Herndon, VA, USA). A CellTiter 96 Aqueous One Solution cell proliferation assay kit was obtained from Promega (Madison, WI, USA). Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) were obtained from BD Biosciences (San Diego, CA, USA).

Plant materials. The root of Panax quinquefolius L. was collected from Roland Ginseng Limited Liability Company (Wausau, WI, USA). The plant material was identified by Dr Chong-Zhi Wang according to the United States Pharmacopoeia NF 21, monograph: American ginseng (Panax quinquefolius L.). The voucher specimen was deposited at the Tang Center for Herbal Medicine Research at the University of Chicago.

Steaming treatment and extraction. The roots of American ginseng were steamed at 120°C for 2 or 4 h. The fresh and steamed roots were lyophilized to obtain dried samples. The extraction process was as follows: The dried roots were ground and extracted with 70% ethanol. The solvent of the extract solution was evaporated under vacuum. The dried extract was dissolved in water and then extracted with water-saturated n-butanol. The n-butanol phase was evaporated under vacuum and then lyophilized.

HPLC analysis. HPLC analysis was conducted on a Waters 2990 instrument with a Waters 996 photodiode array detector (Milford, MA, USA). The separation was carried out on an Alltech UltraspHERE C18 column (5 μ, 250x3.2 mm I.D.) (Deerfield, IL, USA) with a guard column (Alltech UltraspHERE C18, 5 μ, 7.5x 3.2 mm I.D.). Acetonitrile (solvent A) and water (solvent B) were used. Gradient elution started with 18% solvent A and 82% solvent B, and was changed to 21% A for 20 min, to 26% A for 3 min, and held for 19 min; then was changed to 36% A for 13 min, to 50% A for 9 min, to 95% A for 2 min and held for 3 min; and finally changed to 18% A for 3 min and held for 8 min. The flow rate was 1.0 ml/min and the detection wavelength was set to 202 nm. All the tested solutions were filtered through Millex 0.2-μm nylon membrane syringe filters (Millipore Co., Bedford, MA, USA) before use. The contents of ginsenosides in each sample were calculated using standard curves of ginsenosides.

Cell line and culture. The human colorectal cancer cell line SW-480 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and grown in Leibovitz’s L-15 medium supplemented with 10% FBS and 50 IU penicillin/streptomycin in a humidified atmosphere without CO2 at 37°C.

Cell proliferation analysis. Unsteamed and steamed American ginseng root extracts and ginsenosides were dissolved in 50% ethanol and were stored in 4°C before use. The SW-480 cells were seeded in 96-well plates. After 1 day, various concentrations of extracts/ginsenosides were added to the wells. The final concentration of ethanol was 0.5%. The controls were exposed to culture medium containing 0.5% ethanol without drugs. All the experiments were performed in triplicate and repeated 3 times. The cell proliferation was evaluated using an MTS assay according to the manufacturer’s instructions. Briefly, at the end of the drug exposure period, the medium was replaced with 100 μl of fresh medium. 20 μl of MTS reagent (CellTiter 96 aqueous solution) was added to each well, and the plate was returned to the incubator for 1-2 h. A 60-μl aliquot of medium from each well was transferred to an ELISA 96-well plate and its absorbance at 490 nm was recorded. The results were expressed as percentage of the control (ethanol controls set at 100%).

Apoptosis assay. The SW-480 cells were seeded in 24-well tissue culture plates. After culturing for 1 day, the medium was changed and extract from American ginseng root steamed for 4 h was added. After treatment for 48 h, the cells floating in the medium were collected. The adherent cells were detached with 0.05% trypsin. Then the culture medium containing 10% FBS (and floating cells) was added to inactivate the trypsin. After being pipetted gently, the cells were centrifuged for 5 min at 1500 x g. The supernatant was removed and the cells were stained with annexin V-FITC or annexin V-FITC/propidium iodide (PI). The cells were analyzed immediately after staining using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) and FlowJo 7.1.0 software (Tree Star, Ashland, OR, USA). For each measurement, at least 20,000 cells were counted.

Reverse transcription and real-time quantitative PCR array analysis. The expression of apoptotic genes was analyzed by reverse transcription-PCR (RT-PCR; Applied Biosystems 7300, Foster City, CA) using an RT2 real-time SYBR Green/ROX gene expression assay kit (SuperArray Bioscience Corp., Frederick, MD). Total RNA was isolated from solvent control and treated SW-480 cells using the RNeasy kit (Qiagen, Hilden, Germany). The first strand of cDNA was synthesized from 2 μg total RNA using a SuperScript II First-strand synthesis system (Invitrogen). Gene profiling was performed as described by the manufacturer using the RT2-profiler PCR array of the human Apoptosis Signaling Pathway including 84 apoptotic-related genes (catalog number: PAHS-012, SuperArray Bioscience). Briefly, quantitative real-time RT-PCR was performed in a reaction volume of 25 μl including 1 μl cDNA. PCR conditions were as follows: 95°C for 15 min followed by 40 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. GAPDH was
used as an internal reference gene to normalize the expression of apoptotic genes. Relative quantification of apoptotic genes was analyzed by the comparative threshold cycle (Ct) method. A Ct cycle was used to determine the expression level in control and in SW-480 cells treated with American ginseng extract steamed for 4 h. Thus, expression levels were expressed as an n-fold difference relative to the calibrator. For each sample, the apoptotic gene Ct value was normalized using the formula: \( \Delta \Delta C_t = \Delta C_t \text{(apoptotic genes)} - \Delta C_t \text{(GAPDH)} \). To determine relative expression levels, the following formula was used: \( \Delta \Delta C_t = \Delta C_t \text{(Treated)} - \Delta C_t \text{(Control)} \). The value was used to plot the expression of apoptotic genes using the expression \( 2^{-\Delta \Delta C_t} \).

Mitochondrial membrane potential (\( \Delta \psi_m \)) analysis. \( \Delta \psi_m \) was estimated using JC-1 (Molecular Probes, Eugene, OR, USA). This fluorescent compound exists as a monomer at low concentrations. At higher concentrations, it forms aggregates. Fluorescence of the JC-1 monomer is green. That of the aggregate is red. Mitochondria with intact membrane potential concentrate JC-1 into aggregates, which fluoresce red. De-energized mitochondria cannot concentrate JC-1 and fluoresce green (12). SW-480 cells were treated for 24 h with steamed extract at 100, 150 or 200 \( \mu \)g/ml. Control cells were grown in medium containing the same amount of ethanol as treated cells. Then, the adherent cells were incubated in 0.5 ml of medium containing JC-1 (2.5 \( \mu \)g/ml) for 20 min at 37˚C, and images were taken with a Nikon microscope ECLIPSE E800 (Nikon Corporation, Champigny-sur-Marne, France).

Statistical analysis. The data are presented as mean ± standard deviation (SD) with \( n=3 \). A one-way ANOVA determined whether the results had statistical significance. In some cases, the Student’s t-test was used for comparing two groups. The level of statistical significance was set at \( P<0.05 \).

Results

Effect of steaming treatment on the ginsenoside composition.

It is believed that the bioactive compounds in American ginseng are saponins, commonly referred to as ginsenosides (13). In our previous studies (10,11), after steaming treatment of American ginseng root, the contents of ginsenosides Rb1, Rc, Rd and Re decreased, while the content of ginsenoside Rg3 increased. Ginsenosides Rb1, Rc and Rd belong to the protopanaxadiol group. The effect of steaming on the protopanaxadiol group is shown in Fig. 1. During steaming, the protopanaxadiol group ginsenosides Re and Rg1 changed to Rg2 and Rh1. Since the ginsenoside content of the protopanaxadiol group of American ginseng is relatively low, we did not show their structures. In this study, we assayed the ginsenoside composition in the extracts.

From the HPLC chromatograms of extracts from unsteamed and steamed American ginseng roots, ginsenosides Rb1 and Re were the major constituents in unsteamed extract (Fig. 2A). After 4 h of steaming, ginsenosides Rg2 and Rg3 became the main constituents (Fig. 2B). During heat-processing, the peak areas of ginsenosides Re, Rb1, Rc and Rd decreased significantly, while ginsenosides Rg2 and Rg3 increased (Fig. 2A and B). The changes of constituents in the extracts are shown in Fig. 2C. We found the following percentages of ginsenosides in unsteamed extract: Re 19.0%; Rb1 34.2%; Rc 3.4% and Rd 6.5%. After 4 h of steaming, the contents were decreased to undetected, 1.6, 0.1 and 0.9%, respectively. In unsteamed extract, Rg2 was 0%; Rg3 0.06% and Rh2 0.06%. In extract steamed for 2 h, Rg2 was 3.2%; Rg3 5.9% and Rh2 0.3%. After 4 h of steaming, values changed to 2.3, 7.8 and 1.2%, respectively. Steaming for 4 h increased the content of Rg3 and Rh2 significantly (Fig. 2C).

Effects of extracts and ginsenosides on SW-480 human colorectal cancer cells. Using human colorectal cancer cell line SW-480, the antiproliferative effect of unsteamed and steamed American ginseng root extracts was evaluated. At 150 \( \mu \)g/ml, unsteamed extract inhibited cell growth by 6.4%. Steamed for 2 h, the extract inhibited cell growth by 13.5%, and steamed for 4 h, the extract inhibited cell growth by 82.4% (\( P<0.01 \) vs. control). At 250 \( \mu \)g/ml, unsteamed extract inhibited cell growth by 13.5%. And steamed for 2 h, the extract inhibited cell growth by 77.6% (\( P<0.01 \) vs. control). Steamed for 4 h, the extract inhibited cell growth absolutely (Fig. 3A). These results show the antiproliferative effects of the extract on human colorectal cancer cells. Steaming for 4 h made the extract more potent than steaming for 2 h.

Three representative ginsenosides were used to test their antiproliferative effects on SW-480 colorectal cancer cells. Among them, ginsenoside Rb1 was a major constituent in unsteamed American ginseng roots. Two other ginsenosides, Rg3 and Rh2, were the main constituents in steamed roots. After 72 h of treatment at 10-100 \( \mu \)M, ginsenoside Rg3 inhibited cell growth by 4.3%, and Rh2 inhibited cell growth by 99.3% (\( P<0.01 \) vs. untreated control). At 100 \( \mu \)M, ginsenosides Rg3 and Rh2 inhibited cell growth by 31.5 and 98.8%, respectively (both \( P<0.01 \) vs. untreated control, Fig. 3B). Ginsenosides Rg3 and Rh2 showed a positive antiproliferative effect on SW-480 cells, and Rh2 showed a stronger antiproliferative effect than Rg3.
To evaluate the contribution of Rg3 and Rh2 to the antiproliferative effects of extract steamed for 4 h, we compared the effects among the extract and ginsenosides. At 200 μg/ml, the extract inhibits cell growth absolutely (Fig. 3A). At 200 μg/ml the extract contains 19.9 μM of Rg3 and 3.9 μM of Rh2. Yet with 30 μM of Rg3 or 10 μM of Rh2, no antiproliferative effects were observed (Fig. 3B), suggesting that other potent antiproliferative compound(s) exist in extract steamed for 4 h.

**Induction of apoptosis of SW-480 cells.** To explore the potential mechanism through which American ginseng extract inhibits cell growth, cell apoptosis was assayed by flow cytometry after staining with annexin V. As shown in Fig. 4A, after treatment with extract steamed for 4 h, annexin V positive cells were observed, and the percentage of apoptotic cells was increased markedly. Compared to control (9.5%), after treatment with 100, 150 or 200 μg/ml of the extract, the percentage of apoptotic cells was increased to 15.8, 31.9 and 55.3%, respectively.

To further characterize the observed apoptotic phenotype, we carried out double staining of annexin V and PI in SW-480 cells treated with extract (Fig. 4B). Annexin V can be detected in both the early and late stages of apoptosis. PI enters the cell in late apoptosis or necrosis. Viable cells were negative for both annexin V and PI (lower left quadrant); early apoptotic cells were positive for annexin V and negative for PI (lower right quadrant); late apoptotic or necrotic cells...
displayed both positive annexin V and PI (upper right quadrant); non-viable cells which underwent necrosis were positive for PI and negative for annexin V (upper left quadrant). After treatment for 48 h, the percentage of early apoptotic cells induced by 100, 150 or 200 μg/ml of steamed extract was 8.0, 22.5 and 42.6%, respectively. Control was 4.8% (Fig. 4B). Steamed American ginseng root extract induced apoptosis in SW-480 cells significantly. The anti-proliferative effect of American ginseng extract steamed for 4 h was mediated by the induction of apoptosis.

**Effects of steamed extract on apoptotic pathways of SW-480 cells.** To identify specific pathway(s) that mediate apoptosis induced by American ginseng extract, we focused on 84 apoptotic-related genes. The expression level was determined with qPCR for the set of genes that are involved in TNF, mitochondria and p53 pathways. Extract steamed for 4 h inhibited 63 genes and induced 21 genes. Gene expression changes two-fold or greater vs. untreated control are listed in Table I. Steamed extract increased the transcripts of caspase 5 (CASP5), α-growth arrest and DNA-damage-inducible (GADD45A), Harakin, BCL2 interaction protein (HRK), and tumor necrosis factor receptor superfamily member 9 (TNFRSF9). Steamed extract decreased the expression of B-cell CCL/lymphoma 2 (BCL2), BCL2-associated transcription factor 1 (BCLAF1), insulin-like growth factor 1 receptor (IGF1R), CD27 molecule (CD27), tumor necrosis factor (ligand) super family, member 10 (TNFSF10), and tumor protein p73 (TP73).

As shown in Fig. 4, steamed extract treatment significantly increased apoptosis in colorectal cancer cells. Apoptosis is the result of death receptor-dependent (extrinsic)
The best-characterized death receptor is the tumor necrosis factor (TNF) superfamily. The corresponding ligands of the TNF superfamily comprise death receptor ligands. Steamed extract increased TNFRSF9, a TNF receptor that contributes to the clonal expansion, survival and proliferation in monocytes. Steamed extract inhibited another protein, TNFSF10, which preferentially induces apoptosis in transformed and tumor cells (15,16), suggesting that apoptosis is determined by cellular context and interactions between multiple regulatory factors. In our study the death receptor-dependent mechanism may not contribute to apoptotic induction.

HRK, BCL2 and BCLAF1 are Bcl-2 family genes that may contribute to the apoptotic induction of cancer cells through the mitochondrial pathway. The Bcl-2 family consists of pro-apoptotic (or cell death) and anti-apoptotic (or cell survival) genes. It is the balance in expression between these gene lineages that may determine the death or survival of a cell. BCL2 is an anti-apoptotic gene. HRK and BCLAF1 are two pro-apoptotic genes (17). As illustrated in Table I, steamed extract increased the expression of HRK. HRK activates Harakin which interacts with death-repressor proteins Bcl-2 (18). Steamed extract decreased the expression of BCL2 markedly. Forced BCL2 down-regulation results in mitochondrial dysfunction and induces apoptosis (18). BCLAF1, was depressed by steamed extract, demonstrating that apoptosis is induced at least partly through a mitochondrial mechanism.

Effects of steamed extract on the mitochondrial membrane potential of SW-480 cells. To validate the qPCR data, we performed a cellular functional assay. The spatial variation in mitochondrial membrane potential ($\Delta \psi_m$) was estimated using the JC-1 probe. This probe accumulates in the mitochondria because of membrane potentials. Organelles with low $\Delta \psi_m$ accumulate a low number of JC-1 molecules and fluoresce green (485 excitation/535 emission). At high concentrations (high $\Delta \psi_m$), the probe aggregates exhibit a red-shifted fluorescence (535 excitation/590 emission). Loss of mitochondrial potential is followed by a red to green shift. As illustrated in Fig. 5, untreated SW-480 cells showed red fluorescence. After treatment with 100 μg/ml of steamed extract, $\Delta \psi_m$ decreased. After treatment with 150 or 200 μg/ml of extract, fluorescence shifted from red to green, indicating loss of mitochondrial function. This result suggests that steamed American ginseng extract induced apoptosis by the mitochondrial pathway.

**Discussion**

Botanical extracts are a complicated mixture of bioactive compounds. The concentrations of these compounds vary significantly depending on genetics, season, geographical distribution, plant growth, and production and extract processes (19,20). Therefore, botanical identification and analysis are important in the quality assurance of botanical products (21,22). In this study, we used American ginseng from Roland Ginseng, LLC because American ginseng from Wisconsin is a reliable ginseng source (23). The content of the major ginsenosides in American ginseng extracts was determined using HPLC to standardize our extracts.

Steaming American ginseng changes the constituent profiles. Previously we have reported the effect of steaming temperatures and times on ginsenoside content in the crude herb (11). In this study, the content of the main ginsenosides in the extracts was evaluated. Ginsenosides Rg3 and Rh2, two previously recognized anticancer compounds (24), were detected in unsteamed and steamed extracts. In the unsteamed extract, Rg3 and Rh2 were only trace saponins (both 0.06%). After steaming for 4 h, Rg3 and Rh2 increased to 7.8% and

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Description</th>
<th>Fold changes vs. control</th>
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<tr>
<td>CASP5</td>
<td>Caspase 5, apoptosis-related cysteine peptidase</td>
<td>2.50</td>
</tr>
<tr>
<td>GADD45A</td>
<td>Growth arrest and DNA-damage-inducible, α</td>
<td>3.20</td>
</tr>
<tr>
<td>HRK</td>
<td>Harakin, BCL2 interaction protein (contains only BH3 domain)</td>
<td>3.76</td>
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<td>TNFRSF9</td>
<td>Tumor necrosis factor receptor superfamily, member 9</td>
<td>2.14</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell CCL/lymphoma 2</td>
<td>-2.89</td>
</tr>
<tr>
<td>BCLAF1</td>
<td>BCL2-associated transcription factor 1</td>
<td>-2.51</td>
</tr>
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<td>IGF1R</td>
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<tr>
<td>TNFSF10</td>
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</tr>
<tr>
<td>TP73</td>
<td>Tumor protein p73</td>
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RNA was extracted from SW-480 cells which were treated for 24 h with 150 μg/ml of American ginseng extract that had been steamed for 4 h.
Apoptosis is considered an important mechanism in the inhibition of cancer cells of many anticancer agents (27, 28). In this study, we assayed the induction of apoptosis by American ginseng extract. Extract steamed for 4 h showed potent apoptotic induction activities on SW-480 cells (Fig. 4).

To explore the apoptotic induction mechanism of steamed extract, we performed expression profiling analyses using an RT²-profile PCR array containing 84 apoptotic-related genes. Through identification of top up- or down-regulated genes, we found that steamed extract induced SW-480 cell apoptosis through the mitochondrial pathway. This result was further confirmed by a cellular function assay of mitochondrial membrane potential. The mitochondrial pathway may contribute to apoptosis in SW-480 cells induced by steamed American ginseng. Mitochondria integrate transcription of cellular apoptotic signals and amplify the apoptotic response (12). Disruption of mitochondrial electron transport and energy metabolism is recognized as an early event in apoptosis and precedes the appearance of morphologic changes characteristic of apoptosis (18).

In addition to genes that involve the mitochondrial pathway, the RT²-profile PCR array also includes TNF and p53 pathways. Since the cell line SW-480 is a p53 mutation, the p53 pathway does not contribute to the apoptosis (29). We found no evidence that the death receptor-dependent mechanism contributed to apoptosis induced by steamed extract. Interestingly, steamed American ginseng extract not only increased other pro-apoptotic gene expression such as that of CASP5, but also decreased the anti-apoptotic gene expression such of IGF1R. Further confirmation is needed to correlate the observed changes at the mRNA level with protein expression.

In conclusion, expression profiling on selected pathways revealed various apoptotic related genes that inhibited growth in SW-480 human colorectal cancer cells by American ginseng. The mitochondrial apoptotic pathway may play a key role in cancer chemoprevention by steamed American ginseng extract. Our expression analysis may lead to the identification of markers that predict the responsiveness of colorectal cancer cells to American ginseng treatment.

Acknowledgements

This work was supported in part by a grant from the U.S. NIH/NCCAM AT003255 and AT004418.

References


