

# Soft-shelled turtle peptide modulates microRNA profile in human gastric cancer AGS cells

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Received April 27, 2016; Accepted July 14, 2017

DOI: 10.3892/ol.2017.7692

**Abstract.** Cancer prevention using natural micronutrition on epigenetic mechanisms primarily revolves around plant extracts. However, the role of macronutrition, including animal peptides, on epigenetic modification in cancer has been elusive. In traditional Chinese medicine, the soft-shelled turtle has a long-history of being a functional food that strengthens immunity through unknown mechanisms. The present study aimed to investigate the impact of soft-shelled turtle peptide on microRNA (miRNA) expression in gastric cancer (GC) cells and to analyze the potential anticancer mechanisms for GC. Affymetrix GeneChip miRNA 3.0 Array and quantitative polymerase chain reaction were used to detect the miRNA expression profile in human GC AGS cells treated with the soft-shelled turtle peptide. The results demonstrated that 101 miRNAs (49 upregulated miRNAs and 52 downregulated miRNAs) were significantly differentially expressed in the AGS cells following soft-shelled turtle peptide treatment. Several tumor suppressor miRNAs were upregulated markedly, including miRNA-375, let-7d, miRNA-429, miRNA-148a/148b and miRNA-34a. Pathway analysis indicated that soft-shelled turtle peptide may function with anticancer properties through the Hippo signaling pathway and the forkhead box O signaling pathway. Therefore, these results demonstrated that soft-shelled turtle peptide has the capacity to influence cancer-related pathways through the regulation of miRNA expression in GC cells.

## Introduction

Cancers result from abnormal cell growth, which is caused by abnormalities in the genome and the epigenome (1).

Alterations of epigenetic modifications are always reversible and can be influenced by external factors, including diet or environmental exposure (2). Given that epigenetic dysregulation occurs early during tumorigenesis, epigenetic modifications have been confirmed as potential targets for cancer prevention or cancer therapy (3). Nutri-epigenetics, which revolves around the impact of micronutritions and macronutritions on epigenetic mechanisms, has renewed the study of traditional methods in the epigenetic field. Epigenetic modifications in response to functional foods consist of DNA methylation, histone modifications and the effect of non-coding RNAs. However, it has been confirmed that a few functional foods not only play a role in health promotion, but also promote anticancer activity for several tumors. Epigallocatechin-3-gallate (EGCG) extracted from green tea inhibits DNA methylation and increases histone acetylation to recover silenced tumor suppressor genes in cancer cells (4,5). Isothiocyanates extracted from cruciferous vegetables suppress the metastasis potential in lung cancer cells by inducing apoptosis and cell cycle arrest, and inhibiting deubiquitinating enzymes associated with tumorigenesis to exert anticancer effects (6,7).

The occurrence of gastric adenocarcinoma is a stepwise process that may follow several years or decades of gastritis, intestinal metaplasia and dysplasia to malignancy with epigenetic alterations (3). Gradual epigenetic dysregulations are strongly associated with the tumorigenesis of certain inflammation-associated cancer types, particularly gastric cancer (GC). Hence, more and more natural anticancer components are being studied. The search for effective and safe natural anticancer factors, and mechanisms to reverse or counteract cancer-associated epigenetic alterations for GC prevention and interventions are required.

To date, nutraceuticals for cancer chemoprevention have primarily been derived from plants, for example, curcumin from ginger plants (8), allicin from garlic (9), resveratrol from grapes and EGCG from green tea (10,11). However, the influence of animal-derived macronutrition, including proteins or peptides, on cancer epigenetic mechanisms remains uninvestigated. Despite the fact that the consumption of red meat is undeniably associated with cancer risk (12-14), molecular mechanisms of animal peptides on epigenetic regulation have not been fully elucidated. The soft-shelled turtle is

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**Key words:** soft-shelled turtle peptide, microRNA profiling, gastric cancer cells, macronutrition, nutri-epigenetics

commonly consumed in China. According to excavations from the Hemudu site (Zhejiang, China), the history of eating soft-shelled turtles in Asia dates back to the Hemudu culture ~6,000 years ago in the neolithic period. The soft-shelled turtle is also used in traditional Chinese medicine where it is believed to strengthen immunity among other benefits (15). Recently, with the development of turtle aquaculture, soft-shelled turtles have been processed into various types of health products, including products in capsule and liquid forms, and are used to improve the prognosis of cancer patients undergoing radiotherapy and chemotherapy, according to the beliefs of traditional Chinese medicine (16). To the best of our knowledge, for the first time, the present study investigates the impact of soft-shelled turtle peptides on epigenetic mechanisms in GC cells. A microarray was used to detect the expression profile of microRNA (miRNA) in the human GC AGS cell line treated with soft-shelled turtle peptide. The target genes of the soft-shelled turtle peptide-specific miRNAs and corresponding pathways were further analyzed to determine the potential anticancer properties of the soft-shelled turtle peptide.

## Materials and methods

**Materials.** The peptides extracted from soft-shelled turtles were offered by Zhejiang Agricultural Group Co., Ltd. (Hangzhou, China) and stored in the form of a dry powder in a brown glass bottle at room temperature. Every unit of the soft-shelled turtle peptide powder consisted of a polypeptide (40.68%), acid soluble protein (47.97%), free amino acids (7.29%) and hydroxyproline (4.06%). The polypeptide was the major functional component and the treatment concentration was dependent on the actual polypeptide content. The peptide powder was dissolved in cell culture medium at the required concentration immediately prior to use.

**Cell culture and cell treatment.** The human GC AGS cell line was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in F12 medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in T-75 flasks (Corning Inc., Corning, NY, USA) at 37°C in a humidified atmosphere supplied with 5% CO<sub>2</sub>. The AGS cells were treated during the exponential phase of growth. The experimental group of cells were incubated with the soft-shelled turtle peptide dissolved in F12 medium (100 mg/l), while the control group of cells were incubated with F12 medium only. The experimental group and the control group were incubated for 72 h, and the medium was renewed every 24 h. After 72 h, the cells were collected by trypsinization.

**RNA extraction, labeling and miRNA expression profiling analysis.** The total RNA of AGS cells with or without soft-shelled turtle peptide treatment was extracted and purified using the mirVana™ miRNA Isolation kit without phenol (Ambion; Thermo Fisher Scientific, Inc.), following the manufacturer's instructions, and checked for an RNA integrity number to inspect RNA integration using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The genome-wide miRNA expression profiling

was detected by the Affymetrix platform with Genechip miRNA 3.0 array (Affymetrix; Thermo Fisher Scientific, Inc.) based on the Sanger miRBase version 17. This chip contained 19,913 probe sets, including 1,789 mature miRNAs, 1,693 precursor miRNAs and 2,336 small nucleolar RNAs and small Cajal body-specific RNAs. After total RNA quality inspection, total RNAs of each sample were tailed with poly A and labeled with biotin by the FlashTag™ Biotin HSR RNA Labeling kit (Affymetrix; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Hybridization of bio-labeled RNA samples was performed according to the manufacturer's instructions for the Affymetrix GeneChip miRNA 3.0 Array with GeneChip Hybridization Wash and Stain kit, and GeneChip Hybridization Oven 645 (all from Affymetrix; Thermo Fisher Scientific, Inc.). After washing and staining with GeneChip Fluidics Station 450, the arrays were scanned by the GeneChip Scanner 3000 (both from Affymetrix; Thermo Fisher Scientific, Inc.).

**Statistical analysis.** Differentially expressed miRNAs were evaluated by the ratio of fluorescence between the control and the soft-shelled turtle peptide treated sample. Fold-change of  $\geq 2$  and  $\leq 0.5$ , used for threshold values, were regarded as upregulation and downregulation, respectively. Hierarchical clustering analysis was performed to display the discrepant miRNA expression profile in two samples through red and green color blocks via Gene Cluster (version 3.0) and Java TreeView software program ([bonsai.hgc.jp/~mdehoon/software/cluster/software.htm](http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm)). The potential target genes of differentially expressed miRNAs in the treated cells were predicted using five databases, including TARGETMINER ([www.isical.ac.in/~bioinfo\\_miu/targetminer20.htm](http://www.isical.ac.in/~bioinfo_miu/targetminer20.htm)), miRDB ([mirdb.org](http://mirdb.org)), microRNA ([www.mirbase.org/](http://www.mirbase.org/)), TarBase ([diana.imis.athena-innovation.gr/DianaTools/index.php?r=tarbase/index](http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=tarbase/index)) and RNA22 ([cm.jefferson.edu/rna22/Interactive](http://cm.jefferson.edu/rna22/Interactive)). Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (<https://david.ncifcrf.gov/>; DAVID Bioinformatics Resources 6.8) were performed to demonstrate corresponding biological processes and the regulatory network in which specific miRNA-genes may have participated (17,18).

**Validation of miRNA array by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Differentially expressed miRNAs were selected and validated according to RT-qPCR analysis with Platinum Quantitative PCR SuperMix-UDG w/ROX (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA were extracted from AGS cells with or without soft-shelled turtle peptide treatment. PCR was performed using an Applied Biosystems 7500 (Foster City, CA, USA). Amplification was performed at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 34 sec. U6 was selected as an internal reference to normalize the miRNA expression levels, and each sample was validated in triplicate. The specific primer sequences of U6 is as follow: 5'-CGCAAGGATGACACGCAAATTC-3'. The reverse transcription of poly-A tailed miRNAs using anchored Oligo dT primers for the candidate miRNAs are presented in Table I. The relative expression of differentially expressed miRNAs was evaluated according to the 2<sup>-ΔΔC<sub>q</sub></sup> method (19).

Table I. Reverse transcription of poly-A tailed miRNAs using anchored Oligo dT primers for the candidate miRNAs.

microRNA	Name in mirBase	Sequence in mirBase	Length	Modified primer sequence	Primer length
hsa-miR-4455	>hsa-miR-4455 MIMAT0018977	AGGGUGUGUGU GUUUUU	17	GCCAGGGTGTGTGTGTTTT	20
hsa-miR-29a	>hsa-miR-29a-3p MIMAT0000086	UAGCACCAUCUGA AAUCGGUUA	22	TAGCACCAATCTGAAATCGGTTA	22
hsa-miR-378b	>hsa-miR-378b MIMAT0014999	ACUGGACUUGGA GGCAGAA	19	ACTGGACTTGGAGGCAGAA	19
hsa-miR-29b	>hsa-miR-29b-3p MIMAT0000100	UAGCACCAUUUGA AAUCAGUGUU	23	GGTAGCACCAATTTGAAATCAG	21
hsa-miR-205	>hsa-miR-205-5p MIMAT0000266	UCCUUCAUUCCAC CGGAGUCUG	22	TCCTTCAITCCACCCGGAGT	19
hsa-miR-100	>hsa-miR-100-5p MIMAT0000098	AACCCGUAGAUC GAACUUUGUG	22	AACCCGTAGATCCGAACTTGTG	22
hsa-miR-7	>hsa-miR-7-5p MIMAT0000252	UGGAAGACUAAGUG AUUUUGUUGU	23	GCCTGGAAGACTAGTGATTTG	21
hsa-miR-375	>hsa-miR-375 MIMAT0000728	UUUGUUCGUUCGG CUCGGGUGA	22	TGTTTCGTTCCGGCTCCGGTGAA	21
hsa-miR-4299	>hsa-miR-4299 MIMAT0016851	GCUGGUGACAUG AGAGGC	18	TAGCTGGTGACATGAGAGG	19
hsa-miR-378h	>hsa-miR-378h MIMAT0018984	ACUGGACUUGGUG UCAGAUGG	21	ACTGGACTTGGTGTGATGATGG	21

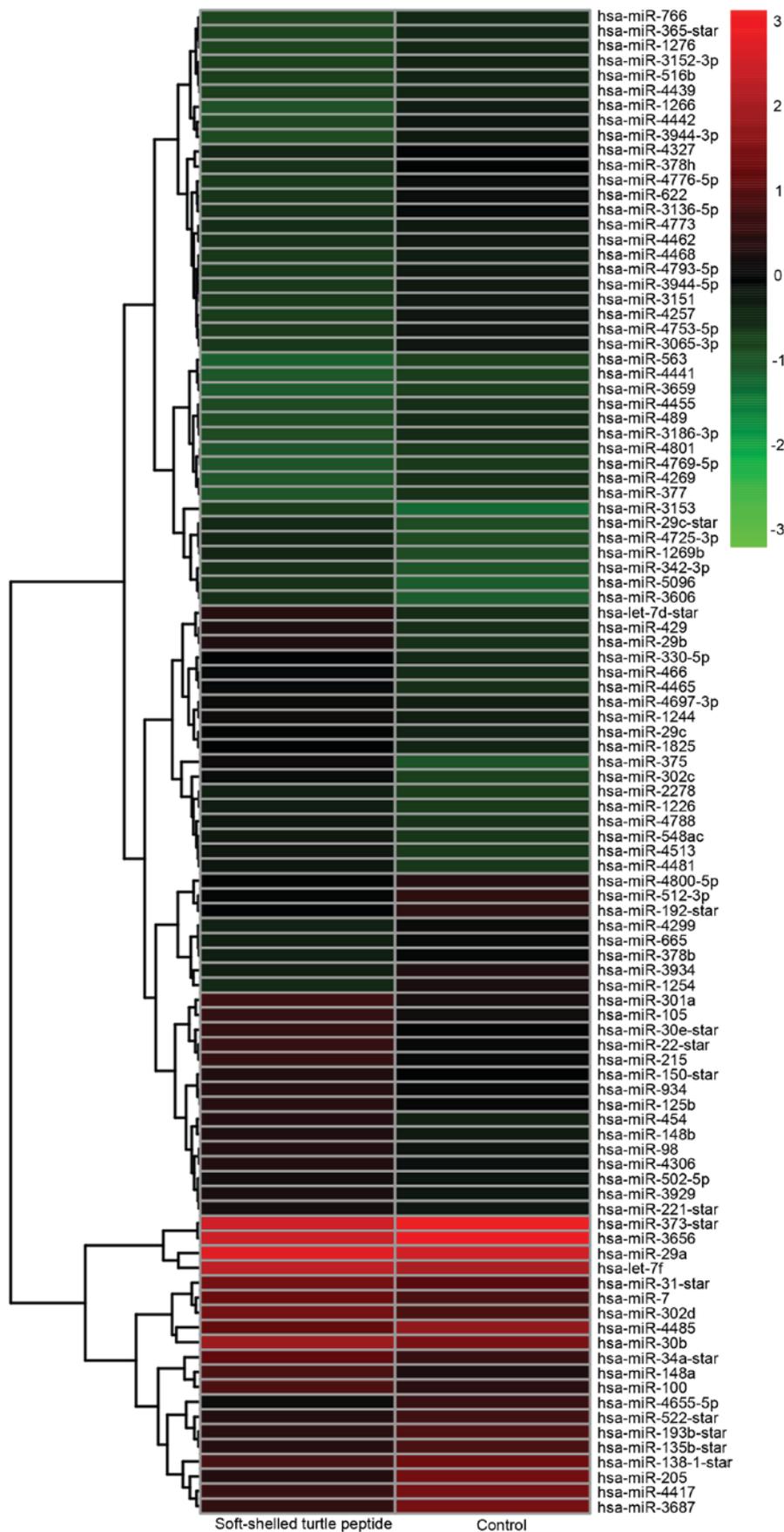


Figure 1. Gene Cluster (version 3.0) and Java TreeView software programs were used to perform the hierarchical cluster analysis of differentially expressed miRNA microarray data in AGS cells treated with soft-shelled turtle peptides. A hierarchical cluster analysis of 101 miRNAs whose expression differed significantly between the soft-shelled turtle peptides-treated group (treatment for 72 h) and the untreated group (fold-change >2 or <0.5;  $P < 0.05$ ). Each row represents a miRNA and each column represents a sample. The miRNA expression levels are illustrated using a color key and histogram. The red and green colors denote high and low expression, respectively. miR/miRNA, microRNA.

## Results

*Alteration of miRNA expression profile in GC cells treated with soft-shelled turtle peptide.* In order to evaluate the soft-shelled turtle peptide-mediated anticancer capabilities in GC cells, the AGS cells were treated with 100 mg/l soft-shelled turtle peptide for 72 h and the medium was renewed every 24 h. A total of 1,744 human miRNAs were screened to analyze the regulation of soft-shelled turtle peptide on miRNA expression in the AGS cells. Compared with the untreated cells, the miRNA expression of AGS cells with soft-shelled turtle peptide treatment varied markedly. According to the hierarchical cluster analysis, there were 101 differentially expressed miRNAs in the AGS cells treated with the soft-shelled turtle peptide (Fig. 1). Among them, 49 miRNAs were upregulated and 52 miRNAs were downregulated after treatment with the soft-shelled turtle peptide (Table II). Particularly, the expression of miRNA-375 was increased intensely by 8.32-fold and other upregulated miRNAs, including let-7d, miRNA-29b, miRNA-429, miRNA-454, miRNA-148a, miRNA-22, miRNA-30e, miRNA-302 and miRNA-148b, were altered markedly by >3-fold. Additionally, the expression of miRNA 205, miRNA-1254, miRNA-3687 and miRNA-1266 was significantly downregulated by <0.25-fold.

Soft-shelled turtle peptides associated with miRNAs in previous studies of GC (20-48) were reviewed and are summarized in Table III. To further validate the microarray results, 10 miRNAs, including hsa-miR-4455, hsa-miR-29a, hsa-miR-378b, hsa-miR-29b, hsa-miR-205, hsa-miR-100, hsa-miR-7, hsa-miR-375, hsa-miR-4299 and hsa-miR-378h, were selected to be verified according to RT-qPCR analysis. Results of the RT-qPCR validation were similar to results of the microarray, indicating that the miRNA data was reliable (Fig. 2).

*Target genes of differentially expressed miRNAs modified by soft-shelled turtle peptide.* With the identification of differentially expressed miRNAs in AGS cells following treatment of the soft-shelled turtle peptide, the target genes of these miRNA were further investigated according to 5 online software programs, including TargetMiner, miRDB, microRNA, TarBase, and RNA-22 (Table IV). As presented in Table V, potential target genes were identified.

*GO analysis and pathway analysis.* To further evaluate the role of soft-shelled turtle peptide influenced miRNAs in physiological functions and pathological processes in GC, interactions between miRNAs and associated target genes were predicted according to GO analysis and pathway analysis. The GO cellular component analysis demonstrated that target genes were mainly clustered into the extracellular region, extracellular space, keratin filament and nucleus categories (Fig. 3). The GO molecular function analysis indicated that the bulk of the target genes participated in olfactory receptor activity and G-protein coupled receptor activity (Fig. 3). In addition, the GO biological process analysis demonstrated that these target genes were closely associated with the detection of chemical stimuli involved in the sensory perception of smell and the G-protein coupled receptor signaling pathway (Fig. 3).

Table II. Differentially expressed miRNAs (upregulated >2-fold or downregulated <0.5-fold) in AGS cells following treatment with soft-shelled turtle peptides.

miRNA	Fold-change
Upregulated	
hsa-miR-375	8.32
hsa-let-7d-star	6.05
hsa-miR-29b	5.82
hsa-miR-429	5.40
hsa-miR-454	4.10
hsa-miR-148a	3.66
hsa-miR-22-star	3.44
hsa-miR-30e-star	3.43
hsa-miR-302c	3.42
hsa-miR-148b	3.23
hsa-miR-215	3.08
hsa-miR-301a	2.82
hsa-miR-302d	2.69
hsa-miR-34a-star	2.66
hsa-miR-4306	2.62
hsa-miR-100	2.59
hsa-miR-330-5p	2.54
hsa-miR-98	2.52
hsa-miR-4465	2.50
hsa-miR-1244	2.49
hsa-miR-30b	2.46
hsa-miR-4513	2.45
hsa-miR-3929	2.44
hsa-miR-105	2.43
hsa-miR-221-star	2.43
hsa-miR-466	2.35
hsa-miR-4481	2.35
hsa-miR-3153	2.32
hsa-miR-3606	2.32
hsa-miR-5096	2.32
hsa-miR-1269b	2.32
hsa-miR-4697-3p	2.29
hsa-miR-4725-3p	2.29
hsa-miR-7	2.21
hsa-miR-1825	2.17
hsa-miR-502-5p	2.14
hsa-miR-4788	2.13
hsa-miR-31-star	2.11
hsa-miR-150-star	2.06
hsa-miR-29c	2.06
hsa-miR-29a	2.05
hsa-miR-29c-star	2.04
hsa-miR-342-3p	2.04
hsa-miR-1226	2.04
hsa-miR-2278	2.04
hsa-miR-548ac	2.04
hsa-let-7f	2.04
hsa-miR-125b	2.02
hsa-miR-934	2.01

Table II. Continued.

miRNA	Fold-change
Downregulated	
hsa-miR-205	0.17
hsa-miR-1254	0.22
hsa-miR-3687	0.23
hsa-miR-1266	0.25
hsa-miR-4442	0.27
hsa-miR-378h	0.27
hsa-miR-3944-3p	0.29
hsa-miR-4776-5p	0.29
hsa-miR-4417	0.29
hsa-miR-3934	0.30
hsa-miR-3136-5p	0.33
hsa-miR-4257	0.33
hsa-miR-4655-5p	0.33
hsa-miR-4753-5p	0.36
hsa-miR-192-star	0.36
hsa-miR-622	0.36
hsa-miR-4299	0.38
hsa-miR-4269	0.39
hsa-miR-3065-3p	0.39
hsa-miR-4327	0.39
hsa-miR-3151	0.39
hsa-miR-512-3p	0.39
hsa-miR-3944-5p	0.40
hsa-miR-377	0.40
hsa-miR-489	0.42
hsa-miR-193b-star	0.42
hsa-miR-665	0.42
hsa-miR-3152-3p	0.42
hsa-miR-4793-5p	0.42
hsa-miR-563	0.43
hsa-miR-766	0.44
hsa-miR-1276	0.44
hsa-miR-4462	0.44
hsa-miR-373-star	0.44
hsa-miR-4468	0.44
hsa-miR-4485	0.45
hsa-miR-365-star	0.45
hsa-miR-3186-3p	0.45
hsa-miR-378b	0.45
hsa-miR-135b-star	0.45
hsa-miR-4800-5p	0.46
hsa-miR-516b	0.46
hsa-miR-522-star	0.48
hsa-miR-4441	0.48
hsa-miR-4439	0.48
hsa-miR-3659	0.48
hsa-miR-4801	0.48

Table II. Continued.

miRNA	Fold-change
hsa-miR-4769-5p	0.48
hsa-miR-3656	0.49
hsa-miR-138-1-star	0.49
hsa-miR-4773	0.49
hsa-miR-4455	0.49
miR/miRNA, microRNA.	

However, the pathway analysis indicated that the target genes were associated with the thyroid hormone signaling pathway, the Hippo signaling pathway, the forkhead box O (FoxO) signaling pathway, the AMP-activated protein kinase signaling pathway, the mechanistic target of rapamycin signaling pathway and transcriptional misregulation in cancer, among others (Fig. 4).

### Discussion

Dietary components function as cancer chemoprevention agents principally through improving the nutritional supplement, enhancing immunity or directly blocking cancer cells (49). Soft-shelled turtle derived proteins can alleviate the side effects of cancer patients undergoing chemotherapy and radiotherapy, including myelosuppression (16). In the present study, the primary focus was upon the direct influence of soft-shelled turtle extract on the miRNA expression profile in human GC AGS cells.

From the results of the miRNA array, a total of 101 differentially expressed miRNAs, including 49 upregulated miRNAs and 52 downregulated miRNAs, were identified. Among the 49 miRNAs with increased expression, the expression of miRNA-375 was altered markedly, with 8.32-fold upregulation. The expression of miRNA-375 has been identified to be frequently downregulated in various types of cancer, including GC, head and neck squamous cell carcinoma, esophageal cancer, pancreatic ductal adenocarcinoma and hepatocellular carcinoma (21,50-53). In GC, miRNA-375 may function as a tumor suppressor. Overexpression of miRNA-375 could inhibit the proliferation of GC cells by targeting Janus kinase 2 (21). Moreover, Snail, a metastasis-associated transcription factor, directly binds to the putative promoter of miR-375 (54). Chang *et al* (55) also identified that the expression of miRNA-375 was dependent on *Helicobacter pylori* infection in GC; the miRNA-375 expression level in *H. pylori*-positive GC tissues was lower than that in the *H. pylori*-negative GC tissues. In the present study, the expression of miRNA-375 was upregulated to the greatest extent following treatment with the soft-shelled turtle peptide. This indicated that the soft-shelled turtle peptide may have the capacity to block the proliferation and metastasis of GC cells by increasing the expression of miRNA-375. Other significantly upregulated miRNAs, including let-7d, miRNA-29b, miRNA-429, miRNA-22, miRNA-148a/148b and miRNA-34a, played an anticancer role in GC. miRNA-429 acts as a tumor suppressor

Table III. Differentially expressed miRNAs induced by soft-shelled turtle peptides in GC.

miRNA	Regulation <sup>a</sup>	Expression in the literature <sup>b</sup>	Source <sup>c</sup> (ref.)
miR-375	Upregulated	Downregulated	GC tissues, gastric epithelial with Hp infection (20) GC cell lines: BGC823, MGC803, SGC7901, AGS, N87 and MKN45 (21,22) Plasma in GC patients (20)
miR-7d	Upregulated	Downregulated	Serum in GC patients with Hp infection (23)
miR-29b	Upregulated	Downregulated	GC tissues (24) GC cell lines: SGC7901, AGS, MKN45, MGC803 and BGC823 (25)
miR-429	Upregulated	Downregulated	GC tissues (26) GC cell lines: BGC823, MGC803 and AGS (27-29)
miR-454	Upregulated	/	/
miR-148a	Upregulated	Downregulated	GC tissues (20,30) GC cell lines: AGS, BGC823, MKN1, MKN45 and MKN74 (30,31) Plasma and serum in GC patients (20,32)
miR-22	Upregulated	Downregulated	GC tissues (33-35) GC cell lines: AGS, BGC823, HGC27 and SGC7901 (33-35)
miR-30e	Upregulated	Downregulated	GC tissues (36)
miR-148b	Upregulated	Downregulated	GC tissues (37) GC cell lines: AGS, MGC803, SGC7901 and BGC823 (37)
miR-34a	Upregulated	Downregulated	GC tissues (38,39) GC cell lines: MGC803, HGC27, NCI-N87 and SGC7901 (39,40) Gastric MALT lymphoma tissues and B-cell lymphoma tissues (41)
miR-205	Downregulated	Downregulated	GC tissues (42) GC cell lines: AGS and NCI-N87 (42)
miR-1254	Downregulated	/	/
miR-3687	Downregulated	/	/
miR-1266	Downregulated	Downregulated	GC tissues (43) GC cell lines: BGC823, MKN28 and SGC7901 (43)
miR-192	Downregulated	Upregulated	GC tissues (44,45) GC cell lines: BGC823, SGC7901 and MKN45 (46) Plasma in GC patients (46)
miR-377	Downregulated	Upregulated	GC tissues (47) GC cell lines: MKN45 (47)
miR-665	Downregulated	Upregulated	Intestinal GC tissues (48)

<sup>a</sup>Regulation means the expression level induced by soft-shelled turtle peptides; <sup>b</sup>literature represents the expression of miRNAs in previous gastric cancer-associated studies; <sup>c</sup>source indicates the expression of miRNAs in different sources presented in the previous literature. miR/miRNA, microRNA; GC, gastric cancer; Hp, *Helicobacter pylori*; MALT, mucosa-associated lymphoid tissue; /, no relevant reference published.

and has been identified to be downregulated in certain cancer types, including GC (26), clear cell renal cell carcinoma (56) and glioblastoma multiforme (57). It has been demonstrated that miRNA-429 induces the apoptosis of glioblastoma cells via B-cell lymphoma 2 (Bcl-2), while the suppression of miRNA-429 promotes Bcl-2-mediated cancer cell survival (57). Other studies indicated that c-Myc was a critical target gene of miRNA-429, and that miRNA-429 significantly downregulated the expression of c-myc to control the growth of GC cells (26). Additionally, miRNA-429 was demonstrated to not only inhibit

GC cell growth, but to regulate GC cell invasiveness through zinc finger E-box-binding homeobox protein (58). miRNA-148a and miRNA-148b play important roles, functioning as tumor suppressors in GC. However, downregulation of miRNA-148a contributes to GC lymph node-metastasis and progression, while upregulation of miRNA-148b suppresses GC cell growth. These two miRNAs are therefore potential therapeutic target candidates (37,59). However, in the study, it was revealed that after treatment with soft-shelled turtle peptide, these suppressor function-associated miRNAs whose expression was

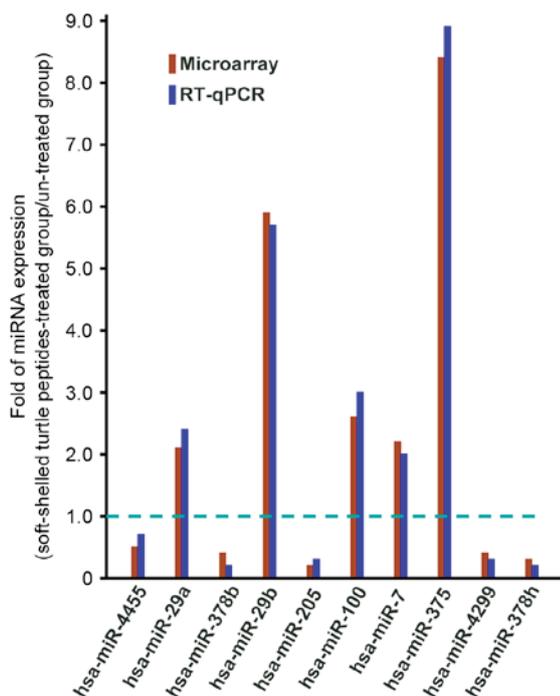


Figure 2. RT-qPCR validation of differentially expressed miRNAs that were screened by the microarray assay. Upregulated and downregulated miRNAs are indicated by bars above and below the dotted line respectively. The RT-qPCR results were representative of three independent experiments. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miRNA, microRNA.

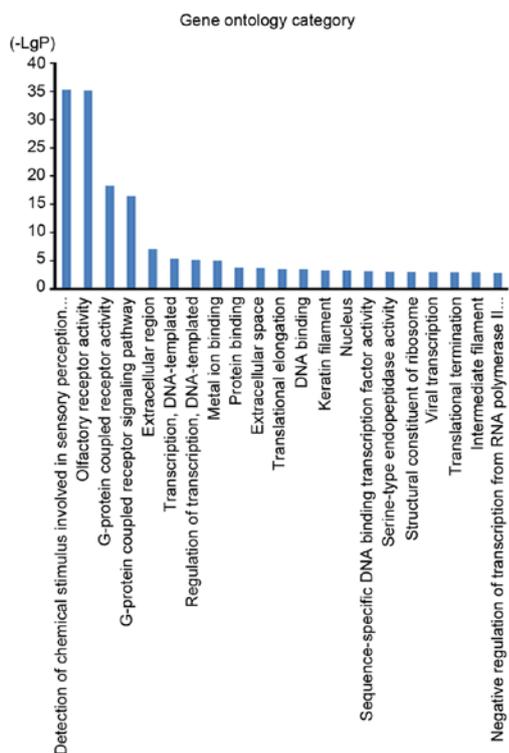


Figure 3. GO analysis for target genes of differentially expressed miRNAs. To investigate the biological functions of the differentially expressed miRNAs, public online databases were used to obtain miRNA-regulated putative target genes. A GO function analysis of miRNA targets was performed to obtain the significantly over-represented GO terms ( $P < 0.01$ ). GO category for target genes indicated that several important GO terms were possibly associated with cell activation, including intracellular signal transduction, protein phosphorylation, metabolic process and the positive regulation of genes. GO, Gene Ontology; miRNA, microRNA.

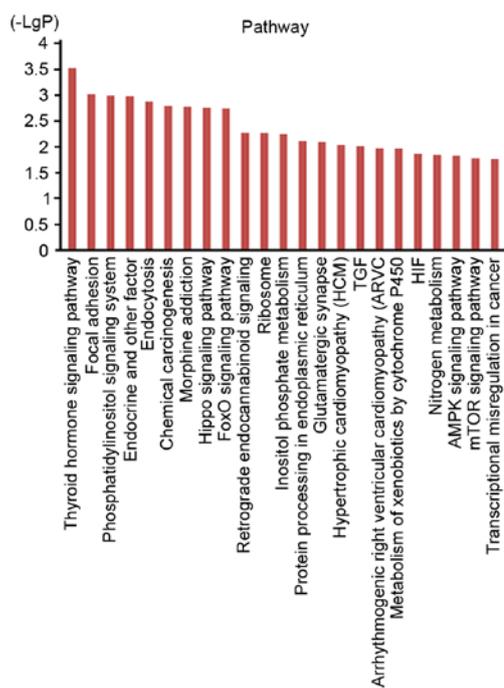


Figure 4. Pathway analysis for target genes of differentially expressed miRNAs. The target genes of soft-shelled turtle peptides related miRNAs were predicted with Kyoto Encyclopedia of Genes and Genomes pathway analysis. miRNA, microRNA; FoxO, forkhead box O; TGF, transforming growth factor; HIF, hypoxia-inducible factor; AMPK, AMP-activated protein kinase; mTOR, mechanistic target of rapamycin.

restrained in GC cells were significantly restored to an upregulated state. This indicated that the soft-shelled turtle peptide not only improves immunity to contend against cancer cells indirectly *in vivo*, but that it also has the capacity to block GC cell growth directly through regulation of associated miRNAs and target genes. Furthermore, certain miRNAs, including miRNA-375, could be detected in plasma and may become potential biomarkers for the dynamic monitoring and evaluation of cancer prognosis (20,60).

Olfactory receptor neurons express olfactory receptor gene to detect odor molecules and transport the action potential to the olfactory bulb of the brain. Olfactory receptors are G protein-coupled receptors that are mainly expressed in the olfactory epithelium to detect odorants (61). However, besides olfactory tissues, non-olfactory tissues have also recently been demonstrated to exhibit the expression of olfactory signaling components (olfactory receptor, olfactory G-protein, adenylate cyclase III and olfactory marker protein) (62,63). The role of olfactory signaling components in non-olfactory tissues has not been clarified. Nevertheless, it has been demonstrated that olfactory receptors are not only involved in monitoring extracellular chemical cues, but that they are also frequently overexpressed in various types of cancer cells (64,65). In the present study, the GO analysis results indicated that target genes of soft-shelled turtle peptide-specific miRNAs were involved in a number of essential biological processes. Moreover, two biological processes, namely detection of chemical stimuli involved in the sensory perception of smell and the G-protein coupled receptor signaling pathway, were closely associated with the target genes. The role of olfactory receptor-associated events in GC has not

Table IV. Predicted target genes of differentially expressed miRNAs from five miRNA databases.

miRNA	miRNA databases					Total
	TargetMiner	miRDB	microRNA	TarBase	RNA22	
<b>Upregulated</b>						
hsa-miR-429	161	767	92	3,283	33	3,625
hsa-miR-454	0	0	380	0	0	380
hsa-miR-4306	3,194	1,689	402	3,560	714	6,716
hsa-miR-98	2,685	0	209	2,426	0	4,504
hsa-miR-4465	2,285	512	0	651	1,020	3,806
hsa-miR-3929	2,763	484	0	3,363	3,169	7,130
hsa-miR-466	72	741	115	3,225	745	3,917
hsa-miR-4697-3p	1,807	247	0	2,111	4,791	7,210
hsa-miR-7	0	0	457	0	0	457
hsa-miR-502-5p	109	216	447	2,456	3,351	5,498
hsa-miR-934	18	133	174	1,125	646	1,893
<b>Downregulated</b>						
hsa-miR-378h	132	171	0	1,976	1,602	3,426
hsa-miR-4417	1,942	218	0	2,175	8,113	9,630
hsa-miR-3934	2,754	0	0	2,772	0	4,829
hsa-miR-4655-5p	278	21	0	221	7,511	7,659
hsa-miR-4299	855	276	447	2,313	2,089	4,754
hsa-miR-4327	1,405	190	396	1,280	7,696	9,158
hsa-miR-512-3p	1,078	383	498	3,066	2,153	5,475
hsa-miR-665	966	612	1,747	3,158	6,901	9,266
hsa-miR-4485	1,275	0	0	191	0	1,438
hsa-miR-378b	132	167	555	2,048	1,926	4,033
hsa-miR-4800-5p	152	83	0	1,296	1,900	3,093
hsa-miR-522-5p	2,001	206	0	460	1,864	4,057

Number of target genes predicted by five miRNA databases for each miRNA was different. miR/miRNA, microRNA.

been reported. In pancreatic cancer cells, the involvement of PI3 kinase  $\gamma$ -dependent signaling pathway in the promotion of tumor cell invasiveness could be triggered by olfactory receptor stimulation (64). Hence, the tight association between olfactory receptors and soft-shelled turtle peptide-specific miRNA-gene indicated that soft-shelled turtle peptide may influence the abilities of growth, metastasis and invasion in cancer cells through the regulation of olfactory receptor-associated events.

In the present study, KEGG analysis revealed that the target genes were associated with several cancer-related pathways. Particularly, the Hippo signaling pathway and the FoxO signaling pathway, which function as tumor suppressors, may be regulated by soft-shelled turtle peptide. The major functions of the Hippo signaling pathway have been defined as restricting cell growth and modulating cell proliferation and differentiation in developing organs (66,67). Transcriptional coactivator YAP1/telomere length regulator Taz1 (YAP1/TAZ) are the most important effectors and are negatively regulated by the Hippo pathway through phosphorylation-dependent and -independent mechanisms. The upregulation of YAP1 and TAZ induces epithelial-mesenchymal transition and increases drug resistance in cancer cells (68-70). Deregulation of the

Hippo signaling pathway is involved in the initiation, progression and metastasis of a number of cancer types (71-73), including GC (74). Hence, the Hippo pathway has been speculated to be a drug target inhibitor of YAP1 and TAZ in cancer therapy (75,76). Additionally, miRNA-375 has been identified to target the Hippo pathway effector YAP to inhibit the proliferation and invasion of liver cancer cells (77). Therefore, we hypothesize that soft-shelled turtle peptide may exert anticancer functions through the miRNA-375-Hippo pathway. FoxO factors not only play an anticancer role in various types of tumors (78,79), but also contribute to extreme longevity and life span (80). miRNA-22 forms a regulatory loop to fine-tune the dynamics of the phosphatase and tensin homolog/AKT/FoxO1 pathway (81). miRNA-30d induces apoptosis and is regulated by the AKT/FoxO pathway in renal cell carcinoma, and is formed as the AKT/FoxO/miR-30d/metastasis adhesion protein signaling transduction pathway (82).

In conclusion, the microarray results of the present study indicated that the soft-shelled turtle peptide has the potential function to influence cancer-related miRNAs and pathways. However, the anticancer properties of soft-shelled turtle peptide in GC require further validation *in vitro* and *in vivo*.

Table V. Potential target genes for miRNAs differentially expressed in response to the soft-shelled turtle peptides.

miRNA	Target gene	Function of the encoded protein
hsa-miR-429	CERS6	Sphingolipid biosynthesis
	MIB1	Ubiquitination
	CBL	Regulation of tyrosine kinases
	HOOK1	Early endosome trafficking
	ZNF148	Regulation of cell growth, apoptosis
hsa-miR-454	SUN2	Interaction with chromatin and the nuclear lamina
	ADAM28	Sperm maturation and immune suppression
	KDM2A	Cell proliferation, differentiation of mesenchymal stem cells derived from dental tissue
hsa-miR-98	COL4A2	Assembled with COL4A1, interaction with growth factors and cell surface receptors
	MAP4K3	Cell growth, viability, cell death, body size and metabolism
	IL13	Mediator of allergic inflammation
	IGF2BP1	Embryo development, oncofetal RNA-binding protein
hsa-miR-7	S100A7A	Inflammation, tumorigenesis
	EGFR	Cell growth, proliferation, differentiation
	SUCNR1	Paired with succinate, cell metabolism
	ZEB2	A key regulator of epithelial mesenchymal differentiation
	PIK3AP1	Regulation of cell proliferation, survival, metabolism, cytoskeletal reorganization and membrane trafficking
	CD86	Immune responses
hsa-miR-502-5p	ADAM9	Upregulation associated with tumorigenesis
	KIF1B	Axonal transport of synaptic vesicles and mitochondria, axon myelination and outgrowth
	RNF14	Mitochondrial transcription, immune function
hsa-miR-378h	GRAP2	Calcium influx and cytokine production
	CBL	Regulation of tyrosine kinases
	PTCH1	A key regulator of cell proliferation, cell surveillance, embryonic development, adult tissue homeostasis and stem cell quiescence
	SULF1	Blocking the binding of growth factors and receptors, regulation of cell growth and differentiation
	TRAF3	Regulation of signaling through B-lymphocyte receptors, inhibition of B-cell homeostatic survival
hsa-miR-665	CELF2	Regulation of mRNA editing, stability and translation
	SRF	A transcription factor for coupling actin dynamics and signaling pathways to gene expression
	SMAD7	An antagonist of TGF- $\beta$ signaling
	BCL7B	Negatively regulating the Wnt-signaling pathway and positively regulating the apoptotic pathway
hsa-miR-4417	ELK1	Regulation of proto-oncogene c-fos
	FGF9	Epithelial-mesenchymal interaction
	PCDH17	Functioning as a tumor suppressor
	FOXO1	Stopping cell cycle progression, promotes apoptosis, and negatively regulating angiogenesis
	IGFBP5	Inducing extracellular matrix production and deposition
	SH2B3	A negative regulator of multiple cytokine signaling pathways
	RAB6A	Regulating intracellular vesicle transport

## Acknowledgements

This study was supported in part by Special Grants from the Zhejiang Agricultural Group Co., Ltd., Scientific Research Grants in Traditional Chinese Medicine, Zhejiang Province of China (no. 2012ZB018), Research Grants from the National Natural Science Foundation of China (nos. 81372332 and 81572822) and Key Projects from the Zhejiang Provincial Natural Science Foundation of China (no. LZ13H160002 and LZ18H160002), and was partly sponsored by Zhejiang Provincial Program for the Cultivation of High-level Innovative Health talents (no. Zjwjw2014-108) and the Major Training Personnel from Zhejiang Provincial Program for the Training and Development Project for 151 talents (no. Zjhrss2014-150).

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