RESEARCH ARTICLE

Clinical Significance of Upregulation of mir-196a-5p in Gastric Cancer and Enriched KEGG Pathway Analysis of Target Genes

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Abstract

Background: miRNAs are relatively recently discovered cancer biomarkers which have important implications for cancer early diagnosis, treatment and estimation of prognosis. Here we focussed on expression of mir-196a-5p in gastric cancer tissues and cell lines so as to analyse its significance for clinicopathologic characteristics and generate enriched KEGG pathways clustered by target genes for exploring its potential roles as a biomarker in gastric cancer. Materials and Methods: The expression of mir-196a-5p in poorly, moderate and well differentiated gastric cancer cell lines compared with GES-1 was detected by RT-qPCR, and the expression of mir-196a-5p in gastric cancer tissues comparing with adjacent non cancer tissues of 58 cases were also assessed by RT-qPCR. Subsequently, an analysis of clinical significance of mir-196a-5p in gastric cancer and enriched KEGG pathways was executed based on the miRWalk prediction database combined with bioinformatics tools DAVID 6.7 and Mirfocus 3.0. Results: RT-qPCR showed that mir-196a-5p was up-regulated in 6 poorly and moderate differentiated gastric cancer cell lines SGC-7901, MKN-45, MKN-28, MGC-803, BGC-823, HGC-27 compared with GES-1, but down-regulated in the highly differentiated gastric cancer cell line AGS. Clinical data indicated mir-196a-5p to beup-regulated in gastric cancer tissues (47/58). Overexpression of mir-196a-5p was associated with more extensive degree of lymph node metastasis and clinical stage (P < 0.05; x2 test). Enriched KEGG pathway analyses of predicted and validated targets in miRWalk combined with DAVID 6.7 and Mirfocus 3.0 showed that the targeted genes regulated by mir-196a-5p were involved in malignancy associated biology. Conclusions: Overexpression of mir-196a-5p is associated with lymph node metastasis and clinical stage, and enriched KEGG pathway analyses showed that targeted genes regulated by mir-196a-5p may contribute to tumorgenesis, suggesting roles as an oncogenic miRNA biomarker in gastric cancer.

Keywords: Gastric cancer - mir-196a-5p - oncogenic gene - KEGG pathway

Introduction

Gastric cancer (GC) is the fifth most common cancer but the third leading cause of cancer death (Fock, 2014), and the common characteristics of GC is high incidence rates, poorly early diagnosis rates and high mortality rates. Epidemiology data indicated that the current trend of GC is severe worldwide, particular in Asia (Katanoda et al., 2013; Liu et al., 2013; Koessler et al., 2014). The major reason of which is associated with lack of specificity of symptoms for early diagnosis and treatment. For example, GC is diagnosed at an early stage in less than 10% of cases in Europe (Saika and Sobue, 2013). The funding and identification of new molecular biomarkers made it easier for GC diagnosis, treatment and clinical outcome prognosis, and microRNAs (miRNAs) were one of new discovered cancer molecular biomarkers relevant to cancer including GC. MiRNAs are 20-to-25 mer noncoding RNAs which incompletely bind to the 3' untranslated regions (UTR) of multiple target mRNAs, enhancing their degradation and inhibiting their translation. MiRNAs participate in multiple carcinogenesis processes, such as cell differentiation, cell cycle, metastasis, progression and apoptosis, and even microRNA polymorphism is closed correlated with cancer (Hao et al., 2013; Du et al., 2014a). Aberrantly expressed miRNAs act as tumor suppressor

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genes or oncogenic genes in cancer tumorigenesis as well as in gastric cancer(Shenouda and Alahari, 2009; Bartels and Tsongalis, 2010; Cortes-Sempere and Ibanez de Caceres, 2011). Mir-196a is a newly reported miRNA biomarkers relevant to multiple cancers, such as breast cancer (Lee et al., 2014), lung cancer (Liu et al., 2012), cervical cancer (Gocze et al., 2013), renal cancer (Du et al., 2014a), head and neck cancer (Christensen et al., 2010), hepatocellular cancer (Hao et al., 2013), pancreatic cancer (Slater et al., 2014), colorectal cancer (Zhan et al., 2011; Du et al., 2014b) and early gastric cancer (Zheng et al., 2014).

In previous study, mir-196a-5p was discovered over expressed in gastric cancer cell lines and gastric cancer tissues (Sun et al., 2012; Tsai et al., 2014). But the clinical significance of mir-196a-5p in cancers of previous reports was different, probably, which may be caused by different representatives of different samples. So expanded study will clarify its clinical significance and understand its roles in gastric cancer, and will make it useful for GC cancer molecular biomarkers discovery. In present study, we decided to validate the expression of mir-196a-5p in seven gastric cancer cell lines comparing with GES-1 cell line, and detect the expression of mir-196a-5p in gastric cancer tissues comparing with adjacent non-cancerous tissues of 58 cases comes from a high gastric cancer incidental rate area. In addition, bioinformatics analysis of its target genes and enriched KEGG pathways will be execute to understand its roles in cancer tumorigenesis, particularly in the case of gastric cancer.

Materials and Methods

Cell lines

Seven gastric cancer cell lines, AGS, SGC-7901, MKN-45, MKN-28, MGC-803, BGC-823, HGC-27 and GES-1 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and Cancer Institute and Hospital, Chinese Academy of Medical Sciences (CAMS) (Beijing, China). All the gastric cancer cell lines were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum in a humidified cell incubator having an atmosphere of 5% $CO2$ at 37°C. Exponentially growing cells were used for experiments. MiRNA PCR primers were purchased from GeneCopoeia™ Inc.

Clinical samples

58 gastric cancer samples were obtained during surgery and used after obtaining informed consent. All patients underwent curative resection of the primary tumor at WuWei city tumor Hospital from the year of 2010 to 2012 (WuWei, China, a high incidental rate area with gastric cancer (Li et al., 2004; ZHANG et al., 2009). All patients had a clear histological diagnosis of gastric cancer, based on the clinicopathologic criteria. All data, including age, sex, histological grade, depth, lymph node metastasis, Local invasion, Depth of tumor invasion, lymph node metastasis, lymphatic invasion, Venous invasion, borrmann type and clinical stage were obtained from clinical and pathologic records. No patients

received neoadjuvant chemotherapy or radiotherapy before surgery and adjuvant radiotherapy after surgery. Resected cancerous tissues (T) and paired noncancerous tissues (N) were immediately cut and stored in, frozen in liquid nitrogen, and kept at- 80°C until RNA extraction.

Written informed consent was obtained from each patient for his or her participation in the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the ethics committee of First Hospital of Lanzhou University.

Total RNA isolation and quality analysis

Total RNA of gastric cancer cell lines and frozen tissues of gastric cancer were extracted using RNeasy mini kit (QIAGEN, Hilgen, Germany) according to the manufacturer's instructions. Concentrations and purity of the RNA samples were assayed by electrophoresis and spectrophotometric methods.

MiRNA quantification by qRT-PCR

MiRNA quantification by real-time qRT-PCR. SYBR green qRT-PCR assay was used for miRNA quantification. In brief, 40 ng of total RNA containing miRNA was polyadenylated by poly(A) polymerase and was reversely transcribed to cDNA using miScript Reverse Transcription kit according to the manufacturer's instructions (GeneCopoeia™, Rockville, USA). miScript SYBR Green PCR kit was used and miscript Universal primer was provided by the manufacturer (GeneCopoeia™, Rockville, USA), qRT-PCR was performed in BIORAD CFX96 Real-time PCR system. Each reaction was performed in a final volume of 10 μl containing 2 μl of cDNA, 0.5 mM of each primer and 1X SYBR Green PCR Master mix (GeneCopoeia™, Rockville, USA). The amplification program was: denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 30 sec and 72°C for 30 sec, in which fluorescence was acquired. At the end of the PCR cycles, melting curve analyses were performed as well as electrophoresis of the products on 2.5% agarose gels in order to validate the specific generation of the expected PCR product. Each sample was run in triplicates for analysis. The expression levels of miRNAs were normalized to RNU6B. Relative gene expression was calculated as $2^{-(CTmiRNA-CTRNU6B\ RNA)}$.

MiRNA Targeted Gene Prediction and KEGG Pathway Analyses by miRWalk and DAVID 6.7

We utilized a miRNA target gene prediction database miRWalk (Dweep et al., 2011) to select predicted and validated targets, and to analysis enriched KEGG pathways by bioinformatics tool DAVID 6.7. The miRWalk prediction database integrated 10 bioinformatics Target Prediction Tools: DIANA-mT, miRanda, miRDB, miRWalk, RNAhybrid, PICTAR4, PICTAR5, PITA, RNA22 and TargetScan. Enriched KEGG pathway analyses of mir-196a-5p targeted genes were performed by bioinformatics tool DAVID 6.7. Prediction Databases Support Number were at least 5, P value of Fisher Test were P<0.05.

MiRNA Targeted Gene Prediction and KEGG Pathway Analyses by mirfocus 3.0

We utilized a miRNA target gene prediction database mirfocus 3.0 (http://mirfocus.org/index.php) to select validated targets of mir-196a-5p to analysis its enriched KEGG pathways and to annotate the molecular function of the miRNA targeted genes. The mirfocus 3.0 integrated 5 bioinformatical Target Prediction Tools: MiRanda, MirTarget2, PicTar, microT and TargetScanS, and the experimental validated Target Tools include miRecords, miR2Disease, TarBase and miRTarBase. Enriched KEGG pathway of mir-196a-5p targeted genes also were performed by mirfocus 3.0. Prediction Databases Support Number was 3, P value of Fisher Test were P<0.05.

Statistical analysis

Student's unpaired t-test was used to compare values of samples of 7 gastric cancer cell lines and samples of GES-1 gastric cell line. Differences between groups were estimated using the X^2 test. A probability level of 0.05 was chosen for statistical significance, and all statistical analyses were performed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

Results

Expression of mir-196a-5p in gastric cancer cell lines

The expression of mir-196a-5p in gastric cancer cell lines comparing with GES-1 was detected by qRT-PCR method. Results showed that mir-196a-5p were up-regulated in 6 gastric cancer cell lines gastric cancer cell lines SGC-7901, MKN-45, MKN-28, MGC-803, BGC-823, HGC-27 compared with GES-1 cell line, but down-regulated in gastric cancer cell line AGS (Figure 1).

Expression of mir-196a-5p in gastric cancer tissues comparing with adjacent non tumor tissues

After detection the expression of mir-196a-5p in gastric cancer cell lines, the levels of mir-196a-5p in 58 cancerous and corresponding non-cancerous tissues were also detected by qRT-PCR method. Results showed that the numbers of mir-196a-5p in high-expression group (T/N>2) and low-expression group (T/N<0.5) amounts to 47 and 11 respectively, according to the median cancer (T)/noncancerous (N) tissue ratio of mir-196a-5p expression (Figure 2, Table 1).

Table 1. mir-196a-5p Level and Clinocopathologic Factors in Patients with Gastric Cancer

\Characteristic	mir-196a-5p High expression (n =47) Number(%)	mir-196a-5p Low expression (n =11) Number(%)	significance x^2	P value
Age			0.113	0.737
<59 years	29(61.7.0%)	8(72.7%)		
≥59 years	18(38.3%)	3(27.3%)		
Gender			0.416	0.519
Males	29(61.7.0%)	5(45.4.0%)		
Females	18(38.3%)	6(54.6%)		
Degree of tumour cell differentiation			0.000	1.000
Moderate-to-well differentiated	20(42.6%)	4(36.4%)		
Poorly differentiated	30(57.4%)	7(63.6%)		
TNM stage			4.958	0.026a
I+II	9(20.0%)	6(76.0%)		
III+IV	38(80.0%)	5(24.0%)		
Tumor size			54.2	0.257
≤5cm	21(20.0%)	7(28.0%)		
>5cm	26(80.0%)	4(72.0%)		
Lymph node metastasis			4.273	0.039a
Positive	35(74.4%)	4(36.4%)		
Negative	12(25.6%)	7(63.6%)		
Lymphatic invasion			0.024	0.878
Positive	42(89.4%)	10(90.9%)		
Negative	5(10.6%)	1(9.1%)		
Venous invasion			0.004	0.951
Positive	4(8.5%)	1(9.1%)		
Negative	43(91.5%)	10(90.9%)		
Depth of tumor invasiona			1.010	0.315
mucosa, submucosa, muscularis propria, subserosa	22(46.8%)	7(63.6%)		
penetration of serosa, adjacent strucures	25(53.2%)	4(36.4%)		
Borrmann type			0.009	0.925
I+II	29(61.7.0%)	6(54.5%)		
III+IV	18(38.3%)	5(45.5%)		

The expression of mir-196a-5p in all cases showed different levels between gastric cancer tissues and adjacent non-cancerous tissues. a P<0.05, lymph node metastasis; TNM stage

Table 2. Enriched KEGG Pathway Clustered by Predicted Targets of mir-196a-5p and Corresponding Target Genes. 'Fisher-P-value' Stands for the Enrichment *p*-value of the Pathway ID using the Fisher's Exact Test. Enrichment Score' Stands for the Enrichment score value of the Pathway ID Qqualing '-log10 (P value)'

KEGG PATHWAY	Fold Enrichment	Fisher Exact	Predicted target genes
Prostate cancer	3.4	6.40E-06	BCL2,CREB3L1,CDKN1A,CDKN1B,FGFR1,FOXO1, HSP90AA1,IKBKB,IGF1,MAPK1,NRAS, NFKB1,NFKBIA,PDGFA,PDGFRA,TCF7,AKT1
Chronic myeloid leukemia	3.1	2.20E-04	CBL,SMAD4,CDK6,CDKN1A,CDKN1B,IKBKB,MA PK1,NRAS,NFKB1,NFKBIA,PTPN11,TGFBR2,AKT1
Acute myeloid leukemia	3.1	1.20E-03	CEBPA,FLT3,IKBKB,MAPK1,NRAS,NFKB1,PML,ST AT3,TCF7,AKT1
Glioma	2.8	2.40E-03	CAMK2G,CALM1,CALM3,CDK6,CDKN1A,IGF1,M APK1,NRAS,PDGFA,PDGFRA,AKT1
Small cell lung cancer	2.5	2.30E-03	BCL2,XIAP,CDK6,CDKN1B,CDKN2B,IKBKB,ITGA V,LAMA4,NFKB1,NFKBIA,RARB,AKT1
Melanogenesis	2.5	1.10E-03	ADCY1,ADCY6,ADCY9,CREB3L1,CAMK2G,CALM1, CALM3,EDNRB,MAPK1,NRAS,PLCB2,PRKX,TCF7, TYRP1,WNT2B
Pancreatic cancer	2.5	6.40E-03	CBL,SMAD4,CDK6,CDKN1A,CDKN1B,IKBKB, MAPK1,NRAS,NFKB1,NFKBIA,PTPN11,TGFBR2, AKT1,VEGFA
TGF-beta signaling pathway	2.5	3.10E-03	SMAD4,SMAD6,ACVR2A,ACVR2B,BMP4,BMPR2, CHRD,CDKN2B,INHBC,MAPK1,ROCK1,TGFBR2
mTOR signaling pathway	2.4	2.50E-02	RICTOR,IGF1,MAPK1,RPS6KA3,TSC1,AKT1,VEGFA
Chemokine signaling pathway	2.3	1.00E-04	RAP1A,WASL,ADCY1,ADCY6,ADCY9,XCL1,CCL17, CCL22,CCL23,CCR10,CXCL10,CXCL12,GNB3,IK, KB,MAPK1,NRAS,NFKB1,NFKBIA,PLCB2,PRKX, STAT2,STAT3
Melanoma	2.3	1.70E-02	CDK6,CDKN1A,FGFR1,IGF1,MAPK1,NRAS,PDGFA, PDGFRA,AKT1
Pathways in cancer	2.1	4.10E-06	BCL2,BAX,CEBPA,CBL,FAS,GLI3,SMAD4,XIAP, BMP4,CDK6,CDKN1A,CDKN1B,CDKN2B, FGFR1,FLT3,FOXO1,HSP90AA1,RALBP1,IKBKB, IGF1,ITGAV,LAMA4,MAPK1,NRAS, NFKB1,NFKBIA,PDGFA,PDGFRA,PML,RET,RARB, STAT3,SHH,TCF7,TGFBR2,AKT1,ETS1,VEGFA, WNT2B
p53 signaling pathway	2.1	3.50E-02	BAX,FAS,CCND2,CDK6,CDKN1A,IGF1,SERPINE1, ZMAT3
Renal cell carcinoma	2	4.10E-02	RAP1A,MAPK1,NRAS,PAK2,PTPN11,AKT1, ETS1,VEGFA

Table 3. Enriched KEGG Pathway Clustered by Validated Targets of mir-196a-5p and Corresponding Target Genes. 'Fisher-P-value' Stands for the Enrichment *p*-value of the Pathway ID using the Fisher's Exact Test. Enrichment Score' Stands for the Enrichment Score value of the Pathway ID Equaling '-log10 (P value) '.

| KEGG PATHWAY	Fold Enrichment	Fisher Exact	Validated target genes
Acute myeloid leukemia	8.9	2.10E-04	CEBPA,FLT3,IKBKB,NFKB1,AKT1
Apoptosis	6	1.40E-03	BAX DFFA IKBKB NFKB1 AKT1
Renal cell carcinoma	5.9	4.40E-03	AKT1, ETS1, VEGFA, RAP1A
Prostate cancer	5.8	1.50E-03	AKT1, IGF1R, CDKN1A, NFKB1, IKBKB
Pancreatic cancer	5.8	4.80E-03	AKT1, VEGFA, NFKB1, IKBKB
Chronic myeloid leukemia	5.5	5.60E-03	AKT1, CDKN1A, NFKB1, IKBKB
Toll-like receptor signaling pathway	5.1	2.70E-03	AKT1, IFNA1, NFKB1, TLR4, IKBKB
Small cell lung cancer	4.9	8.30E-03	AKT1, ITGAV, NFKB1, IKBKB
Cell cycle	4.2	6.70E-03	CDKN1A, CCND2, ESPL1, MCM2, SMC3
Pathways in cancer	4.1	8.30E-06	BMP4, CEBPA, FLT3, NFKB1, SHH, AKT1, IGF1R, CDKN1A, ETS1, ITGAV, BAX, VEGFA, IKBKB

Clinical significances of mir-196a-5p in gastric cancer tissues comparing with adjacent non tumor tissues

All clinicopathologic factors were analyzed in relation to mir-196a-5p level, The mir-196a-5p high-expression group showed more lymph node metastasis and clinical stage than the high-expression group ($p<0.05$; X^2 test). However, no significant differences were observed among age, gender, extensive degree of tumor cell differentiation, tumor size, depth of tumor invasion, venous invasion, borrmann type, and lymphatic invasion (Table 1).

Table 4. Enriched KEGG Pathway Clustered by Predicted and Validated Targets of mir-196a-5p and Corresponding Target Genes. Genes enrichment were Performed using the Fisher's exact test. Enrichment score' Stands for the Enrichment Score value of the Pathway ID equaling '-log10 (P value)

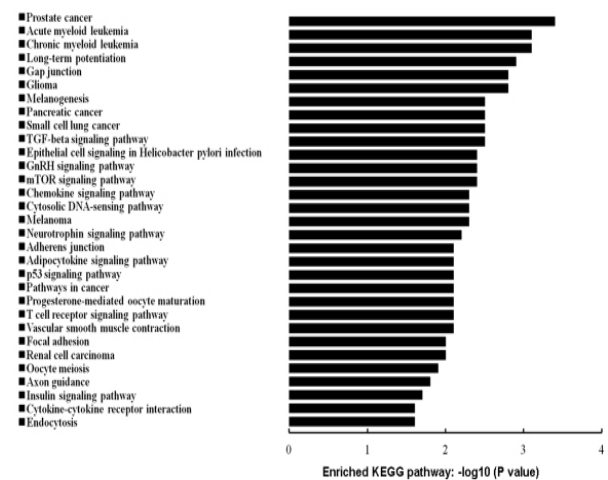| KEGG PATHWAY	Fold Enrichment	Predicted target genes	Validated target genes
MicroRNAs in cancer	3.44	HMGA2, NRAS,PDGFRA	CASP3,CCND1,CCND2,CCNE2, CDKN1B,HMOX1,IGF2BP,IKBKB, NOTCH2, PDCD4
Transcriptional misregulation in cancer	2.76	ERG, HMGA2, PBX1, PBX3	CCND2,CCNT2,CDKN1B,EWSR1, FOXO1,HOXA9,IGF1R,
Prostate cancer	2.66	NRAS, PDGFRA	CCND1,CCNE2,CDKN1B,FOXO1, IGF1R,IKBKB
Glioma	2.11	CALM1,CALM3,PDGFRA	CCND1,IGF1R,NRAS
PI3K-Akt signaling pathway	1.68	COL1A1, COL1A2, COL24A1, OSMR,COL3A1, NRAS, PDGFRA	CCND1,CCND2,CCNE2,CDKN1B, IGF1R,IKBKB,ITGAV, NR4A1
Cell cycle	1.36		BUB1,CCND1,CCND2,CCNE2, CDKN1B,ESPL1,SMC3
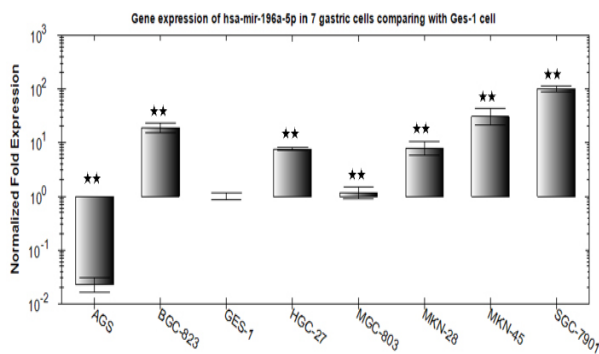
Figure 1. RT-qPCR Experiment Validation Showed that mir-196a-5p were up-regulated in 6 Gastric Cancer Cell Lines SGC-7901, MKN-45, MKN-28, MGC-803, BGC-823, HGC-27 Compared with GES-1 Cell line, but Down-regulated in Gastric Cancer Cell Line AGS. Relative gene expression was calculated as 2^−ΔΔCT method, and each sample was analyzed in triplicate. there were statistically significant difference between 7 gastric cancer cell lines and GES-1 cell line (*p*<0.05, indicated as ★★
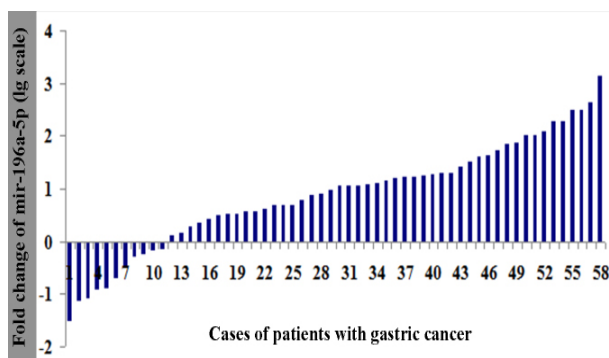
Figure 2. Expression of mir-196a-5p in Gastric Cancer Tissue Comparing with Adjacent Non Tumor Tissues of 58 Gastric Cancer Samples

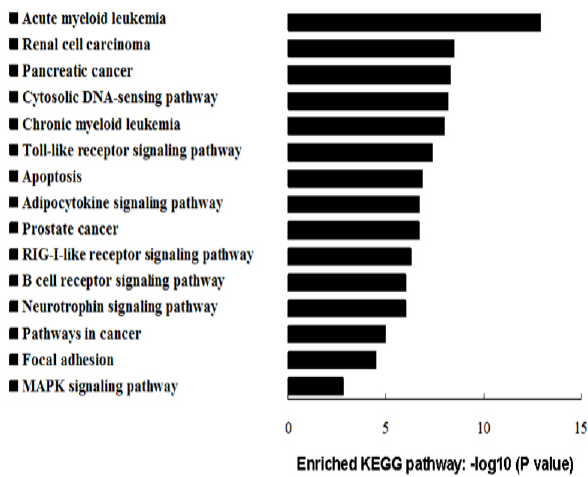*KEGG pathway analyses of mir-196a-5p targeted genes by miRWalk and DAVID 6.7*

In order to investigate the possible regulation mechanisms of mir-196a-5p in the process of gastric cancer, we utilized an online bioinformatics database miRWalk to select plausible targets and validated targets of this miRNA and to analysis Enriched KEGG pathways by DAVID 6.7.
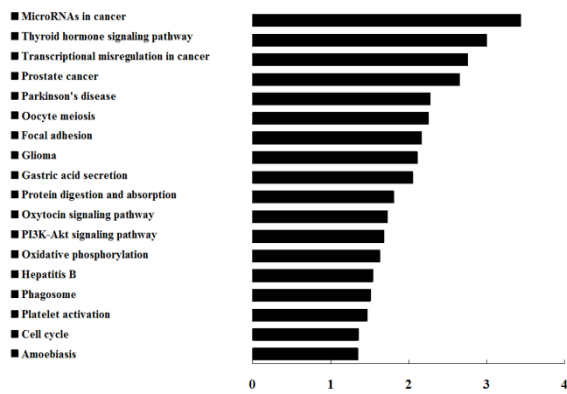

Figure 3. The 31 Enriched KEGG Pathways from Predicted Target Genes of mir-196a-5p which were Searched in MiRWalk Database and Analysed by DAVID 6.7

Based on predicted targets of miRWalk, a total of 1081 target genes were searched as the target genes of mir-196a-5p. Enriched KEGG pathway analyses showed that the targeted genes which were regulated by mir-196a-5p were involved 31 pathways, and 13 of which were centralized in cancer associated terms, which were as follows: prostate cancer, chronic myeloid leukemia, acute myeloid leukemia, glioma, small cell lung cancer, melanogenesis, pancreatic cancer, TGF-beta signaling pathway, mTOR signaling pathway, chemokine signaling pathway, Melanoma, Pathways in cancer and p53 signaling pathway (Table 2, Figure 3).

Based on validated targets of miRWalk, a total of 209 target genes were searched as the target genes of mir-196a-5p. Enriched KEGG pathway analyses showed that the targeted genes which were regulated by mir-196a-5p were involved 14 pathways, and 10 of which were centralized in cancer associated terms, which were as follows: acute myeloid leukemia, apoptosis, renal cell carcinoma, prostate cancer, pancreatic cancer, chronic myeloid leukemia, toll-like receptor signaling pathway, small cell lung cancer, cell cycle and pathways in cancer (Table 3, Figure 4).

Figure 4. The 14 Enriched KEGG Pathways from Validated Target Genes of mir-196a-5p which were Searched in miRWalk Database and analysed by DAVID 6.7

Figure 5. The 18 Enriched KEGG Pathways from Predicted and Validated Target Genes of mir-196a-5p which were Searched and Analysed in miRfocus 3.0 Database

KEGG pathway analyses of mir-196a-5p targeted genes by Mirfocus 3.0

In order to investigate the possible regulation mechanisms of mir-196a-5p in the process of gastric cancer, we utilized a bioinformatical database mirfocus 3.0 to select plausible targets of this miRNA. A total of 348 target genes including predicted and validated genes were choosen as the target genes of mir-196a-5p.

Results of enrichment KEGG pathway indicated that the targeted genes which were regulated by mir-196a-5p were involved 18 pathways, and 6 of which were centralized in cancer associated terms, which were as follows: MicroRNAs in cancer, transcriptional misregulation in cancer, prostate cancer, glioma, cell cycle and PI3K-Akt signaling pathway ((Table 4, Figure 5).

Discussion

Aberrantly expressed miRNAs are unique cancer biomarkers which will bring benefit to early diagnosis, treatment guidelines and prognosis estimation of cancer as well as mRNA and protein biomarkers. In recent years, more and more miRNAs were found up-regulated or down-regulated in gastric cancer, so that they were potential biomarkers which will supplement classic cancer biomarkers in clinical applications, such as miR-21(Guo et al., 2013; Ma et al., 2013). But an ideal biomarker not only must be associated with cancer clinical significance, but also functional experiments of over expression or silence should prove it having great impact on cancer behavior. Mir-196a-5p was newly discovered promising gastric cancer biomarker, more studies should be carried out to fully understand its multiple roles and functions and to clarify any previous inconsistent clinical significance. This study employed 58 gastric cancer cases comes from WuWei city tumor Hospital from the year of 2010 to 2012 which belong to WuWei, Gan su, China, an area is famous for its high incidental rate with gastric cancer. So the clinical data is typical for clinical significance study, and is qualified for biomarker evaluation.

In this study, RT-qPCR experiment showed that mir-196a-5p were up-regulated in 6 gastric cancer cell lines SGC-7901, MKN-45, MKN-28, MGC-803, BGC-823, HGC-27 compared with GES-1 cell line, but down-regulated in gastric cancer cell line AGS. Among these seven gastric cancer cell lines, MGC-803 and HGC-27 were undifferentiated carcinoma cells, SGC-7901, MKN-45, MKN-28 and BGC-823 were poorly and moderate differentiated carcinoma cells, only AGS was highly differentiated carcinoma cell. So mir-196a-5p may be responsible for the differentiation of gastric cancer cell.

Clinical data analysis indicated that the numbers of mir-196a-5p in high-expression group (T/N>2) and low-expression group (T/N<0.5) amounts to 47 and 11 respectively. The mir-196a-5p high-expression group showed more extensive degree of lymph node metastasis and clinical stage than the high-expression group (P < 0.05; x2 test). However, no significant differences were observed among age, gender, depth of tumor invasion, venous invasion, tumor cell differentiation, tumor size, Borrmann type, and lymphatic invasion.

Previous study discovered that overexpression of miR-196a was significantly associated with tumor progression and poorer 5-year survival outcomes, and overexpression of miR-196a enhanced GC cell migration and invasion (Tsai et al., 2014). In another study (Sun et al., 2012), higher expression of miR-196a in gastric cancer tissues was reported associated with tumor size, higher clinical stage, and shorter overall survival, and downregulation of miR-196a expression significantly suppressed the cell-cycle progression, proliferation, and colony formation of gastric cancer cells in vitro, and ectopic miR-196a expression significantly enhanced the development of tumors in nude mice. Result of our study is partly consistent with these studies.

Bioinformatics analysis of plausible targets and validated targets of miRNA and following analysis of KEGG pathway clustered by target genes is a promising way to give insights on potential biomarkers and key events involved in cancer tumorigenesis and relevant pathways. In recent years, this kind of combination analysis of any identified miRNA, its corresponding target

genes, enriched KEGG pathways, and even miRNA-mRNA network were performed in cancer studies (Satoh, 2012; Liu et al., 2014), particularly in cancer biomarker discoveries (Romero-Cordoba et al., 2012; Beta et al., 2013). In this study, based on miRWalk database combined with DAVID 6.7 tool and mirfocus 3.0, bioinformatics prediction and Enriched KEGG pathways analysis of miR-196a-5p indicated that both predicted target genes and validated target genes of miR-196a were clustered in cancer associated KEGG pathways.

In conclusion, our study fully reinforced the expressions of mir-196a-5p in gastric cancer lines and gastric cancer tissues were up regulated. These results, combined with bioinformatics analysis of Enriched KEGG pathways clustered by target genes provide ways to identify mir-196a-5p as new biomarkers in gastric cancer, and new light should be shed on its clinical significances of gastric cancer diagnosis and prognosis.

Acknowledgements

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