Matrine alters microRNA expression profiles in SGC-7901 human gastric cancer cells

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Abstract. Matrine, a major alkaloid extracted from *Sophora flavescens*, has been reported to possess antitumor properties in several types of cancers, including gastric cancer. However, its mechanisms of action on gastric cancer remain poorly understood. Dysregulation of microRNAs, a class of small, non-coding, regulatory RNA molecules involved in gene expression, is strongly correlated with cancer. The aim of the present study was to demonstrate that matrine treatment altered miRNA expression in SGC7901 cells. Using miRCURY™ microarray analysis, we identified 128 miRNAs substantially exhibiting >2-fold expression changes in matrine-treated cells relative to their expression levels in untreated cells. RT-qPCR was used to show that the levels of 8 miRNAs whose target genes were clustered in the cell cycle pathway increased, while levels of 14 miRNAs whose target genes were clustered in the MAPK signaling pathway decreased. These results were consistent with those from the miRNA microarray experiment. Bioinformatical analysis revealed that the majority of 57 identified enrichment pathways were highly involved in tumorigenesis. In conclusion, the results demonstrated that matrine induces considerable changes in the miRNA expression profiles of SGC7901 cells, suggesting miRNA microarray combined with RT-qPCR validation and bioinformatical analysis provide a novel and promising approach to identify anticancer targets and the mechanisms of matrine involved.

Introduction

Matrine is a major alkaloid extracted from *Sophora flavescens* that has been used clinically in the treatment of gastric cancer. Matrine exhibits antitumor activity in several types of cancer including gastric (1-5), lung cancer (6,7), acute myeloid leukemia (8,9), breast (10), prostate (11), hepatocellular (12), pancreatic (13), colon cancer (14) and gallbladder carcinomas (15). The anticancer properties of matrine are associated with its ability to suppress proliferation and induce apoptosis in tumor cell lines through a variety of different pathways (2,7-10,16). However, in the case of gastric cancer the mechanism of action has yet to be clarified.

Matrine injections have been found to inhibit the migration, invasion and adhesion capacity of SGC-7901 gastric cancer cells *in vitro*. This inhibition may be correlated with the downregulation of CD44 (V6) protein expression (2). Matrine injections are also able to induce apoptosis of SGC-7901 cells by upregulating Fas/FasL expression and activating caspase-3 (3). Furthermore, matrine can induce apoptosis in MKN45 gastric cancer cells via an increase in pro-apoptotic molecules from the Bcl-2 family (5). Matrine has been found to modulate NF-κB, XIAP, CIAP and p-ERK protein expression in the MNK45 cell line, resulting in antitumor effects (4). In addition, matrine inhibits the adhesion and migration of BGC823 gastric cancer cells by affecting the structure and function of the vasodilator-stimulated phosphoprotein (VASP) (17).

It has recently been reported that matrine suppresses breast cancer by downregulating miR-21, leading to the dephosphorylation of Akt and resulting in an accumulation of Bad, p21(/WAF1/CIP1) and p27(/KIP1) (10). Thus, miRNA may act as a mediator, contributing to the therapeutic efficacy of matrine. The aim of the present study was to examine the effects of matrine on miRNA expression profiles of the SGC7901 gastric cancer cell line and to identify the putative target genes by analyzing differentially expressed miRNAs and the enrichment pathways of target genes. To the best of our knowledge, this is the first study to examine the effect of treatment with matrine on miRNA expression profiles in gastric cancer cells.

Materials and methods

Cell line and cell culture. The SGC7901 cell line was obtained from the Cancer Institute and Hospital, Chinese

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Academy of Medical Sciences (CAMS) (Beijing, China). The cells were grown in 90% RPMI-1640, supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin and incubated at 37˚C in a humidified atmosphere of 5% CO_2.

Matrine treatment. Matrine (Baoji Fangsheng Pharmaceutical Co., Ltd., Xian, China) was analyzed by HPLC assay and dissolved in phosphate-buffered saline (PBS; Invitrogen-Gibco, Grand Island, NY, USA). SGC-7901 cell cultures were then treated for 24 h with 0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml concentrations of matrine. Subsequently, 20 μl of 5 mg/ml MTT in PBS was added to each well and the plate was incubated at 37˚C for 4 h. The plate was then centrifuged at 1,000 x g for 2 min and followed by removal of the medium. One-hundred and fifty microliter of dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA) was then added. After incubation at 37˚C for 5 min, absorbance in the control and matrine-treated cells was measured spectrophotometrically at 570 nm using a benchmark microtiter plate reader (Bio-Rad Laboratories, Hercules, CA, USA). Microarray array was subsequently conducted on the control (blank) vs. 1.5 mg/ml matrine (MA) treatment groups.

miRNA microarray analysis. Total RNA, including miRNA, was isolated using the miRNeasy mini kit (Qiagen, Hilgen, Germany), according to the manufacturer's instructions. RNA quality and quantity was measured via a NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, DE, USA) and RNA integrity was determined by gel electrophoresis. The concentration and purity of total RNA were assessed at absorbance readings of 260 and 280 nm, using an ultraviolet spectrophotometer. Only the RNA samples with ratios of A260/A280 >1.8 were used in the present study. After isolating the RNA, the miRCURY Hy3™/Hy5™ Power labeling kit (Exiqon, Copenhagen, Denmark) was used for miRNA labeling, according to the manufacturer's instructions. One microgram of each sample was 3'-end-labeled with a Hy3™ fluorescent label using T4 RNA ligase, as follows: RNA was placed in 2.0 μl of water and combined with 1.0 μl of CIP buffer and CIP (Exiqon, Vedbaek, Denmark). After incubating for 30 min at 37˚C the reaction was terminated by 5-min incubation at 95˚C. A volume of 3.0 μl labeling buffer, 1.5 μl fluorescent label (Hy3™), 2.0 μl DMSO, and 2.0 μl labeling enzyme were then added. The mixture was incubated for 1 h at 16˚C for labeling, followed by 15-min incubation at 65˚C to terminate the labeling reaction. After labeling, the Hy3™-labeled samples were hybridized on the miRCURY™ LNA Array v.18.0 (Exiqon, Copenhagen, Denmark), according to the array manual. A 25 μl volume of the Hy3™-labeled sample mixture and 25 μl hybridization buffer were denatured for 2 min at 95˚C, incubated on ice for 2 min and then hybridized to the microarray for 16-20 h at 56˚C in a 12-Bay hybridization system (NimbleGen Hybridization System 12; Roche Applied Science, Madison, WI, USA), which provides an active mixing action and constant incubation temperature to improve hybridization uniformity and signal enhancement. Following hybridization, slides were prepared, washed several times using a wash buffer kit (Exiqon), and dried via centrifugation for 5 min at 400 rpm. The slides were scanned using an Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA, USA).

miRNA target gene prediction and signaling pathway analyses. miRBase, miRanda and TargetScan were used to predict the target genes of differentially expressed miRNAs following matrine treatment. The prediction information from the three databases that overlapped was considered the final result. The miRNA target gene prediction databases miRFocus 2.0 (http://mirfocus.org/index.php) and miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) were also used to analyze plausible KEGG pathway enrichment of dysregulated miRNA targets. The miRFocus 2.0 prediction tools included miRanda, MirTarget2, PicTar, microT and TargetScanS. The experimental validated target gene tools included miRecords, miR2Disease, TarBase and miRTarBase. The prediction databases support number was ≥3 and the Fisher test P-value cut-off was 0.01.

miRNA quantification by RT-qPCR. A SYBR-Green RT-PCR assay was used for miRNA quantification. In brief, 1 μg of total RNA, containing miRNA, was polyadenylated using poly(A) polymerase and was reverse transcribed to cDNA using a miScript Reverse Transcription kit, according to the manufacturer's instructions (GeneCopoeia, Rockville, MD, USA). The miScript Universal primer was provided by the manufacturer (GeneCopoeia) and the miScript SYBR-Green PCR kit was used. RT-PCR was performed using the Bio-Rad CFX96 real-time PCR system. Each reaction was performed in a final volume of 10 μl containing 2 μl cDNA, 0.5 mM of each primer and 1X SYBR-Green PCR Master Mix (GeneCopoeia). The amplification program was as follows: denaturation at 95˚C for 10 min, followed by 45 cycles at 94˚C for 15 sec, 55˚C for 30 sec and 70˚C for 30 sec, in which fluorescence was obtained. After completion of the PCR cycles melting curve analyses and electrophoresis, on 2.5% agarose gels were performed to validate the specificity of the expected PCR product. Each sample was analyzed in triplicate. The expression levels of miRNAs were normalized to RNU6B and the relative gene expression was calculated as $2^{-(CTmiRNA\text{-}CTRNU6B\ RNA)}$. Each sample was analyzed in triplicate. All the miRNA PCR primers were purchased from GeneCopoeia.

Statistical analysis. Scanned images of the miRNA Array were imported into GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. Replicated miRNAs were averaged and miRNAs with intensities of ≥50 in all the samples were used to calculate the normalization factor. Expressed data were normalized using the Median normalization. After normalization, differentially expressed miRNAs were identified through fold change filtering. Hierarchical clustering was performed using MeV software (v4.9, TIGR). Results of real-time RT-PCR experiments are expressed as means ± SD.

Results

MTT results. The antitumor reducing activity of matrine cytotoxicity (0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml) to SGC-7901 cells culture is shown in Fig. 1. Matrine showed a dose-dependent inhibition of the growth of SGC-7901 cells.

Effects of matrine on miRNA expression profiling of SGC-7901 gastric cancer cells. After SGC-7901 cells were treated with

Table I. Expression of 8 upregulated miRNAs altered by microarray experiment and RT-qPCR experiment validation after matrine treatment (1.50 mg/ml).

miRNA	Fold change, microarray experiment	Fold change compared with control; mean ± SD, RT-qPCR experiment
miR-192-5p	6.352408	5.30±0.70
miR-194-5p	3.258979	3.71±0.29
miR-299-5p	4.094039	5.22±0.37
miR-300	4.702027	5.53±0.59
miR-302a-3p	2.215804	4.28±0.81
miR-483-5p	2.921674	3.60±0.82
miR-498	3.790496	5.34±0.40
miR-513a-5p	2.628494	3.24±0.96

(Fold change, mean ± SD, treated with matrine vs. untreated). Eight upregulated miRNAs found by miRNA microarray experiment were consistent with qPCR experiment validation. Relative gene expression was calculated as $2^{-(CTmiRNA-CTRNU6B\ RNA)}$, and each sample was analyzed in triplicate.

Table II. Expression of 14 downregulated miRNAs altered by microarray experiment and qRT-PCR experiment validation after matrine treatment (1.50 mg/ml).

miRNA	Fold change, microarray experiment	Fold change compared with control; mean ± SD, qRT-PCR experiment
let-7b-5p	0.458117	0.74±0.10
let-7c	0.376421	0.67±0.07
let-7d-5p	0.445587	0.09±0.01
let-7f-5p	0.145997	0.76±0.13
miR-19a-3p	0.327783	0.56±0.07
miR-19b-3p	0.498536	0.60±0.07
miR-20b-5p	0.218928	0.66±0.12
miR-21-5p	0.341840	0.58±0.08
miR-27a-3p	0.300471	0.56±0.04
miR-27b-3p	0.432703	0.51±0.05
miR-29c-3p	0.466145	0.78±0.07
miR-30e-5p	0.489503	0.82±0.05
miR-98-5p	0.386457	0.69±0.07
miR-106b-5p	0.475087	0.53±0.08

(Fold change, treated with matrine vs. untreated). Results indicating the trend of 14 downregulated miRNAs found by miRNA microarray experiment was consistent with qPCR experiment validation. Relative gene expression was calculated as $2^{-(CTmiRNA-CTRNU6B\ RNA)}$, and each sample was analyzed in triplicate.

1.5 mg/ml matrine for 24 h, 60 miRNAs were downregulated (fold change, ≤0.5) and 68 miRNAs were upregulated (fold change, ≥2.0) (Fig. 2). By searching the miRFocus 2.0 and miRWalk miRNA databases, only 38 of the 60 downregulated miRNAs and 8 of 68 upregulated miRNAs were annotated with their respective expressions in cancer tissues or cells or the predicted and validated target genes. Of the 46 annotated miRNAs, 25 were upregulated and 6 were downregulated in gastric cancer tissue or gastric cancer cell lines by miRNA microarray or RT-PCR (Tables I-III).

Table III. Matrine-regulated miRNAs and the related documentation of expression in gastric cancer tissue or gastric cancer cell lines identified by microarray analysis or RT-qPCR experiments.

miRNA name	Matrine treatment response	Expression in gastric cancer tissue or cell lines	(Refs.)
let-7b-5p	↓	↑	(18)
miR-10a-5p	↓	↑	(19,20)
miR-15b-5p	↓	↑	(18)
miR-18a-5p	↓	↑	(21,22)
miR-18b-5p	↓	↑	(21)
miR-19a-3p	↓	↑	(22,23)
miR-19b-3p	↓	↑	(18,20,24)
miR-20b-5p	↓	↑	(20,21,24,25)
miR-21-5p	↓	↑	(20,21,24,26-30)
miR-23a-3p	↓	↑	(20,26)
miR-26b-5p	↓	↑	(5,31)
miR-27a-3p	↓	↑	(20,25,26,32,33)
miR-29b-3p	↓	↓	(18,24)
miR-29c-3p	↑	↓	(18,24,34,35)
miR-30e-5p	↓	↓	(20)
miR-32-5p	↓	↑	(26)
miR-34a-5p	↓	↑	(20,22,36)
miR-98	↓	↑	(22)
miR-101-3p	↓	↑	(37)
miR-106b-5p	↓	↑	(20,22,30,36)
miR-130a-3p	↓	↓	(30)
miR-130b-3p	↓	↓	(22,30)
miR-148b-3p	↓	↓	(24,37)
miR-181a-5p	↓	↑	(22,38,39)
miR-338-3p	↓	↑	(25)
miR-340-5p	↑	↑	(21,22)
miR-424-5p	↑	↑	(40)
let-7a-5p	↓	↑	(26,32,41-43)
miR-192-5p	↑	↑	(20,38,44)
miR-194-5p	↑	↑	(20,45)
miR-335-5p	↑	↑	(37,45)

Of 38 miRNAs that were downregulated after matrine treatment, 20 have been reported to be upregulated in human gastric cancers and gastric cancer cell lines and act as oncogenes. Additionally, 5 matrine-upregulated miRNAs were found to be elevated in human gastric cancers and gastric cancer cell lines, whereas 6 matrine-downregulated miRNAs were found to be reduced in human gastric cancers or cell lines.

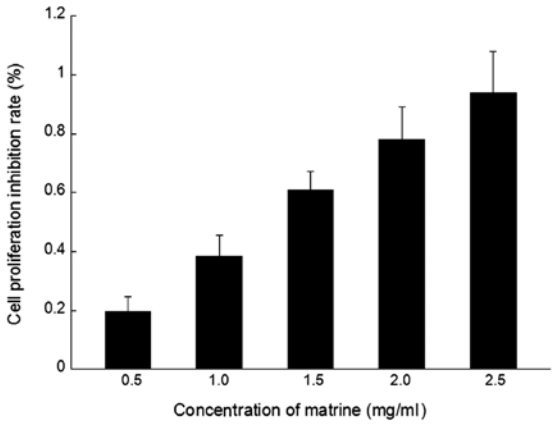

Figure 1. Matrine inhibited SGC-7901 cell proliferation in a dose-dependent manner. Cells were treated with 0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml of matrine for 24 h. Cell proliferation was determined by an MTT assay as described in 'Materials and methods'. The inhibition rate is expressed as the percentage of the cell inhibition rate compared with the control. Matrine inhibits SGC-7901 cell proliferation. A statistically significant difference within different dosage groups is evident (P<0.01).

Signaling pathway analyses of differentially expressed miRNA target genes. The miRBase, miRanda and TargetScan bioinformatical databases were used to predict the target genes of 46 aberrantly expressed miRNAs. The results showed that there are 4,159 overlapped target genes as predicted by the three tools for all the downregulated miRNAs and 367 overlapped target genes for the upregulated miRNAs that exhibited a >2-fold expression change after matrine treatment (Fig. 3). Possible regulation mechanisms of differentially expressed miRNAs following matrine treatment were investigated using the bioinformatics database, miRFocus 2.0, to select clustered enrichment KEGG pathways of plausible targets for each miRNA. A total of 57 enrichment pathways for predicted target genes were identified, including 36 enrichment signaling pathways for downregulated miRNAs. According to the Enrichment Score, the top 10 signaling pathways were the MAPK, focal adhesion, neurotrophin, axon guidance, ECM-receptor interaction, Wnt, amino sugar and nucleotide sugar metabolism, glycosaminoglycan biosynthesis-keratan

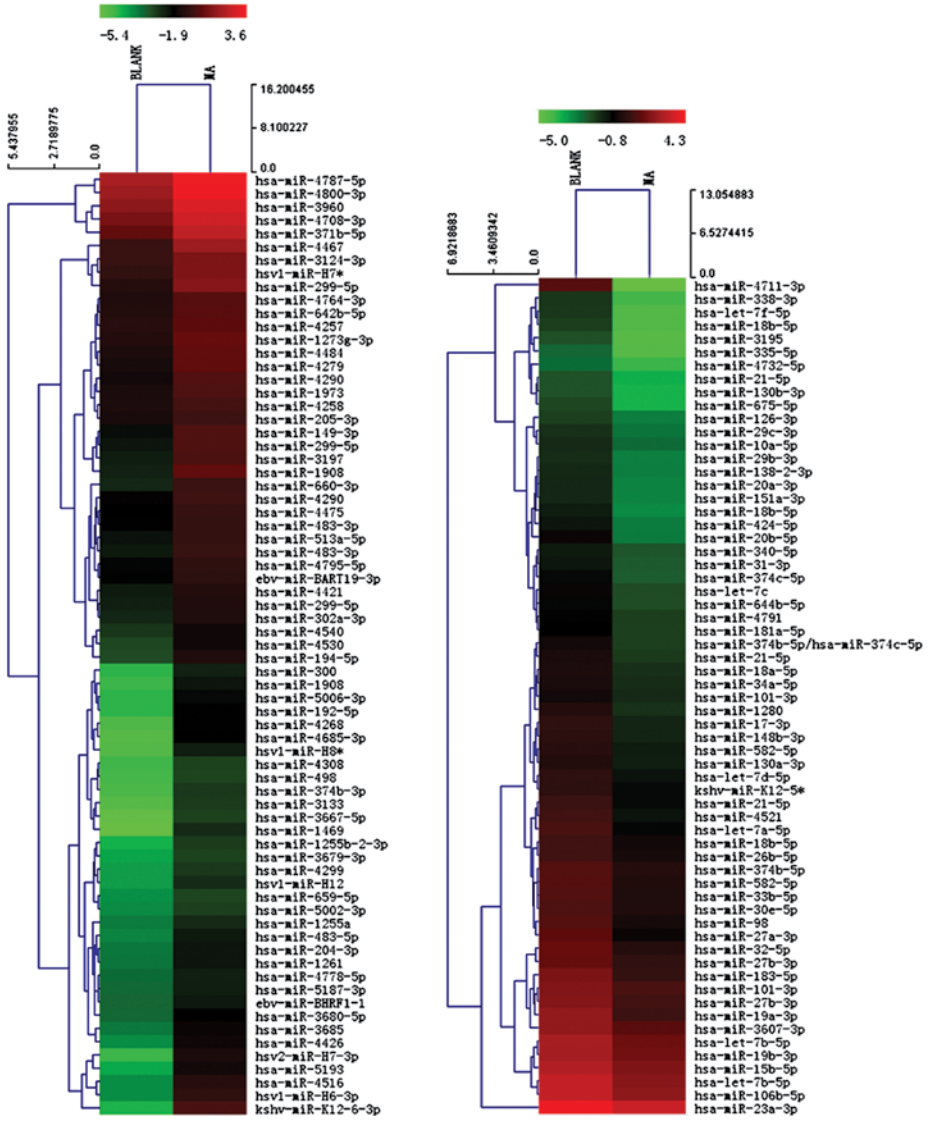

Figure 2. The miRNA expression profiles of SGC-7901 human gastric cancer cells after matrine treatment. A total of 128 human miRNAs including 60 upregulated and 68 downregulated exhibited ≥2-fold change in expression, relative to the control cells. Relative miRNA expression is represented on a scale from -5.4 to 3.6, and -5.0 to 4.3 of up- and downregulated miRNAs, respectively, for each treatment group [control (blank), 1.5 mg/ml matrine (MA)].

Table IV. Enrichment KEGG pathway analysis of downregulated miRNA target genes after matrine treatment.

ID	Pathway definition	Fisher-P-value	FDR	Enrichment score
hsa04010	MAPK signaling pathway, *Homo sapiens*	8.71694E-07	0.000218795	6.059636
hsa04510	Focal adhesion, *Homo sapiens*	6.70214E-06	0.000841118	5.173787
hsa04722	Neurotrophin signaling pathway, *Homo sapiens*	4.59225E-05	0.00366527	4.337974
hsa04360	Axon guidance, *Homo sapiens*	6.48307E-05	0.00366527	4.18822
hsa05200	Pathways in cancer, *Homo sapiens*	7.30133E-05	0.00366527	4.136598
hsa04512	ECM-receptor interaction, *Homo sapiens*	0.000645354	0.02684024	3.190202
hsa04310	Wnt signaling pathway, *Homo sapiens*	0.000748533	0.02684024	3.125789
hsa00520	Amino sugar and nucleotide sugar metabolism	0.001141903	0.03582719	2.942371
hsa00533	Glycosaminoglycan biosynthesis, keratan sulfate	0.001916047	0.05343643	2.717594
hsa04115	p53 signaling pathway, *Homo sapiens*	0.003151918	0.07764395	2.501425
hsa04930	Type II diabetes mellitus, *Homo sapiens*	0.003402723	0.07764395	2.468173
hsa04920	Adipocytokine signaling pathway, *Homo sapiens*	0.003740387	0.07823643	2.427083
hsa05211	Renal cell carcinoma, *Homo sapiens*	0.004416889	0.08527994	2.354883
hsa04141	Protein processing in endoplasmic reticulum	0.007499238	0.1175604	2.124983
hsa04330	Notch signaling pathway, *Homo sapiens*	0.007665283	0.1175604	2.115472
hsa04120	Ubiquitin mediated proteolysis, *Homo sapiens*	0.008175015	0.1175604	2.087511
hsa04070	Phosphatidylinositol signaling system	0.008771364	0.1175604	2.056933
hsa05014	Amyotrophic lateral sclerosis (ALS)	0.008897519	0.1175604	2.050731
hsa05202	Transcriptional misregulation in cancer	0.008898995	0.1175604	2.050659
hsa04974	Protein digestion and absorption	0.0100545	0.126184	1.99764
hsa04114	Oocyte meiosis, *Homo sapiens*	0.01317126	0.1534031	1.880373
hsa04728	Dopaminergic synapse, *Homo sapiens*	0.01344569	0.1534031	1.871417
hsa05164	Influenza A, *Homo sapiens*	0.01526479	0.1599239	1.816309
hsa03015	mRNA surveillance pathway, *Homo sapiens*	0.01529153	0.1599239	1.815549
hsa00770	Pantothenate and CoA biosynthesis, *Homo sapiens*	0.0196612	0.1973984	1.70639
hsa05219	Bladder cancer, *Homo sapiens*	0.02059202	0.1987922	1.686301
hsa05410	Hypertrophic cardiomyopathy (HCM)	0.02616797	0.2405538	1.58223
hsa04912	GnRH signaling pathway, *Homo sapiens*	0.02683468	0.2405538	1.571304
hsa04350	TGF-β signaling pathway, *Homo sapiens*	0.02926174	0.2532654	1.5337
hsa05216	Thyroid cancer, *Homo sapiens*	0.03390439	0.2836667	1.469744
hsa04020	Calcium signaling pathway, *Homo sapiens*	0.03627392	0.2937018	1.440405
hsa04664	Fc ε RI signaling pathway, *Homo sapiens*	0.04012037	0.3046442	1.396635
hsa04012	ErbB signaling pathway, *Homo sapiens*	0.04018206	0.3046442	1.395968
hsa04110	Cell cycle, *Homo sapiens*	0.04126655	0.3046442	1.384402
hsa04720	Long-term potentiation, *Homo sapiens*	0.04408158	0.3161279	1.355743
hsa00051	Fructose and mannose metabolism, *Homo sapiens*	0.04561514	0.3180389	1.340891

'Fisher-P-value' stands for the enrichment P-value of the Pathway ID using the Fisher's exact test. 'FDR' stands for the false discovery rate of the Pathway ID. 'Enrichment score' stands for the Enrichment score value of the Pathway ID equaling '-log10 (P-value)'.

sulfate, and the p53 signaling pathway, as well as pathways in cancer (Table IV).

The remaining 21 enrichment signaling pathways were associated with the target genes of upregulated miRNAs. According to the Enrichment Score, the top 10 signaling pathways were the Legionellosis, notch, neurotrophin, protein processing in endoplasmic reticulum, TGF-β, small cell lung cancer, epithelial cell signaling in *Helicobacter pylori* infection, cell cycle, RIG-I-like and pertussis signaling pathways (Table V).

The miRFocus 2.0 database was thoroughly examined for target genes of pathways for MAPK signaling and cell cycles. The results showed that 196 genes are associated with MAPK signaling pathways regulated by the 14 downregulated miRNAs, while 53 genes are associated with cell cycle pathways regulated by the 8 upregulated miRNAs. In this study, of the 159 genes 14 miRNAs were downregulated (Figs. 4 and 5).

Quantification of dysregulated miRNAs whose target genes are clustered in MAPK signaling pathway and cell cycle by RT-qPCR. After SGC-7901 cells were treated with 1.5 mg/ml matrine for 24 h, 14 miRNAs whose target genes were clustered in the MAPK signaling pathway and 8 miRNAs whose

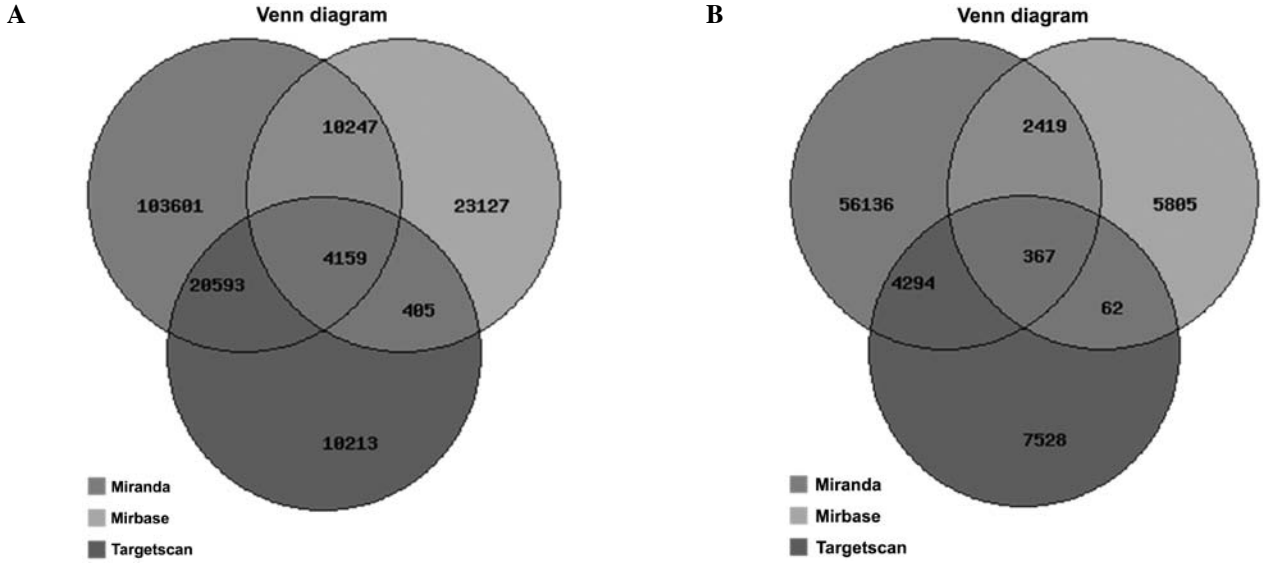

Figure 3. The Venn diagram shows the predicted miRNA target genes using the miRBase, miRanda and TargetScan bioinformatical databases. There are (A) 4,159 overlapped target genes as predicted by the three tools for all the downregulated miRNAs and (B) 367 overlapped target genes for the upregulated miRNAs that exhibited a >2-fold expression change after matrine treatment.

Table V. Enrichment KEGG pathway analysis of upregulated miRNA target genes after matrine treatment.

ID	Pathway definition	Fisher-P-value	FDR	Enrichment score
hsa05134	Legionellosis, *Homo sapiens*	0.000904176	0.2269482	3.043747
hsa04330	Notch signaling pathway, *Homo sapiens*	0.002738301	0.3436568	2.562519
hsa04722	Neurotrophin signaling pathway, *Homo sapiens*	0.004751378	0.3842182	2.32318
hsa04141	Protein processing in endoplasmic reticulum	0.007446489	0.3842182	2.128048
hsa04350	TGF-β signaling pathway, *Homo sapiens*	0.00777451	0.3842182	2.109327
hsa05222	Small cell lung cancer, *Homo sapiens*	0.009184498	0.3842182	2.036945
hsa05120	Epithelial cell signaling in *Helicobacter pylori* infection	0.01317173	0.4225359	1.880357
hsa04110	Cell cycle, *Homo sapiens*	0.01437375	0.4225359	1.84243
hsa04622	RIG-I-like receptor signaling pathway	0.01566376	0.4225359	1.805104
hsa05133	Pertussis, *Homo sapiens*	0.01845303	0.4225359	1.733932
hsa04620	Toll-like receptor signaling pathway	0.01905906	0.4225359	1.719898
hsa05166	HTLV-I infection, *Homo sapiens*	0.02046761	0.4225359	1.688933
hsa04010	MAPK signaling pathway, *Homo sapiens*	0.0231678	0.4225359	1.635115
hsa04120	Ubiquitin mediated proteolysis, *Homo sapiens*	0.02356774	0.4225359	1.627682
hsa05110	Vibrio cholerae infection, *Homo sapiens*	0.02532895	0.4238377	1.596383
hsa04512	ECM-receptor interaction, *Homo sapiens*	0.03141567	0.4711115	1.502854
hsa04621	NOD-like receptor signaling pathway, *Homo sapiens*	0.03190795	0.4711115	1.496101
hsa04310	Wnt signaling pathway, *Homo sapiens*	0.03732464	0.494149	1.428004
hsa05131	Shigellosis, *Homo sapiens*	0.03744232	0.494149	1.426637
hsa05200	Pathways in cancer, *Homo sapiens*	0.03937442	0.494149	1.404786
hsa04721	Synaptic vesicle cycle, *Homo sapiens*	0.04349567	0.5198769	1.361554

'Fisher-P-value' stands for the enrichment P-value of the Pathway ID using the Fisher's exact test. 'FDR' stands for the false discovery rate of the Pathway ID. 'Enrichment score' stands for the Enrichment score value of the Pathway ID equaling '-log10 (P-value)'.

target genes are clustered in pathways of the cell cycle were selected to verify their expression using RT-qPCR. The results showed the alterations of 8 upregulated miRNAs, including miR-192-5p, miR-299-5p, miR-194-5p, miR-300, miR-302a-3p, miR-483-5p, miR-498 and miR-513a-5p whose target genes were clustered in the cell cycle pathway following matrine treatment (1.5 mg/ml), were consistent with alterations identified by miRNA microarray. However, for the 38 down-regulated

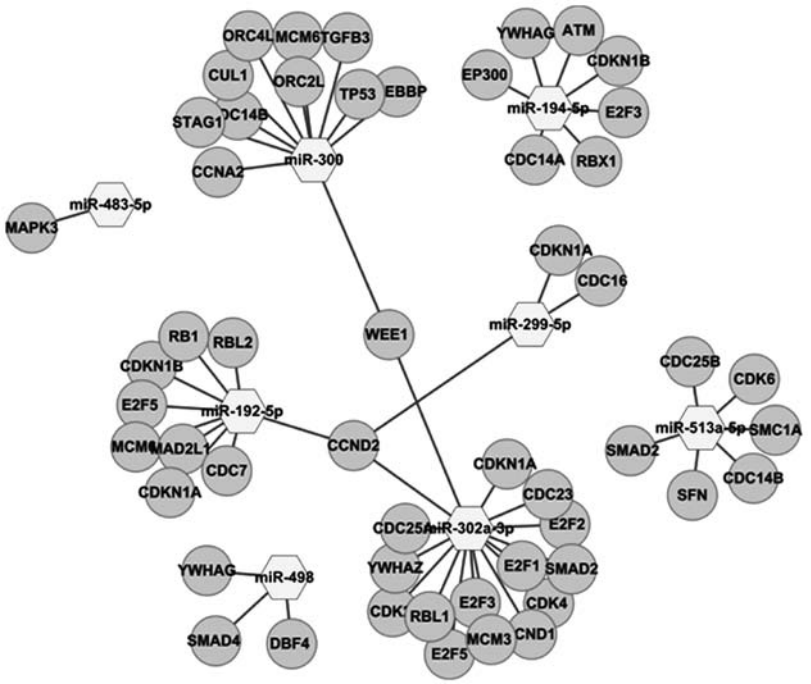

Figure 4. Predicted and validated cell cycle pathway (hsa04110) targets of 8 upregulated miRNAs that exhibited a >2-fold expression change in response to matrine, following a search in the miRfocus 2.0 and miRWalk databases. This graph was generated by Cytoscape 2.8. The hexagon node represents miRNAs, and the elliptical node represents target genes.

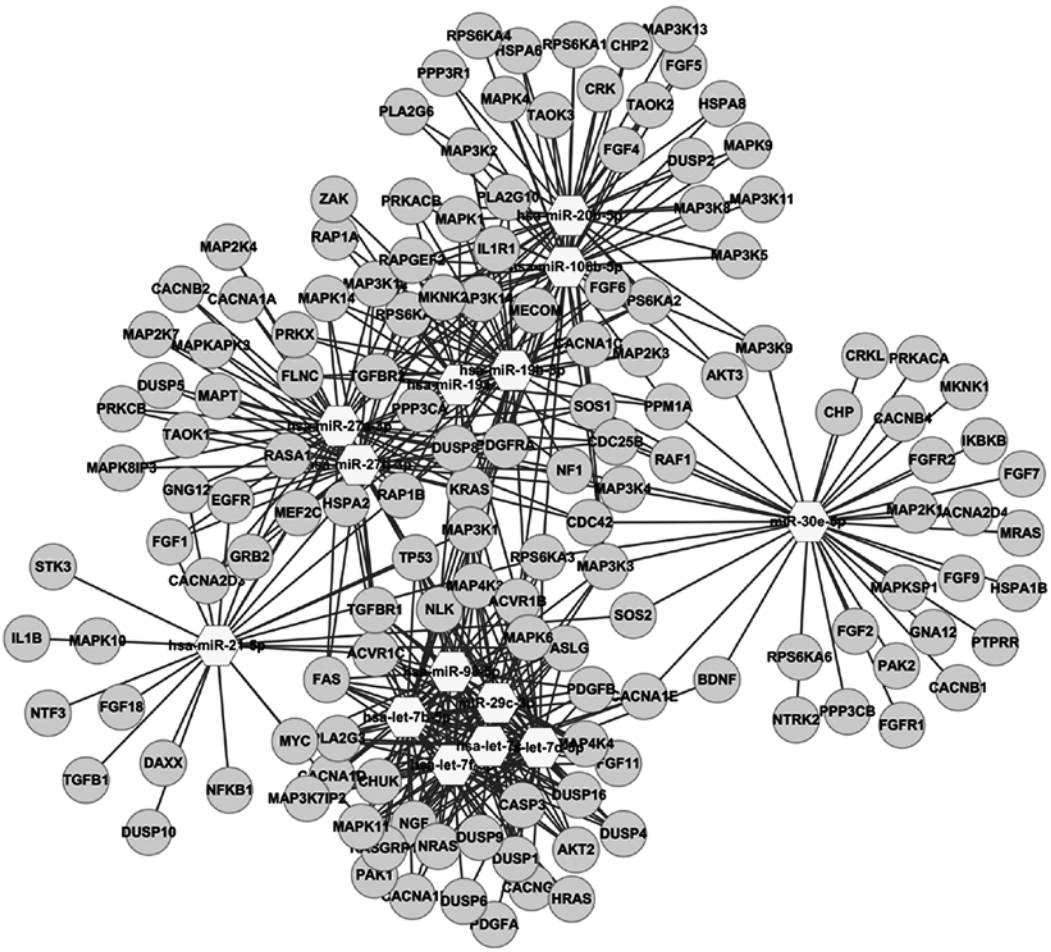

Figure 5. Predicted and validated MAPK signaling (hsa04010) pathway targets of 14 downregulated miRNAs that exhibited a >2-fold expression change in response to matrine, following a search in the miRfocus 2.0 and miRWalk databases. This graph was generated by Cytoscape 2.8. The hexagon node represents miRNAs, and the elliptical node represents target genes.

miRNAs, the alterations of 14 miRNAs following matrine treatment (1.5 mg/ml), including let-7b-5p, let-7c, let-7d-5p, let-7f-5p, miR-19a-3p, miR-19b-3p, miR-20b-5p, miR-21-5p, miR-27a-3p, miR-27b-3p, miR-29c-3p, miR-30e-5p, miR-98-5p and miR-106b-5p whose target genes were clustered in the MAPK signaling pathway, were consistent with alterations identified by miRNA microarray (Tables I and II).

Discussion

Based on the miRNA microarray analysis, 128 miRNAs with differential expression were identified after 24 h of treatment with 1.5 mg/ml matrine. Of these, 68 were upregulated and 60 were downregulated following matrine treatment. The functions of 8 of the upregulated miRNAs and 38 of the downregulated miRNAs have been annotated with their target genes in the miRbase miRNA database. Of these 46 differentially expressed miRNAs with annotations, results of RT-qPCR showed that 8 miRNAs whose target genes were clustered in the cell cycle pathway were upregulated after matrine treatment, while 14 miRNAs whose target genes were clustered in the MAPK signaling pathway were downregulated after matrine treatment.

The majority of the miRNAs that were affected by matrine treatment were associated with the pathogenesis and development of human gastric cancers. Of the 38 miRNAs that were downregulated after matrine treatment, 20 were upregulated in human gastric cancers and gastric cancer cell lines and act as oncogenes. These include let-7b-5p, miR-10a-5p, miR-15b-5p, miR-18a-5p, miR-18b-5p, miR-19a-3p, miR-19b-3p, miR-20b-5p, miR-21-5p, miR-23a-3p, miR-26b-5p, miR-27a-3p, miR-27b-3p, miR-32-5p, miR-34a-5p, miR-98, miR-106b-5p, miR-181a-5p, miR-183-5p and miR-338-3p. Notably, matrine upregulated 5 miRNAs that have been reported to be elevated in human gastric cancers and gastric cancer cell lines. These include let-7a-5p, miR-192-5p, miR-194-5p, miR-335-5p and miR-424-5p. In addition, matrine downregulated 6 miRNAs that have been reported to be reduced in human gastric cancers and gastric cancer cell lines. These include miR-29b-3p, miR-29c-3p, miR-30e-5p, miR-130a-3p, 130b-3p and 148b-3p (Table III) (7-34). Bioinformatical analysis of differentially expressed miRNAs revealed that the target genes were associated with cancer and cell cycle pathways, as well as the MAPK, p53, TGF-β, mTOR, Wnt and notch signaling pathways, which are involved in tumorigenesis. The 8 upregulated miRNAs and 14 downregulated miRNAs, in particular, are involved in the cell cycle and MAPK signaling pathways and were found to be regulated by matrine. Thus, gastric, as well as other cancer cells may be mediated by miRNAs.

Since matrine promotes the apoptosis of Raji cells by activated p38MAPK (46), it suppressed the activity of ERK and increased the activities of p38 and JNK when inhibiting the proliferation of the U937 cell line, inducing apoptosis *in vitro* (47). Furthermore, matrine inhibited the migration of HUVECs induced by A549 by suppressing MAPK/ERK signaling transduction (47). In addition, matrine has been reported to reduce the percentage of cells in the G2/M phase, and increase the percentage of cells arrested in the G0/G1 phase of the cell cycle, resulting in blocking of the cell cycle at the G0/G1 stage when treating cancer cells, such as human SGC-7901 gastric cancer cell line (1), HT29 colon cancer cell line (14), human rhabdomyosarcoma cells (48), PC-3 prostate cancer cell line (11), HepG2 human hepatoma cell line (12), human A549 adenocarcinoma lung cancer cell line (49) and the human K562 leukemia cell line (50). Therefore, the aberrantly expressed miRNAs whose target genes were clustered in the MAPK signaling pathway and pathways selected for analysis of their predicted and validated target genes by miRFocus 2.0 and miRWalk provide further explanations of the underlying mechanism of action and mechanisms involved in the SGC-7901 gastric cancer cell line following matrine treatment.

Putative target miRNAs, along with target genes and enriched KEGG pathways identified through analysis and validation of the differentially expressed miRNAs in human SGC-7901 gastric cancer cells that had been treated with matrine has facilitated the possibility of finding the target of natural, chemopreventive agents. This may provide insight into the antitumor mechanism of action of matrine, particularly in the case of gastric cancer. In recent years, there have been several reports of miRNA regulation via natural, non-toxic, chemopreventive agents, including curcumin, resveratrol, isoflavones, (-)-epigallocatechin-3-gallate (EGCG), lycopene, 3,3'-diindolylmethane (DIM) and indole-3-carbinol (I3C) (51,52). Thus, natural agents, including matrine, may inhibit cancer progression, increase drug sensitivity, reverse epithelial-mesenchymal transition (EMT), and prevent metastasis though the modulation of miRNAs, providing promising new therapies for the treatment of cancer.

In conclusion, matrine affects miRNA expression in the SGC-7901 gastric cancer cell line as evidenced by miRNA microarray and analysis of differentially expressed miRNA target genes and enrichment pathways. These results, combined with RT-qPCR validation and bioinformatical analyses, provide a novel and promising approach to identify targets for matrine and/or other natural substances, when treating cancer.

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