Resistant starch prevents tumorigenesis of dimethylhydrazine-induced colon tumors via regulation of an ER stress-mediated mitochondrial apoptosis pathway

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Abstract. Resistant starch is as common soluble fiber that escapes digestion in the small intestine and can regulate intestinal function, metabolism of blood glucose and lipids, and may prevent tumorigenesis of gastrointestinal cancer. Epidemiology and other evidence have suggested that resistant starch may prevent colon cancer development. The aim of the current study was to explore the ameliorative effects and potential mechanisms of resistant starch in the tumorigenesis of colon tumors induced by dimethylhydrazine in C57BL/6 mice. Western blot analysis, ELISA, microscopy, immunofluorescence and immunohistochemistry were used to analyze the efficacy of resistant starch on the metabolic balance in the colon and tumorigenesis of colon tumors. The results demonstrated that a diet containing resistant starch decreased the animal body weight and reduced free ammonia, pH and short chain fatty acids in feces compared with mice that received a standard diet. Resistant starch reduced the incidence of colon tumors and suppressed the expression of carcinogenesis-associated proteins, including heat shock protein 25, protein kinase C-d and gastrointestinal glutathione peroxidase in colon epithelial cells compared with standard starch and control groups. Colon tumor cells proliferation and dedifferentiation were significantly decreased by a resistant starch diet. The results also demonstrated that resistant starch increased the apoptosis of colon tumor cells through regulation of apoptosis-associated gene expression levels in colon tumor cells. Oxidative stress and endoplasmic reticulum stress were upregulated, and elevation eukaryotic translation initiation factor 2α (eIF2α), activating transcription factor-4 and secretase-β expression levels were increased in the resistant starch diet group. Additionally, the activity of eIF2α and PERK were increased in colon tumor cells from mice that had received resistant starch. Increasing DNA damage-inducible transcript 3 protein (CHOP), binding immunoglobulin protein (BIP) and caspase-12 expression levels upregulated by resistant starch diet may contribute to the resistant starch-induced apoptosis of colon tumor cells induced by 1,2-dimethylhydrazine. *In vitro* assays demonstrated that knockdown of eIF2α inhibited apoptosis of colon tumor cells isolated from mice fed with resistant starch, which also downregulated CHOP, BIP and caspase-3 expression levels compared with controls. Furthermore, long-term survival of experimental mice was prolonged by the resistant starch diet compared with the standard diet group. In conclusion, the results indicate that resistant starch in the diet may prevent carcinogenesis of colon epithelial cells, mediated by enhancing apoptosis through an endoplasmic reticulum stress-mediated mitochondrial apoptosis pathway.

Introduction

Colon cancer is one of the most common gastrointestinal tumors; it is the second most common cancer in women and third in men worldwide (1). In recent years, diagnosis, treatments and prognosis of patients with colon cancer have been improved (2). Systematic review has provided various targeted therapies for the treatment of advanced colorectal cancer and explored the potential of predictive biomarkers (3). However, no satisfactory therapies for colon cancer have been developed clinically due to local migration and long distance metastasis (4). Colon cancer metastasis and invasion is a major issue for clinicians and detrimental for patients with colon cancer (5,6). The underlying molecular mechanisms of colorectal cancer metastasis and invasion have attracted research to develop targeted therapies for suppressing metastasis and invasion (7-9). Given that regulation of tumor cell growth and metastasis is imperative for patients with colon cancer and for future development of clinical strategies, molecular bioinformatics has enabled biopharmaceutical researchers to screen for targeted molecules that could be useful for diagnosis and therapy protocols, and offer the possibility of individual tailored medicine for patients with cancer or other human diseases (10,11).

Resistant starch is widely present in carbohydrate starch material and has miscellaneous effects in colon metabolism (12). The structure-physiological function of resistant starch is associated with the extent of digestion and absorption in the colon (13). Systematic review and meta-analysis of randomized controlled trials have demonstrated the beneficial effects of resistant starch supplementation on bowel function in healthy adults by increasing fecal wet weight, butyrate concentration,

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fecal pH and defecation frequency (14). Dronamraju *et al* (15) investigated the effects of resistant starch on cell kinetics and gene expression changes in patients with colorectal cancer given resistant starch in a randomized controlled trial (15). Studies have suggested that dynbiotic intervention of *Bifidobacterium lactis* and resistant starch are protective against colorectal cancer development in a rat-azoxymethane model, and long-term consumption of resistant starch markedly decreased the risk of colorectal cancer in a randomized controlled trial (16,17).

Increasing apoptosis of tumors cells has benefits for prevention and treatment of colon cancer through regulation of the expression of apoptosis-associated proteins (18). It has been reported that endoplasmic reticulum (ER) stress is associated with apoptosis of colon cancer cells (19). A previous study has reported that upregulation of the ER stress pathway can reduce γ-tocotrienol-induced apoptosis in mammary tumor cells (20). Edagawa *et al* (21) investigated the function of activating transcription factor-3 (ATF-3) in ER stress-induced apoptosis in human colon cancer cells. These studies indicated that ER stress-mediated apoptosis may be associated with tumorigenesis and development of colon cancer.

The current study investigated the anticancer effects and potential mechanisms of resistant starch in the tumorigenesis, formation and development 1,2-dimethylhydrazine-induced colon cancer. The colon physiological functions of experimental mice were analyzed following consumption of a diet containing resistant starch. Notably, this analysis investigated whether resistant starch induces apoptosis of colon tumor cells following treatment with 1,2-dimethylhydrazine.

Materials and methods

Ethics statement. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (22). All experimental protocols were performed in accordance with National Institutes of Health and approved by the Committee on the Ethics of Animal Experiments Defence Research (Northeast Agricultural University, Harbin, China).

Animal study. A total of 20 C57BL/6 mice, 6-8 weeks old, were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and housed in a temperature-controlled room (25±1°C) with artificial 12/12 h light/dark cycle. All mice could access water containing 1,2-dimethylhydrazine (3 mg/kg) to induce colon cancer. The incidence of colon tumor induced by 1,2-dimethylhydrazine was calculated by histopathology as described in immunohistochemistry assay. Experimental mice were divided into two groups (n=10/group) with free access to a regular diet (5 mg/kg) or a resistant starch diet (5 mg/kg). All mice were sacrificed for further analysis on day 120.

Analysis of ammonia, pH and short chain fatty acids. Ammonia in experimental mice was measured using ionization constant. pH was determined by pH meter (Mettler). Short chain fatty acids were analyzed using High Performance Liquid Chromatography (Takara, Tokyo, Japan).

Cells culture and reagents. Colon epithelial cells and colon tumor cells were isolated from experimental mice and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KgaA, Darmstadt, Germany). Cells were cultured at 37°C and 5% CO_2.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was reverse transcribed into cDNA at 42°C for 2 h using the High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. PCR amplification had preliminary denaturation at 94°C for 2 min, followed by 45 cycles of 95°C for 30 sec; the annealing temperature was reduced to 56.8°C for 30 sec and 72°C for 10 min. The reaction volume was a total of 20 μl containing 50 ng genomic cDNA, 200 μM dNTPs, 200 μM primers, and Taq DNA polymerase and SYBR-Green (both 2.5 U; Thermo Fisher Scientific, Inc.). Total RNA was extracted from colon epithelial cell and colon tumor cells by using RNAeasy mini kit (Qiagen, Inc., Valencia, CA, USA). mRNA expression levels of heat shock protein 25 (HSP25), protein kinase C-d (PKC-d), gastrointestinal glutathione peroxidase (GI-GPx), c-myc, Ras, p53, proliferating cell nuclear antigen (PCNA), claudin 1, claudin 2, mechanistic target of rapamycin kinase (mTOR), hexokinase-2 (HK-II), caspase-3, caspase-9, p53, Bcl-2 apoptosis regulator (Bcl-2), superoxide dismutase (SOD) and glutathione synthetase (GSH) in colon epithelial cell and/or colon tumor cells were measured by RT-qPCR with β-actin as an endogenous control (23) (Invitrogen; Thermo Fisher Scientific, Inc.). All the forward and reverse primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.) (Table I). Relative mRNA expression changes were calculated by $2^{-\Delta\Delta Cq}$ (24). The results are expressed as the n-fold change compared with control.

Western blot analysis. Colon tumor cells were homogenized in a radioimmunoprecipitation assay buffer (Sigma-Aldrich; Merck KgaA) and centrifuged at 6,000 x g at 4°C for 10 min. Protein concentration was measured with a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). A total of 10 μg/lane protein was were separated in a 12% SDS assay and then transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billierica, MA, USA). Membranes were blocked in 5% BSA (Sigma-Aldrich; Merck KgaA) for 1 h at 37°C and subsequently incubated with the following primary antibodies: HSP25 (ab202846), PKC-d (ab182126), GI-GPx (ab137431), c-myc (ab32071), Ras (ab52939), P53 (ab1431), PCNA (ab18197), claudin1 (ab15098), claudin2 (ab53032), mTOR (ab2732) and HK-II (ab24937), caspase-3 (ab13847), caspase-9 (ab202068), Bcl-2 (ab59348), SOD (ab13533), GSH (ab26255), DDIT3 (ab179823) , Beclin1 (ab62557), CHOP (ab10444), BIP (ab108615), caspase-12 (ab62484), ATF-4 (ab23760), BACE1 (ab2077), eIF2α (ab5369) and β-actin (ab8227) for 12 h at 4°C. All primary antibodies were used at a dilution of 1:1,000 and purchased from Abcam (Cambridge, UK). The membranes were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) monoclonal secondary antibodies (1:2,000; cat. no. PV-6001; OriGene Technologies, Inc., Beijing, China) for 24 h at 4°C. An enhanced chemiluminescence substrate ECL Select™ (Roche Diagnostics, Basel, Switzerland) was used to analyze the protein expression (Olympus BX51; Olympus Corporation, Tokyo, Japan).

Table I. Primer sequences for RT-qPCR.

Gene name
Sequences

HSP25
  F: 5'-ATCGAGATCTAATGGAGCCAGGGGAGGCG-3'
  R: 5'-ATCGAGATCTGGAGAAGGCGGAGGGCGCGG-3'
PKC-d
  Forward: 5'-GCATCCTCTTCAGTTACGTCC -3'
  Reverse: 5'-AAGAGAGCTTCCGTAAGGCG-3'
GI-GPx
  Forward: 5'-GAGGATATTTCGTGCCGCGC -3'
  Reverse: 5'-GGAAGCTCCTGAAGATCTGT-3'
c-myc
  Forward: 5'-ATTGGGACAGCTTGGATCAC-3'
  Reverse: 5'-AGTCACACGTCATCGACACC-3'
Ras
  Forward: 5'-CGCATCCTCAAAGGAGACATTCC-3'
  Reverse: 5'-CACATCGAGGTGAACGGGAGTAAG-3'
p53
  Forward: 5'-AAGGATCCTCAGTCTGAGTCAGGCC-3'
  Reverse: 5'-ACCACCATCCACTACAACTAC-3'
PCNA
  Forward: 5'-AGCACGGTAACGTAGGGTGT-3'
  Reverse: 5'-CATTGGAGGCATAAGCTG-3'
Claudin1
  Forward: 5'-AGAACCAGTGAGCCTGATACATACAG-3'
  Reverse: 5'-GCAACTCCATCGGCCTTTCCTACCAG-3'
Claudin2
  Forward: 5'-GGAGTAGAAGTCCCGCAGGAT-3'
  Reverse: 5'-AGGCCTCCTGGGCTTCAT-3'
m-TOR
  Forward: 5'-CGTACATGTCAGCCAGCTTC-3'
  Reverse: 5'-TGGAGGAATTCTTGCTTTGC-3'
HK
  Forward: 5'-GACGCAATCAATGTTTACTCG-3'
  Reverse: 5'-TATTTGGTTGGTCAGCACAGG-3'
Caspase-3
  Forward: 5'-TTGAGGTAGCTGCACTGTGG-3'
  Reverse: 5'-GGGCGTGTTTCTGTTTTGTT-3'
Caspase-9
  Forward: 5'-CCAACCAAATGAAGCCAAGT-3'
  Reverse: 5'-GCCCTTGCCTCTGAGTAGTG-3'
CPI
  Forward: 5'-GGAACACCTCGCTCTCCA-3'
  Reverse: 5'-GGGATTCCCTGGACCTAAAG-3'
Bcl-2
  Forward: 5'-CGTCTTCAGAGACAGCCAGGAG-3'
  Reverse: 5'-TGAACCGGCATCTGCACAC-3'
SOD
  Forward: 5'-TTTGCCAGCAGTCACATTGC-3'
  Reverse: 5'-GTACCAGTGCAGGTCCTCAC-3'
GSH
  Forward: 5'-CCGATCCAATCTGTTCTGGT-3'
  Reverse: 5'-CCAGGGCTTTTCAAAAATGA-3'
β-actin
  Forward: 5'-AGCCTTCTCCATGGTCGTGA-3'
  Reverse: 5'-CGGAGTCAACGGATTTGGTC-3'

Transfection of small interfering RNA (siRNA). Colon tumor cells (1×10^6) were transfected with 100 pmol of siRNA targeting eukaryotic translation initiation factor 2α (eIF2α) with siRNA-vector as control (both from Applied Biosystems; Thermo Fisher Scientific, Inc.) using a Cell Line Nucleofector kit L. (Lonza Group, Ltd., Basel, Switzerland). Cells were cultured in 2.5 ml DMEM containing 10% FBS 6-well plates for 24 h. All siRNAs were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.) including siRNA-eIF2α (eIF2α, L-015389) or siRNA-vector (scramble, D-001810). Cells were used for the subsequent assays after 48-h transfection.

Cell differentiation. Colon tumor cells isolated from experimental mice and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KgaA) at a 37°C humidified atmosphere of 5% CO_2. Cell colonies growing on Matrigel® were loosely detached by dispase treatment for 5 min, washed 3 times with PBS. Cells were resuspended in DMEM medium containing 20% FBS. Cells were maintained on 1% agar-coated and allowed to differentiate for another 18 days. Cells were then fixed with 10% formalin for 1 h at 37°C. Next following stained with the 60% Oil Red O in isopropanol as working solution for 10 min. The proportion of Oil Red O-positive cells was determined by counting stained cells under a light microscope.

Activity assays. Eukaryotic translation initiation factor 2-α kinase 3 (PERK) activity in colon tumor cells was analyzed by recombinant glutathione S-transferase-PERK (536-1,116 amino acids) with 6-His-full-length human eIF2α as a substrate (25). eIF2α activity was analyzed by stimulation of eIF-2-α kinase GCN2 in colon tumor cells (26). AMPK activity was determined by transient transfection assays as described previously (27).

Immunohistochemistry and immunofluorescent staining. Immunohistochemistry and immunofluorescent staining were performed according to the standard procedures (28). Paraffin-embedded colon tumor tissues sections were prepared and epitope retrieval was performed for further analysis. The paraffin sections were treated with hydrogen peroxide (3%) for 10-15 min, which subsequently blocked by a regular blocking solution for 10-15 min 37°C for immunohistochemistry. Colon tumor cells were cultured and stained with microtubule associated protein 1 light chain 3 α (MAP1LC3A), translocase of outer mitochondrial membrane 20 (TOMM20) for observation of microtubules and/or microvessels, Neo (Nase), and calreticulin (Invitrogen; Thermo Fisher Scientific, Inc.), NRP-2 (ab129050; Abcam), Apaf-1 (ab2001; Abcam), Bad (ab32445; Abcam) for immunofluorescent staining. All antibodies were used at a dilution of 1:1,000. Cells were then incubated with goat anti-rabbit IgG H&L (HRP) (1:2,000; ab205718; Abcam) for 1 h at 37°C. All fluorescent samples were visualized with a confocal fluorescence microscope (Leica TCS SP8; Leica Corporation, Wetzlar, Germany).

Cell cycle analysis. Cells (1×10^7) were collected from the experimental mice, and fixed with 70% ethanol for 2 h at 30°C. Fixed cells were rehydrated in PBS for 5 min and incubated in RNase A (1 mg/ml) for 30 min at 37°C. The cells were then subjected to
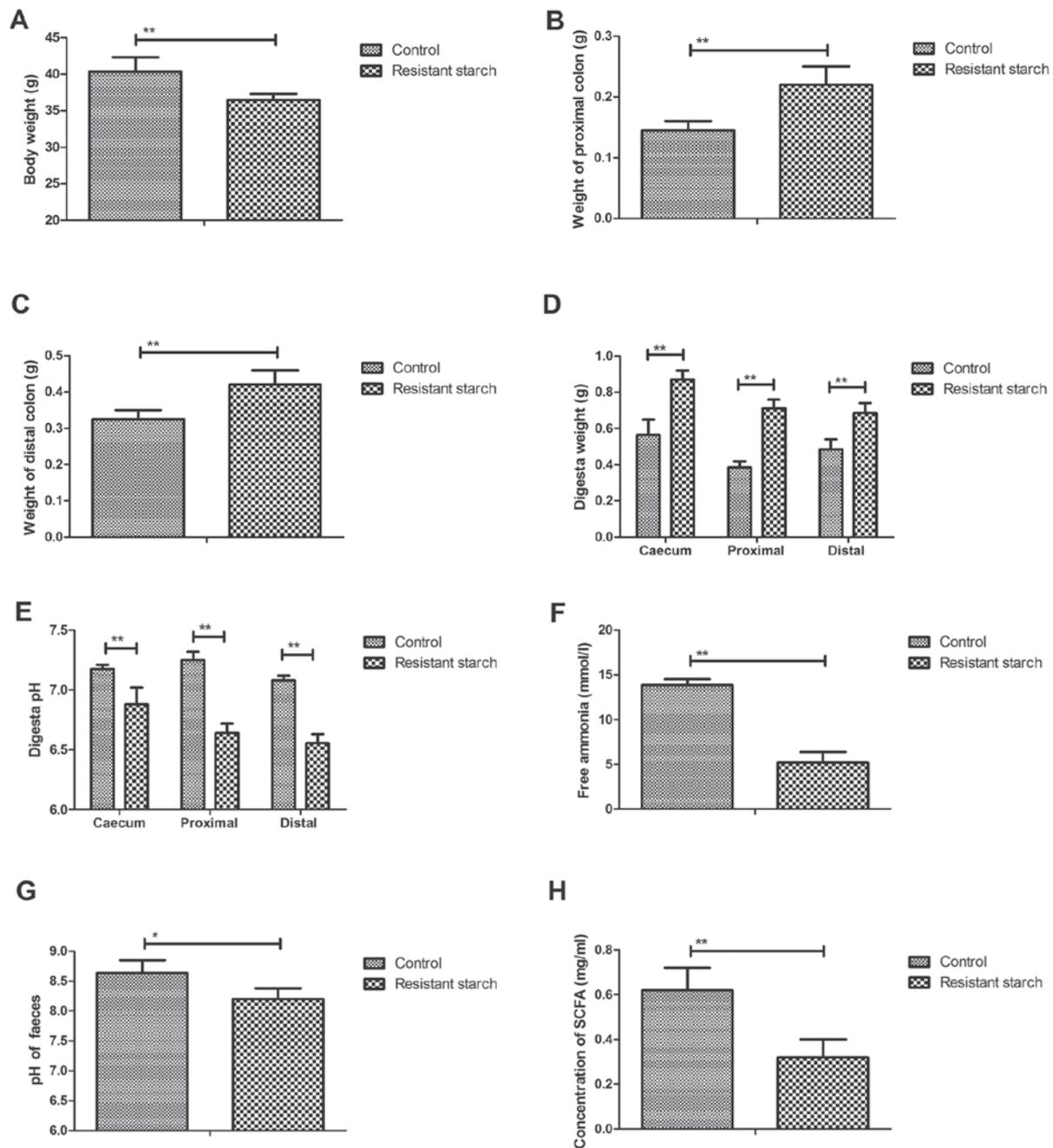
Figure 1. Effects of resistant starch on body weight and metabolic characteristic of colon tissues. (A) Body weight of experimental mice fed with resistant starch after induced by 1,2-dimethylhydrazine. Resistant starch improves weight of (B) proximal colon and (C) distal colon compared to regular diets. (D) Effects of resistant starch on digesta weight in caecum, proximal colon and distal colon in experimental mice induced by 1,2-dimethylhydrazine. (E) Digesta pH in caecum, proximal colon and distal colon in experimental mice fed with resistant starch after induced by 1,2-dimethylhydrazine. Effects of resistant starch on (F) free ammonia, (G) pH and (H) SCFA in faeces in mice fed with resistant starch. *$P < 0.05$ and **$P < 0.01$. SCFA, short chain fatty acids.

PI/RNase staining followed by flow cytometric analysis using a FACScan instrument (Becton Dickinson, Mountain View, CA, USA) and Cell Quest software (Becton Dickinson).

Apoptosis assay. Colon tumor cells were isolated from experimental mice and trypsinized and collected for apoptosis analysis. The cells were adjusted to 5×10^6 cells/ml with phosphate-buffered saline, labeled with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) using an Annexin V-FITC kit, and analyzed with a FACScan flow cytometer (both from BD Biosciences, Franklin Lakes, NJ,

USA). The treatments were performed in triplicate, and the percentage of labeled cells undergoing apoptosis in each group was determined and calculated using FCS Express™ 4 IVD software 1.0 (De Novo Software, Glendale, CA, USA).

Statistical analysis. All data were presented as mean + standard error with three independent experiments. Statistical significance was analyzed using two tailed Student's t-test between groups. Unpaired data was analyzed by variance. $P < 0.05$ was considered to indicate a statistically significant difference.
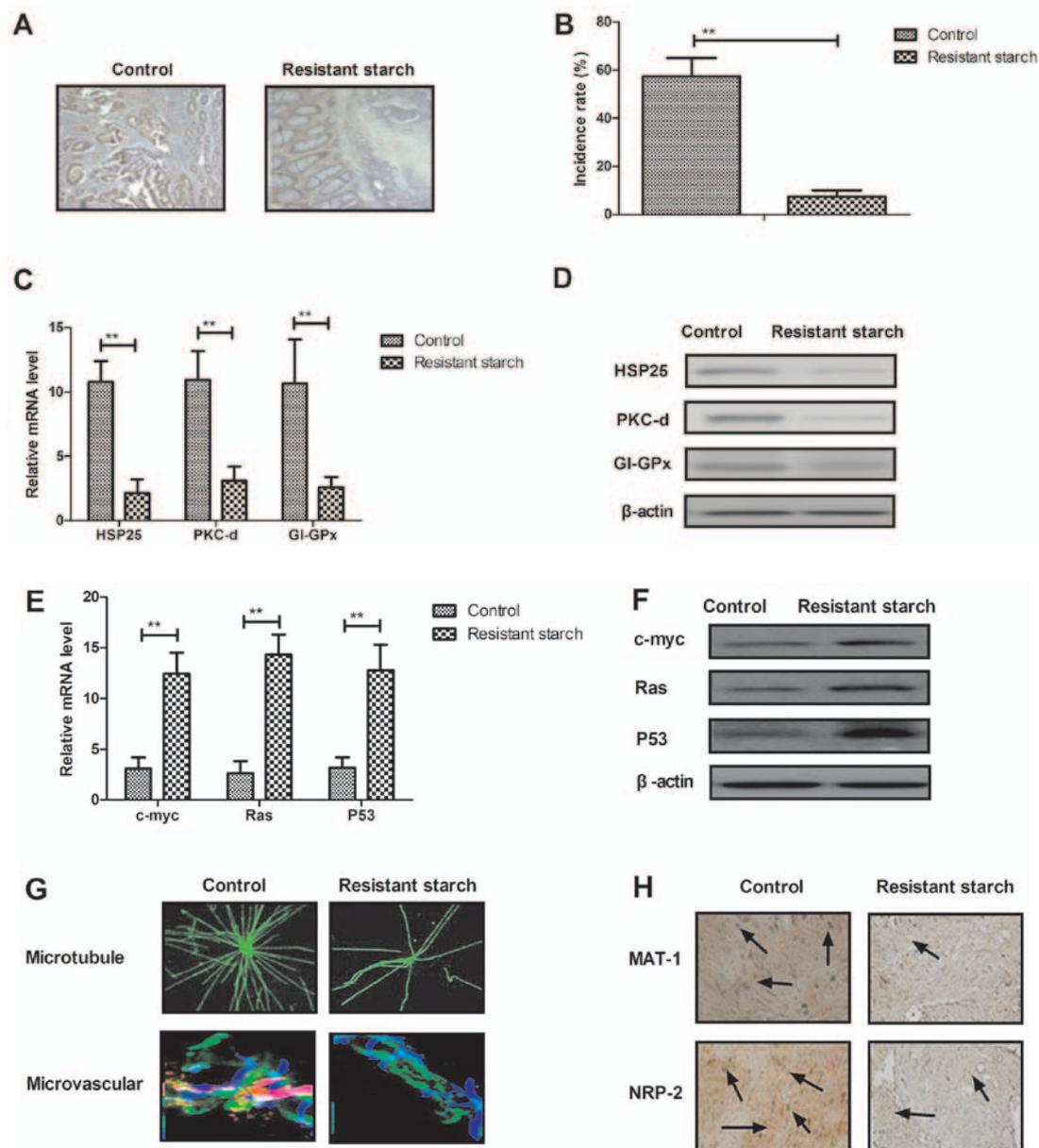
Figure 2. Effects of resistant starch on tumorigenesis in colon tissues induced by 1,2-dimethylhydrazine. (A) Resistant starch inhibits tumor formation in experimental mice treated by 1,2-dimethylhydrazine. (B) Effects of resistant starch decreases incidence of colon tumor in a mouse model induced by 1,2-dimethylhydrazine. (C) Gene and (D) protein expression levels of HSP25, PKC-d and GI-GPx in colon epithelial cells in experimental mice fed with resistant starch after induced by 1,2-dimethylhydrazine. Effects of resistant starch on (E) gene and (F) protein expression levels of c-myc, Ras and p53 in colon epithelial cells. (G) Resistant starch diet inhibits formation of microtubule and tumor vessel in colon tissues in experimental mice. (H) Resistant starch diet inhibits protein express levels of MAT-1 and NRP-2 in colon tissues in experimental mice. $^{**}P < 0.01$. HSP25, heat shock protein 25; PKC-d, protein kinase C-d; GI-GPx, gastrointestinal glutathione peroxidase; MAT-1, CDK-activating kinase assembly factor MAT1; NRP-2, neuropilin-2 (magnification, x40).

Results

Resistant starch diet improves body weight and the metabolic characteristics of colon tissues. In order to investigate the benefits of resistant starch diets for metabolism of experimental mice, body weight and metabolic characteristic of colon tissues were analyzed. A resistant starch diet decreased body weight compared with a regular diet (Fig. 1A). Weight of the proximal and distal colon was also increased by resistant starch diets in experimental mice (Fig. 1B and C). The results demonstrated that digesta weight in the caecum, proximal colon and distal colon was significantly increased by the resistant starch diet compared with a regular diet (Fig. 1D). The digesta pH in the caecum,

proximal colon and distal colon was downregulated in mice fed with resistant starch compared with a normal diet (Fig. 1E). Free ammonia, pH and short chain fatty acids (SCFA) in feces were decreased in mice fed with resistant starch (Fig. 1F-H). These results suggest that a resistant starch diet improves body weight and metabolic characteristics of colon tissues.

Resistant starch diet inhibits tumorigenesis in colon tissues induced by 1,2-dimethylhydrazine. Anti-tumorigenesis efficacy of resistant starch was investigated in colon tissues induced by 1,2-dimethylhydrazine. Results showed that resistant starch suppressed tumor formation in experimental mice treated by 1,2-dimethylhydrazine (Fig. 2A). Histopathology
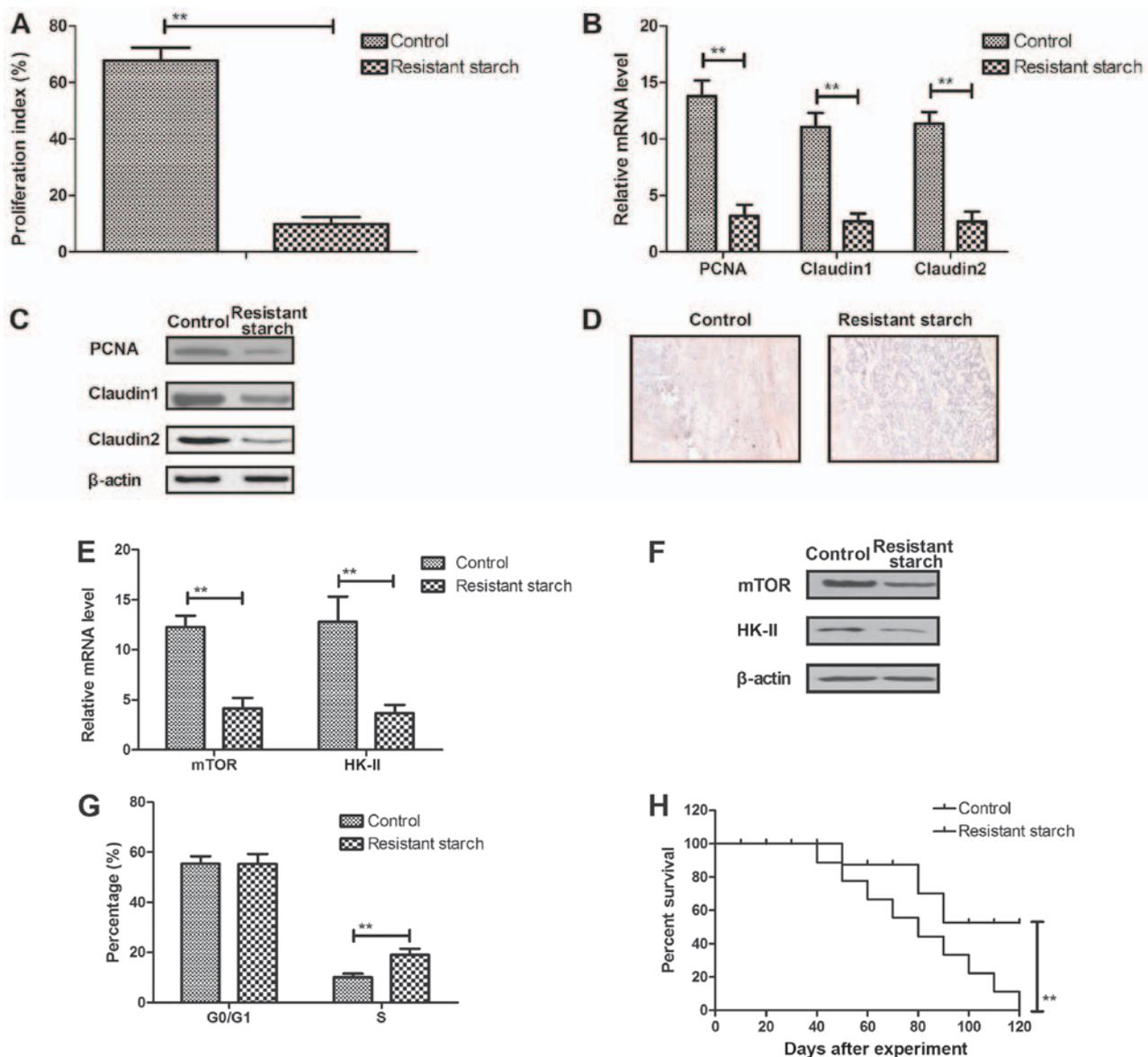
Figure 3. Effects of resistant starch on inhibition of colon tumor cells proliferation and differentiation. (A) Resistant starch diet inhibits colon tumor cells proliferation in colon tissues induced by 1,2-dimethylhydrazine. Effects of resistant starch on (B) gene and (C) protein expression levels of PCNA, claudin 1 and claudin 2 in colon tumor cells. (D) Resistant starch diet inhibits colon tumor cells differentiation in colon tissues induced by 1,2-dimethylhydrazine. Resistant starch diet blocks (E) gene and (F) protein expression levels of mTOR and HK-II in colon tumor cells. (G) Resistant starch diet arrests S phase of colon tumor cells in experimental mice. (H) Long-term survival rate of experimental mice between resistant starch diet and standard diet groups determined by Kaplan-Mrier. **P<0.01. PCNA, proliferating cell nuclear antigen; mTOR, mechanistic target of rapamycin kinase; HK-II, hexokinase-2 (magnification, x40).

demonstrated that resistant starch significantly inhibited incidence of colon tumor in mice model induced by 1,2-dimethylhydrazine (Fig. 2B). The gene and protein expression levels of carcinogenesis-associated genes, HSP25, PKC-d and GI-GPx, were downregulated in colon epithelial cells from mice receiving resistant starch compared with the standard starch group (Fig. 2C and D). However, the gene and protein expression levels of oncogenes c-myc, Ras and pro-apoptosis gene p53 were upregulated by resistant starch in colon epithelial cells (Fig. 2E and F). Immunofluorescence demonstrated that microtubule and tumor vessels were inhibited in colon tissues in mice in the resistant starch group compared with the control group (Fig. 2G). Immunohistochemistry demonstrated that the expression levels of MAT-1 and NRP-2 were downregulated by resistant

starch in colon tumor tissues (Fig. 2H). These results indicated that a resistant starch diet inhibits tumorigenesis in colon tissues induced by 1,2-dimethylhydrazine.

Resistant starch diet inhibits the proliferation and differentiation of colon cells. Tumor cell proliferation and differentiation has a vital role in tumorigenesis. Thus, the effect of resistant starch on colon tumor cell proliferation and differentiation was determined. Colon tumor cell proliferation was suppressed by resistant starch compared with the control group in 1,2-dimethylhydrazine-induced mice (Fig. 3A). RT-qPCR and western blot analysis demonstrated that the expression levels of PCNA, claudin 1 and claudin 2 were decreased in colon tumor cells in resistant starch-fed mice compared with the control group (Fig. 3B and C). The results also demonstrated that
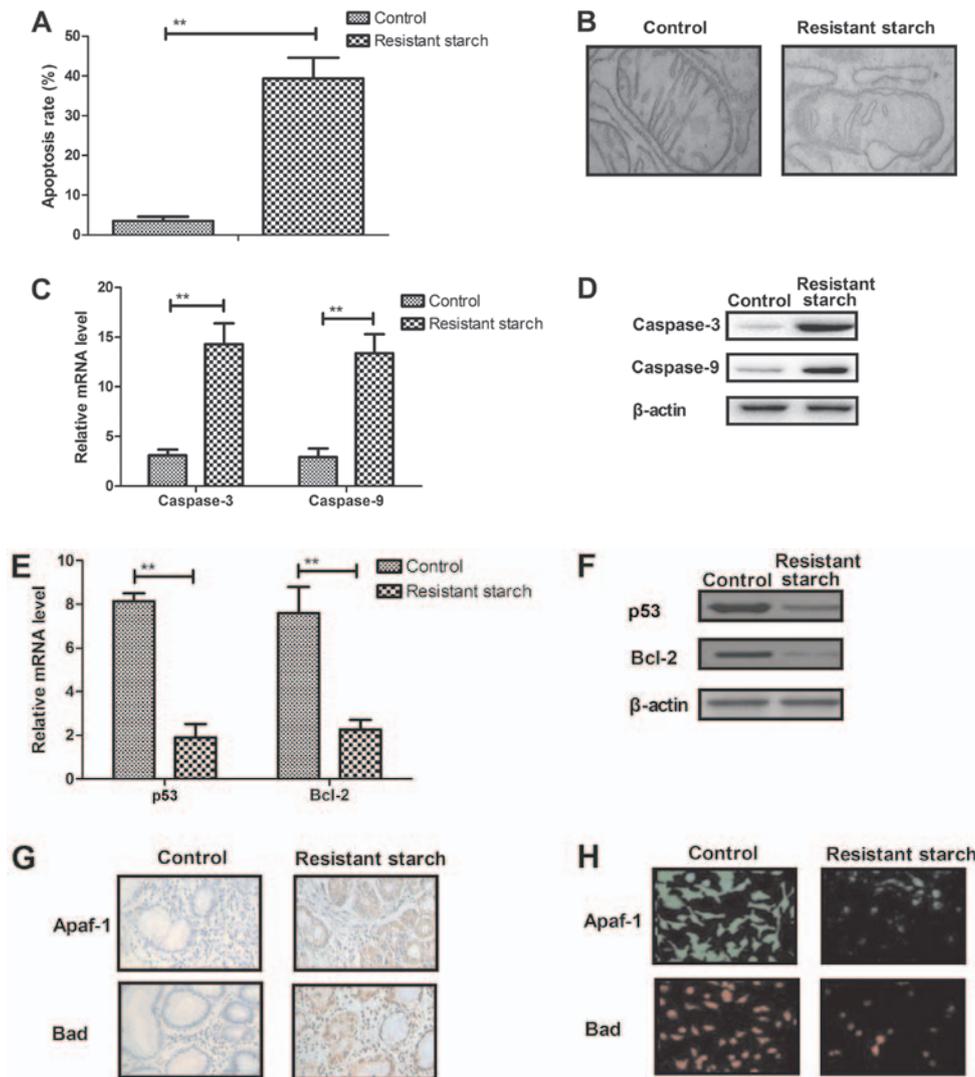
Figure 4. Resistant starch diet induces apoptosis of colon tumors through mitochondrial apoptotic pathway. (A) Resistant starch diet promotes apoptosis of colon tumor cells in mice treated by 1,2-dimethylhydrazine. (B) Effects of resistant starch diet on mitochondria damage in colon tumor cells in experimental mice (magnification, x100). Resistant starch diet increases pro-apoptosis (C) gene and (D) protein expression levels of cleaved caspase-3 and caspase-9 in colon tumor cells induced by 1,2-dimethylhydrazine. Resistant starch diet increases anti-apoptosis (E) gene and (F) protein expression levels of p53 and Bcl-2 in colon tumor cells induced by 1,2-dimethylhydrazine. Resistant starch diet promotes expression levels of Apaf-1 and Bad in colon tumor tissue in mice treated by 1,2-dimethylhydrazine determined by (G) immunohistochemistry (tissue) and (H) immunofluorescence (cells) (magnification, x40). **$P < 0.01$. Bcl-2, apoptosis regulator Bcl-2; Apaf-1, apoptotic protease-activating factor 1; Bad, Bcl-2-associated agonist of cell death.

that resistant starch inhibited colon tumor cell differentiation in colon tissues (Fig. 3D). RT-qPCR and western blot analysis demonstrated that expression levels of mTOR and HK-II were reduced by resistant starch compared with the control diet in colon tumor cells (Fig. 3E and F). The resistant starch diet increased S phase arrest of colon tumor cells, and downregulation of mTOR and HK-II expression levels compared with the normal diet group (Fig. 3G). Long-term survival of experimental mice was prolonged by the inclusion of resistant starch in the diet compared with the control diet group (Fig. 3H). These results suggest that resistant starch diets can inhibit the proliferation and differentiation of colon tumor cells by arresting the cell cycle.

Resistant starch promotes apoptosis of colon tumor cells through the mitochondrial pathway. The anti-apoptosis effects of resistant starch were also analyzed in experimental mice with 1,2-dimethylhydrazine-induced colon tumors. The resistant starch diet promoted apoptosis of colon tumor cells (Fig. 4A). Cellular structure demonstrated that mitochondria exhibited different degrees of damage in colon tumor cells (Fig. 4B). Pro-apoptosis gene and protein expression levels of caspase-3 and caspase-9 were upregulated in colon tumor cells (Fig. 4C and D). However, anti-apoptosis gene and protein expression levels of p53 and Bcl-2 were downregulated in colon tumor cells in resistant starch-fed experimental mice compared with the control treatment (Fig. 4E and F). Immunohistochemistry and immunofluorescence demonstrated that resistant starch increased Apaf-1 and Bad expression levels in colon tumor tissues in experimental mice compared with control mice (Fig. 4G and H). These results suggest that resistant starch in the diet can enhance apoptosis of colon tumors through mitochondrial apoptotic pathway in mice with colon tumors induced by 1,2-dimethylhydrazine.
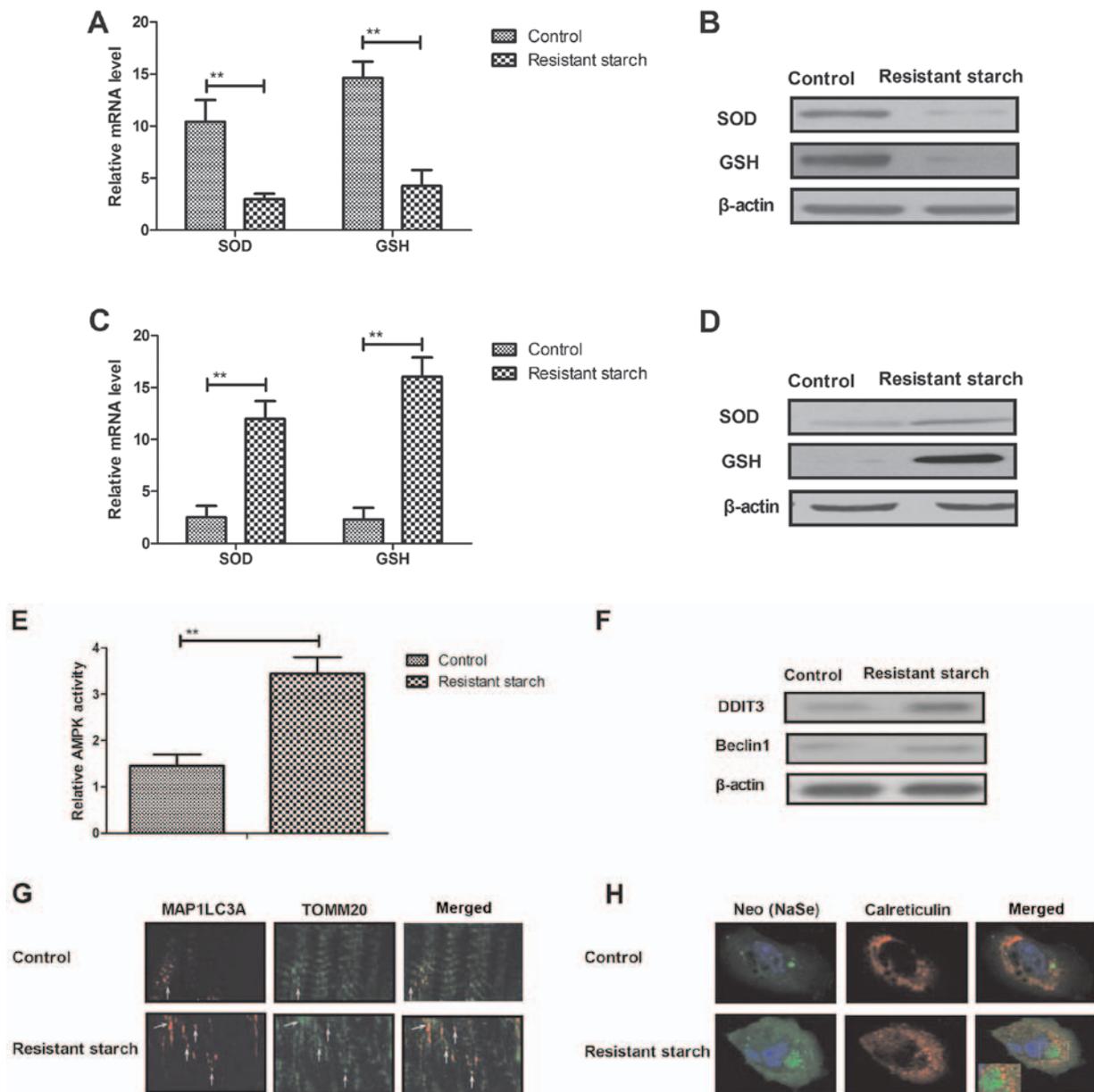
Figure 5. Resistant starch diet increases oxidative stress in colon tumors through regulation of autophagy progression. Effects of resistant starch on (A) mRNA and (B) protein levels of SOD and GSH in colon tumor cells. Effects of resistant starch on (C) mRNA and (D) protein levels of SOD and GSH in colon epithelial cells. (E) Resistant starch upregulates AMPK activity in colon tumor cells. (F) Resistant starch upregulates expression levels of DDIT3 and Beclin 1 in colon tumor cells in mice treated by 1,2-dimethylhydrazine. Resistant starch diet promotes (G) mitophagy and (H) reticulophagy of colon tumor cells in mice treated by 1,2-dimethylhydrazine (magnification, x40). **P<0.01. SOD, superoxide dismutase; GSH, glutathione synthetase; AMPK, AMP-activated protein kinase; DDIT3, DNA damage-inducible transcript 3 protein; MAP1LC3A, microtubule associated protein 1 light chain 3 α; TOMM20, translocase of outer mitochondrial membrane 20; Neo (Nase), 5'-Nase-ALPase.

Resistant starch diet enhances oxidative stress in colon tumors through regulation of autophagy progression. Changes of oxidative stress in colon tumors and colon epithelial cells was investigated. Resistant starch diets reduced the mRNA and protein levels of SOD and GSH in colon tumor cells compared with mice that received the control diet (Fig. 5A and B). However, mRNA and protein levels of SOD and GSH were increased by resistant starch in colon epithelial cells compared to regular diet (Fig. 5C and D). AMP-activated protein kinase (AMPK) activity was increased in colon tumor cells from mice fed with resistant starch compared with the regular diet (Fig. 5E). Potential mechanisms were demonstrated as resistant starch increased the expression levels of DNA

damage-inducible transcript 3 protein and Beclin 1 in colon tumor cells (Fig. 5F). The results also demonstrated that resistant starch promoted mitophagy and reticulophagy of colon tumor cells (Fig. 5G and H). Collectively, the results indicated that the resistant starch diet enhanced-activated autophagy in colon tumors through upregulation of autophagy genes.

Resistant starch diet alters ER stress-dependent PERK activity through upregulation of eIF2α phosphorylation in colon tumor cells in 1,2-dimethylhydrazine-induced mice. The effect if resistant starch on ER stress and its potential mechanism in inhibition of tumor cells growth was investigated. The resistant starch diet increased the expression levels of eIF2α, ATF-4 and
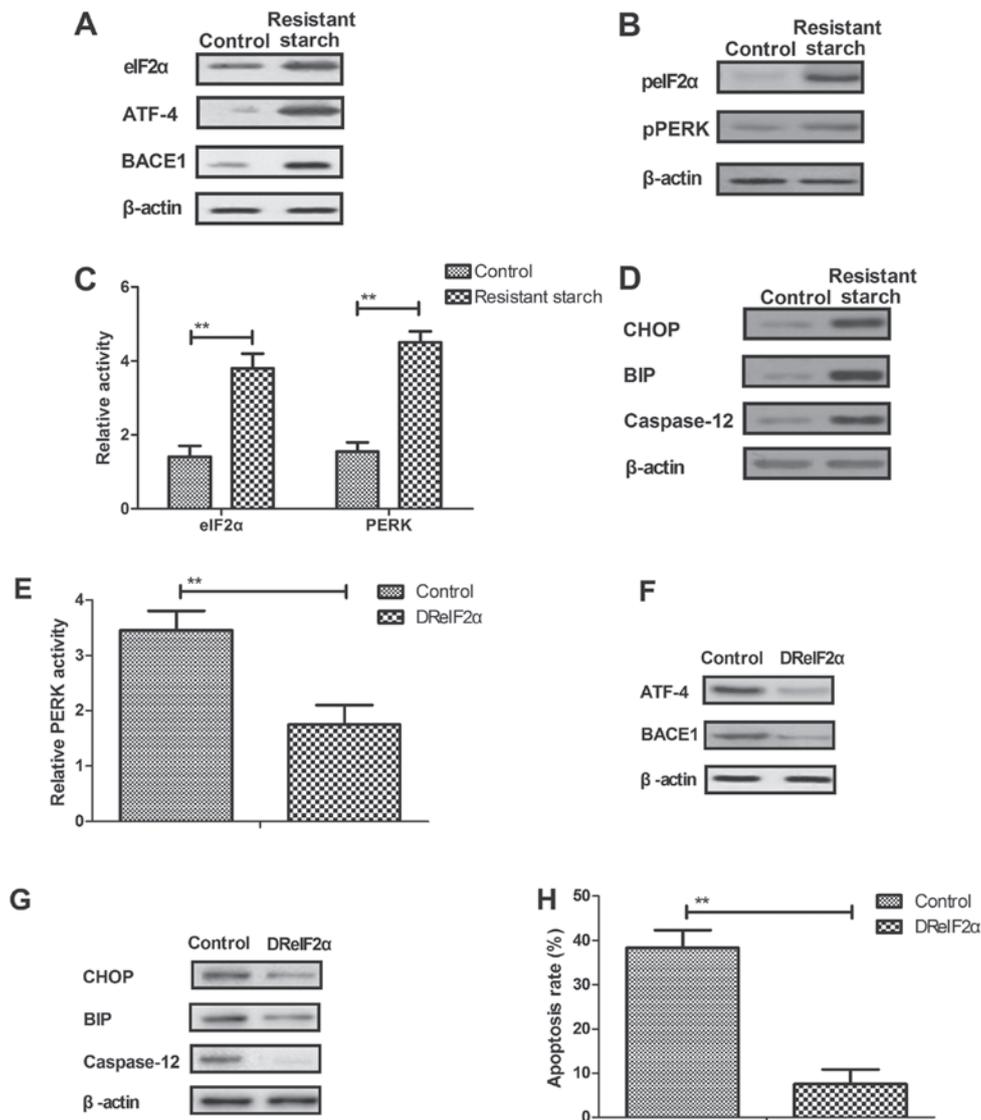
Figure 6. Resistant starch diet regulates endoplasmic reticulum stress-dependent PERK activity through upregulation of eIF2α phosphorylation in colon tumor cells. (A) Resistant starch diet increases expression levels of eIF2α, ATF-4 and BACE1 in colon tumor cells. (B) Effects of resistant starch diet on phosphorylation levels of eIF2α and PERK in colon tumor cells in mice treated by 1,2-dimethylhydrazine. (C) Effects of resistant starch diet on activity of eIF2α and PERK in colon tumor cells in mice treated by 1,2-dimethylhydrazine. (D) Resistant starch diet increases expression levels of CHOP, BIP and caspase-12 in colon tumor cells in mice treated by 1,2-dimethylhydrazine. (E) Effects of DReIF2α on PERK activity in colon tumor cells in mice induced by 1,2-dimethylhydrazine. (F) eIF2α knockdown inhibits resistant starch-suppressed ATF-4 and BACE1 expression levels in colon tumor cells in mice fed with resistant starch. (G) Knockdown of eIF2α suppresses expression levels of CHOP, BIP and caspase-12 in colon tumor cells in mice fed with resistant starch. (H) Knockdown of eIF2α inhibits resistant starch-induced apoptosis of colon tumor cells isolated from mice fed with resistant starch. **P<0.01. eIF2α, eukaryotic translation initiation factor 2α; ATF-4, activating transcription factor-4; BACE1, secretase-β; p, phospho; PERK, eukaryotic translation initiation factor 2-α kinase 3; CHOP, DNA damage-inducible transcript 3 protein; BIP, binding immunoglobulin protein; AMPK, AMP-activated protein kinase; DReIF2α, downregulation of eIF2α.

secretase-β (BACE1) in colon tumor cells compared with cells from control mice (Fig. 6A). Total protein expression levels of eIF2α and PERK were increased by resistant starch in colon tumor cells (Fig. 6B). eIF2α and PERK activity was increased by the resistant starch diet compared with the regular diet in mice induced by 1,2-dimethylhydrazine (Fig. 6C). The resistant starch diet increased the expression levels of DNA damage-inducible transcript 3 protein (CHOP), binding immunoglobulin protein (BIP) and caspase-12 in colon tumor cells (Fig. 6D). *In vitro* assays demonstrated that downregulation of expression levels of eIF2α using siRNA suppressed PERK activity in colon tumor cells (Fig. 6E). Expression levels of ATF-4 and BACE1 were reduced by knockdown of eIF2α compared with control siRNA in colon tumor cells (Fig. 6F). Knockdown of eIF2α

exhibited inhibitory effects on CHOP, BIP and caspase-12 expression in colon tumor cells isolated from mice fed by resistant starch (Fig. 6G). Result also demonstrated that knockdown of eIF2α inhibited resistant starch-induced apoptosis of colon tumor cells (Fig. 6H). Collectively, these results suggest that the resistant starch diet regulated ER stress-dependent PERK activity through upregulation of eIF2α activity in colon tumor cells in mice induced by 1,2-dimethylhydrazine.

Discussion

Colon cancer is one of the most common gastrointestinal tumors, with highly invasive ability characterized by rapid invasion of lymphatics, flow transfer and local

invasion (29,30). Various studies have indicated that most cases of sporadic colon cancer can be attributed to diet (31,32). Studies have suggested that resistant starch fermentation modulated colonic bacterial metabolism and reduced the risk of colon cancer tumorigenesis (33). The current study investigated the benefits and potential mechanism of resistant starch-mediated anti-cancer efficacy in experimental mice induced by 1,2-dimethylhydrazine. Compared with a regular diet, the resistant starch diet inhibited tumorigenesis, proliferation and differentiation in colon tissues induced by 1,2-dimethylhydrazine, and increased the animal body weight and improved the metabolic characteristics of the colon tissues. Analysis of the molecular mechanisms indicated that the resistant starch diet promotedapoptosis of colon tumor cells through the mitochondrial apoptotic pathway, enhanced oxidative stress through regulation of autophagy progression, and regulated ER stress-dependent PERK activity through upregulation of $eIF2\alpha$ activity in the colon tumor cells of mice induced by 1,2-dimethylhydrazine.

Tumorigenesis mechanisms are crucial for initiation and progression of tumor formation (34). Previous studies have reported that elevation of mRNA expression levels of PKC-d, HSP25 and GI-GPx are associated with tumorigenesis (35). The resistant starch diet downregulated expression levels of PKC-d, HSP25 and GI-GPx in colon epithelial cells that contributed to inhibition of tumor lesions formation. Studies have clearly demonstrated that tumor blood vessels and higher expression levels of MAT-1 and NRP-2 hindered the therapeutic efficacy of anti-cancer drugs (36-38). The data of the current study revealed that the structure of microtubules and tumor vessels in colon tumor cells were altered and gene expression of MAT-1 and NRP-2 were downregulated in resistant starch-fed mice induced by 1,2-dimethylhydrazine.

Proliferation and differentiation of colon tumor cells contribute to tumor migration and invasion mediated by molecular signaling through effector pathways (39). In the present study, the resistant starch diet inhibited proliferation and differentiation of colon tumor cells by decreasing PCNA, claudin 1 and claudin 2 gene and protein expression levels. The results also indicated that resistant starch arrested the cell cycle of colon tumor cells and downregulated mTOR and HK-II expression levels, which may have contributed to the increased long-term survival of experimental mice in the resistant starch group compared with the control diet group.

Induction of apoptosis by tumor therapeutic agents is essential for cancer therapy (40). In this study, the anti-cancer efficacy of resistant starch was investigated in mice induced with 1,2-dimethylhydrazine. The results demonstrated that resistant starch promoted apoptosis via the mitochondrial apoptotic pathway, and also increased the expression of tumor suppressor genes in colon epithelial cells. These results suggest that resistant starch contributes to apoptosis of colon tumor cells and anti-apoptosis efficacy of colon epithelial cells in experimental mice induced by 1,2-dimethylhydrazine. However, we observed that p53 was reduced by resistant starch, which is one of the most important tumor suppressor gene. Therefore, further study should perform to identify the inhibitory effects of resistant starch on tumor cells. Additionally, we found that oncogenes c-myc and Ras were upregulated by resistant starch, which also need to further analyzed in our future work.

Autophagy is a cellular progression of materials conversion that promotes the obliteration of metabolic precursors (41,42). A previous study demonstrated that autophagy contributes to the inhibition of oncogenesis and suppression of tumor growth in colon cancer (43). Jang *et al* (44) reported that promotion of autophagy can inhibit tumorigenesis and induce apoptosis of human cancer cells by modulating sphingolipids, and suppress colon tumor development in mice. In the current study, the resistant starch diet increased oxidative stress and increased SOD and GSH levels in colon tumors. AMPK activity, mitophagy, reticulophagy and Beclin 1 expression were also increased by resistant starch in colon tumor cells (45,46).

Studies have indicated that ER stress is associated with apoptosis of colon tumor cells (47). PERK activation has an important role in promoting tumorigenesis by attenuating apoptosis of premalignant granule cell precursors (48). The current study demonstrated that the resistant starch diet promoted the expression levels of $eIF2\alpha$, ATF-4 and BACE1, and increased $eIF2\alpha$ activity in colon tumor cells. Mechanistic analysis indicated that knockdown of $eIF2\alpha$ exhibited inhibitory effects on CHOP, BIP and caspase-12 expression, and apoptosis in colon tumor cells isolated from mice fed with the resistant starch diet. The results of the present study indicated that the resistant starch diet promoted apoptosis by regulating ER stress-dependent PERK activity via upregulation of $eIF2\alpha$ activity in colon tumor cells from mice induced by 1,2-dimethylhydrazine, which contributes to tumor suppression.

In conclusion, resistant starch improved bowel function, and the outcomes indicated that the resistant starch diet improved body weight and the metabolic characteristic of colon tissues. The results indicated that resistant starch inhibited tumorigenesis in the colon by promoting apoptosis and autophagy by upregulation of ER stress-dependent PERK activity, which mediated by $eIF2\alpha$ in colon tumor cells from mice induced by 1,2-dimethylhydrazine. These investigations indicated that resistant starch prevents tumorigenesis of colon tumors induced by dimethylhydrazine via regulation of an ER stress-mediated mitochondrial apoptosis pathway, which may useful in the future for the prevention of tumorigenesis of colon tissues.

Competing interests

The authors declare that they have no competing interests.

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