

An integrated approach of predicted miR-34a targets identifies a signature for gastric cancer

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Abstract. microRNA-34a (miRNA/miR-34a) functions as a tumor suppressor gene in gastric cancer and may be involved in system-wide regulatory networks. To clarify the expression of all predicted target genes of this miRNA, a comprehensive and systematic analysis of miR-34a-target genes in gastric cancer was conducted in the present study. In the initial analysis, the potential functions, pathways and networks of gastric cancer-associated molecules and miR-34a targets were identified. In the final integrative analysis of gastric cancer-associated miR-34a targets, 30 hub genes were identified using overlap calculations, indicating that miR-34a may be significant in the development and progression of gastric cancer through the Smad signaling pathway, the cell cycle, the mitogen-activated protein kinase signaling pathway, apoptosis, the Notch signaling pathway and other pathways. The present study provides a bioinformatic analysis of miR-34a-targets in gastric cancer, describes numerous target genes and novel coregulatory networks, and may provide an opportunity to identify a critical regulatory network for predicting the molecular mechanisms of miR-34a in the development and progression of gastric cancer.

Introduction

Gastric cancer undergoes genetic and epigenetic alterations during its progression, and is the fourth most common malignancy and the second leading cause of cancer mortality worldwide (1,2). Surgical intervention remains as the preferred treatment for gastric cancer; however, even with intervention, the 5-year survival rate is only ~40% (3). Cisplatin-based chemotherapy is currently one

of the most frequently used therapies. However, numerous patients do not respond to this chemotherapy and must tolerate the associated toxic and adverse effects. Therefore, it is clinically important to distinguish the mechanisms that underlie chemoresistance and the malignant phenotypes of gastric cancer (3). The identification of novel and reliable diagnostic biomarkers and therapeutic strategies is also of the utmost importance (2). Previous studies that sought to identify convincing candidate genes that characterize the heterogeneity of gastric cancer, although far from complete or conclusive, may provide the foundation for systematic analyses of their genetic contributions to this type of tumor, and their regulatory pathways and networks may offer insight into the molecular basis of the pathological or clinical characteristics (1-3).

microRNAs (miRNA/miR) are a class of naturally occurring, small regulatory RNAs that function as negative gene regulators and modulate numerous biological processes, including cellular differentiation, proliferation, apoptosis and metabolism, by targeting varying genes (4). miRNAs have become a major focus in the field of cancer research (5) and the theory that miRNA profiles may reflect the developmental lineage and differentiated state of tumors has been extensively studied in a number of different types of cancer, including gastric cancer (6-8). Notably, miR-34a, which possesses anti-oncogenic activity in certain types of cancer, is downregulated in gastric cancer and cisplatin-resistant cell lines (9,10). A previous study has demonstrated that miR-34a is involved in the sensitivity of gastric cancer to chemotherapies (9). However, the exact molecular mechanism of miR-34a downregulation and its role in gastric cancer development and progression has not been established. Furthermore, it is predicted that a series of factors are involved in the cancer-associated molecular signatures of miR-34a (11). Thus, a comprehensive and systematic analysis of miR-34a-target genes in gastric cancer is of great significance and may provide an opportunity to identify a critical regulatory network for diagnosing and predicting prognosis in gastric cancer.

The present study aimed to systematically analyze the expression of miR-34a predicted target genes associated with tumorigenesis, chemoresistance to cisplatin-based chemotherapy and prognosis in gastric cancer.

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Materials and methods

Natural language processing (NLP) analysis of gastric cancer. Data selection, extraction and filtering was conducted as previously described (12). The search was performed using the PubMed database (Medline; www.ncbi.nlm.nih.gov/pubmed), attempting to cover all papers published between January 1980 and March 2012, with a combination of the following keywords: 'gastric cancer' AND 'cisplatin' OR 'resistance' OR 'carcinogenesis' OR 'tumorigenesis' OR 'prognosis'; and '1980/01/01' [program delivery assessment tool (PDAT)]: '2012/03/20' (PDAT) (12). All the associated genes and proteins reported in each of the studies were compiled into a list, followed by gene mention tagging using a biomedical named entity recognizer (ABNER; http://pages.cs.wisc.edu/~bsettles/abner/). In addition, the gene symbol in the Entrez gene database of NCBI was considered to be the most common and was therefore used for the study (13). The flow of the NLP analysis was as follows: i) Document searching and formatting; ii) gene mention tagging using ABNER; iii) conjunction resolution; iv) gene name normalization based on the Entrez database; and v) statistical analysis.

Statistical analysis, gene ontology (GO) analysis, pathway analysis and network analysis were also performed as previously described (12).

Statistical analysis. The frequency of the occurrence of each gene was calculated. The higher the frequency of the gene, the greater the likelihood of the association between gastric cancer and that specific gene. The following formulae were used:

$$p = 1 - \sum_{i=0}^{k-1} p(i | n, m, N)$$

$$p(i | n, m, N) = \frac{n!(N-n)!m!(N-m)!}{(n-i)!i!(n-m)!(N-n-m+i)!N!}$$

N represents the total number of studies in the literature from the PubMed database; m and n represent the frequency of genes and gastric cancer, respectively, in the literature from the PubMed database; and k represents the assumption of the actual concomitant occurrence of a gene and a disease. All statistical analyses were performed using GraphPad Prism version 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.01$ was considered to indicate a statistically significant difference.

Gene ontology. The analysis was conducted using the GSEA-Base package from the R Project for Statistical Computing platform (www.r-project.org/), and the genes were classified according to biological processes, cellular components and molecular functions.

Pathway analysis. Genes were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database using GenMAPP software version 2.1 (www.genmapp.org/), and the enrichment P-value was calculated for each pathway (14).

Network analysis. A total of 3 different interaction associations were integrated as previously described (12). Briefly, the pathway data were downloaded from the KEGG database and

were then used to analyze the genomic interaction between genes with the KEGGSOAP package from The R Project for Statistical Computing platform (www.bioconductor.org/packages/2.4/bioc/html/KEGGSOAP.html), including 3 types of associations: Enzyme-enzyme interactions, protein-protein interactions and gene expression interactions (15). The protein-protein interaction data were downloaded from the The MIPS Mammalian Protein-Protein Interaction Database (mips.helmholtz-muenchen.de/proj/ppi/) (16). For interactions that had been previously reported, the co-citation algorithm in the PubMed abstracts was used: The study analyzed whether a gene term and all its term variants co-occurred within the sentences, calculated the frequency of the co-citation gene, and performed a statistical analysis using the same method as described in the NLP analysis. The resulting network was displayed by using the Medusa software (17).

Prediction of miR-34a target genes. The analysis of the miR-34a predicted targets was subsequently determined using a combination of 3 independent software packages as described previously (12,18): i) PicTar2005 (pictar.mdc-berlin.de/cgi-bin/PicTar_vertebrate.cgi); ii) miRandaV5 (www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/); and iii) TargetScan 5.1 (www.targetscan.org/); GO, pathway and network analyses of miR-34a targets were performed as described in the NLP analysis.

Integrative analysis of miR-34a target genes and NLP results. The overlap of the miR-34a target genes and gastric cancer-associated genes and gene network analysis was subsequently performed.

Results

NLP analysis of gastric cancer. The initial computerized search identified 22,885 primary studies and a total of 1,183 gastric cancer-associated genes, using the aforementioned search strategies. The 20 most frequently cited genes are listed in Table I. The 1,183 genes were categorized in GO according to biological process, cellular component and molecular function (Fig. 1). Pathway analysis was then performed and indicated that there were 148 pathways available. Among these pathways, the representation in 33 signaling pathways was statistically significant ($P < 0.01$; Table II). It has previously been hypothesized that gene networks reflect the physiological situation as a whole, in addition to the stability of gene regulatory networks and the highly connected hub genes, which are crucial to the stability of the network. Thus, the gene network analysis of the 1,183 identified genes was conducted and is shown in Fig. 2, which presents the relationships between the genes as a whole. A connectivity analysis was also performed. As demonstrated in Fig. 3, the *PIK3CA* gene has the most interaction gene counts.

Analysis of miR-34a predicted targets. Considering that miRNAs exert biological effects via their numerous targets, the predicted target genes of miR-34a were analyzed using 3 commonly used computational algorithms: TargetScan4.0, PicTar and miRanda. A total of 460 potential unique gene symbols targeted by miR-34a were obtained, and all these genes were categorized by GO analysis (Fig. 4). The gene ontology analysis results for the biological process category revealed

Table I. List of the 20 most frequently cited genes in studies reporting on gastric cancer.

Gene	Count	P-value	Description
<i>TP53</i>	189	1.00×10^{-14}	Tumor protein p53
<i>ERBB2</i>	133	1.00×10^{-14}	v-erb-b2 erythroblastic leukemia viral oncogene
<i>VEGFA</i>	112	1.00×10^{-13}	Vascular endothelial growth factor A
<i>BCL2</i>	106	1.00×10^{-13}	B-cell CLL/lymphoma 2
<i>PTGS2</i>	96	1.00×10^{-12}	Prostaglandin-endoperoxide synthase 2 (COX-2)
<i>EGFR</i>	94	1.00×10^{-12}	Epidermal growth factor receptor
<i>JAG1</i>	57	7.54×10^{-9}	Jagged 1 (Alagille syndrome)
<i>CCND1</i>	54	1.02×10^{-8}	Cyclin D1
<i>TCEAL1</i>	53	1.00×10^{-11}	Transcription elongation factor A (SII)-like 1
<i>MMP9</i>	47	3.44×10^{-9}	Matrix metalloproteinase 9
<i>IL10</i>	45	1.00×10^{-11}	Interleukin 10
<i>MAPK8</i>	43	1.00×10^{-11}	Mitogen-activated protein kinase 8
<i>DPYD</i>	40	1.00×10^{-11}	Dihydropyrimidine dehydrogenase
<i>IL6</i>	40	1.00×10^{-11}	Interleukin 6 (interferon, β 2)
<i>CDKN2A</i>	39	1.00×10^{-11}	Cyclin-dependent kinase inhibitor 2A (p16)
<i>TNF</i>	39	1.00×10^{-11}	Tumor necrosis factor (TNF superfamily, member 2)
<i>CD44</i>	38	1.00×10^{-10}	CD44 molecule (Indian blood group)
<i>MLH1</i>	35	1.00×10^{-10}	MutL homolog 1, colon cancer, nonpolyposis type 2
<i>MAPK3</i>	35	1.00×10^{-10}	Mitogen-activated protein kinase 3
<i>STAT3</i>	28	1.00×10^{-9}	Signal transducer and activator of transcription 3

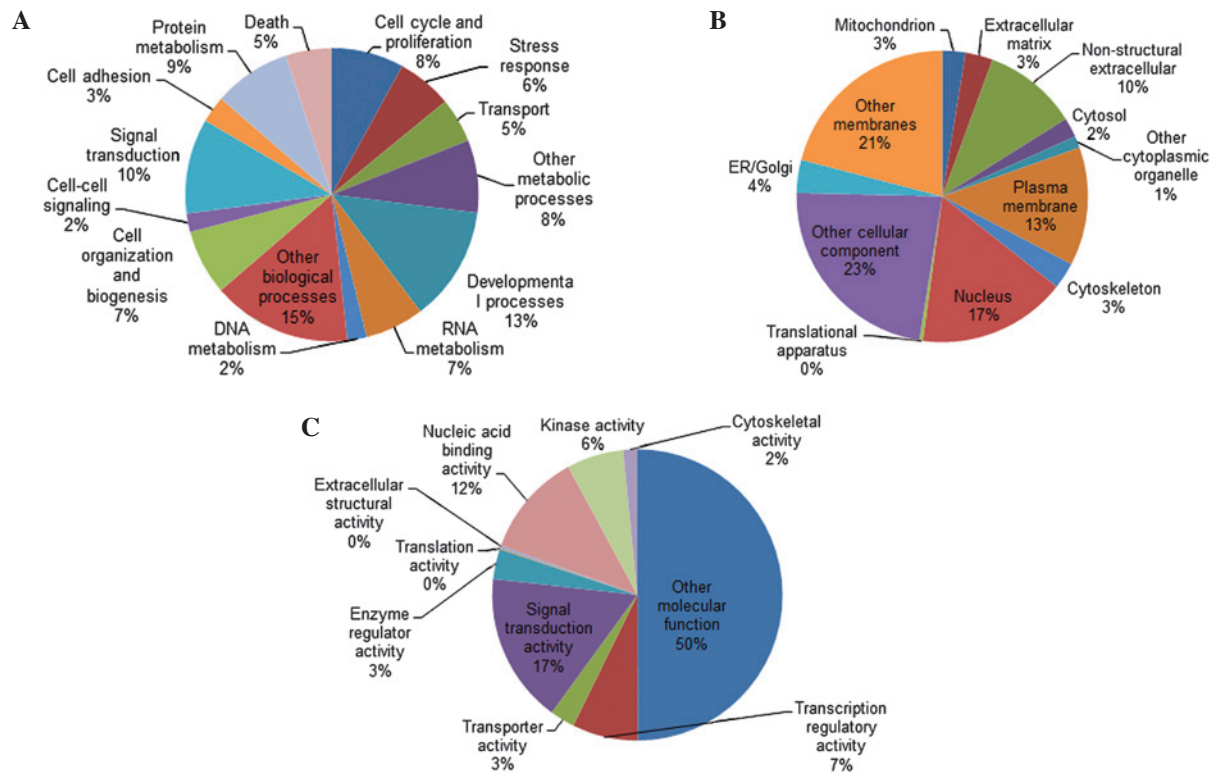


Figure 1. Gene ontology analysis of 1,183 gastric cancer-related genes. (A) Biological process, (B) cellular component and (C) molecular function.

that miR-34a-target genes were predominantly associated with nucleotide metabolic processes, multicellular organism development and cellular component organization. In the pathway analysis, 98 pathways were obtained in the miR-34a

targets-pathway. Specific pathways that were identified by the analysis included the PI3K-Akt signaling pathway, the p53 signaling pathway, the notch signaling pathway, adherens junctions, the cell cycle, galactose metabolism and the

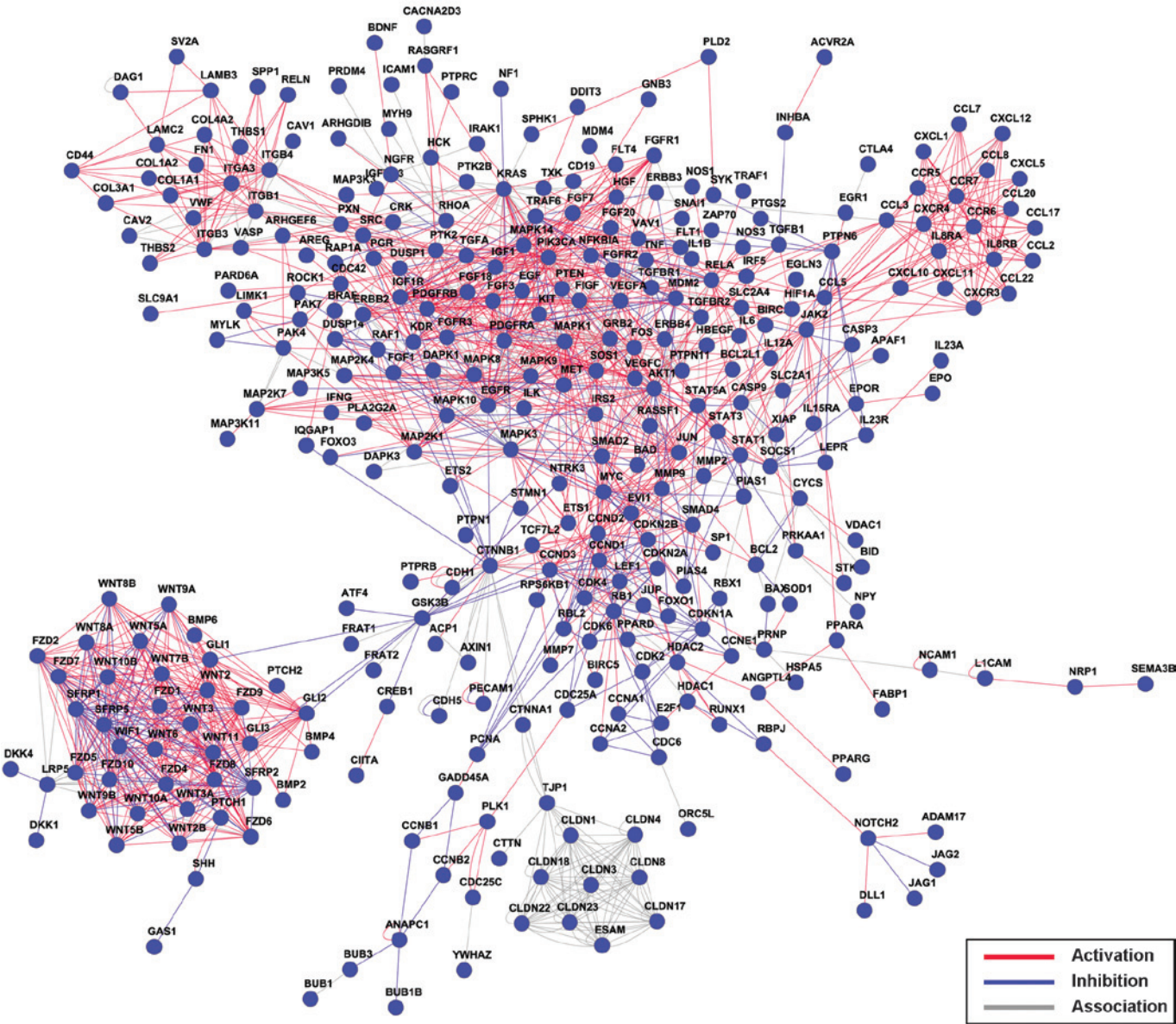


Figure 2. Network analysis of gastric cancer-related genes. Red represents activation, green represents inhibition and gray represents association.

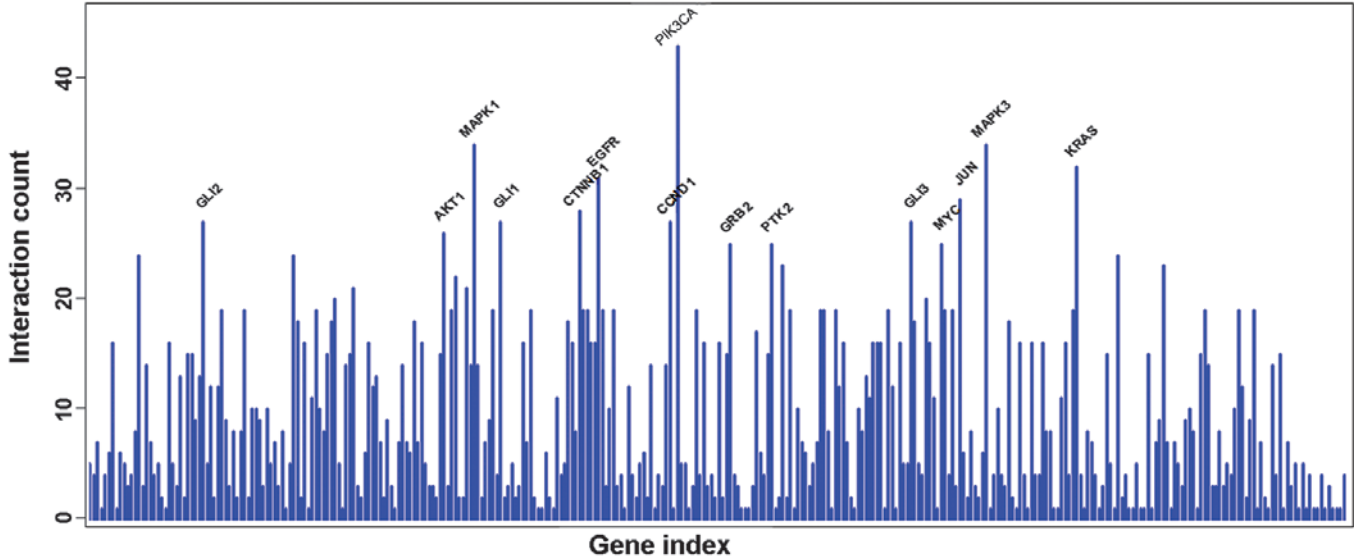


Figure 3. Interaction gene counts for the gastric cancer-related genes.

Table II. Signaling pathways represented by gastric cancer-associated genes ($P < 0.01$).

Title	Count	P-value
p53 signaling pathway	41	2.41×10^{-12}
Wnt signaling pathway	56	3.25×10^{-12}
Focal adhesion	72	2.55×10^{-11}
Cytokine-cytokine receptor interaction	82	3.17×10^{-11}
ErbB signaling pathway	35	3.02×10^{-10}
Hedgehog signaling pathway	27	4.02×10^{-10}
Cell cycle	40	2.04×10^{-12}
Melanogenesis	37	2.92×10^{-9}
Neurotrophin signaling pathway	42	6.21×10^{-9}
T-cell receptor signaling pathway	37	1.75×10^{-8}
Toll-like receptor signaling pathway	34	1.10×10^{-7}
Adherens junction	27	5.00×10^{-7}
Cell adhesion molecules	39	7.98×10^{-7}
Chemokine signaling pathway	50	1.24×10^{-6}
Leukocyte transendothelial migration	34	5.12×10^{-6}
MAPK signaling pathway	62	6.68×10^{-6}
Apoptosis	27	1.63×10^{-5}
Hematopoietic cell lineage	26	3.90×10^{-5}
Jak-STAT signaling pathway	38	1.01×10^{-4}
Dorso-ventral axis formation	11	1.07×10^{-4}
Natural killer cell mediated cytotoxicity	34	1.82×10^{-4}
B-cell receptor signaling pathway	22	2.06×10^{-4}
TGF-beta signaling pathway	24	2.50×10^{-4}
FcεRI signaling pathway	22	3.81×10^{-4}
Regulation of actin cytoskeleton	46	4.84×10^{-4}
VEGF signaling pathway	21	5.73×10^{-4}
Base excision repair	12	9.71×10^{-4}
ECM-receptor interaction	22	1.15×10^{-3}
Adipocytokine signaling pathway	18	2.32×10^{-3}
mTOR signaling pathway	15	2.48×10^{-3}
GnRH signaling pathway	24	2.95×10^{-3}
Antigen processing and presentation	21	4.98×10^{-3}
Axon guidance	28	5.54×10^{-3}

MAPK, mitogen-activated protein kinase; STAT, signal transducers and activators of transcription; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; ECM, extracellular matrix; mTOR, mammalian target of rapamycin; GnRH, gonadotropin-releasing hormone.

HIF-1 signaling pathway. These pathways have already been demonstrated to be involved in the development, progression and chemosensitivity of gastric cancer. Additionally, in the network analysis of the miR-34a predicted targets (Fig. 5), the connectivity of the *CCND1* gene was the highest among the 110 hub genes that were obtained.

Integrative analysis of miR-34a target genes and the NLP results. The overlap between the 460 miR-34a target genes and the 1,183 prognosis-associated genes in gastric cancer obtained from the NLP analysis was calculated. A total of

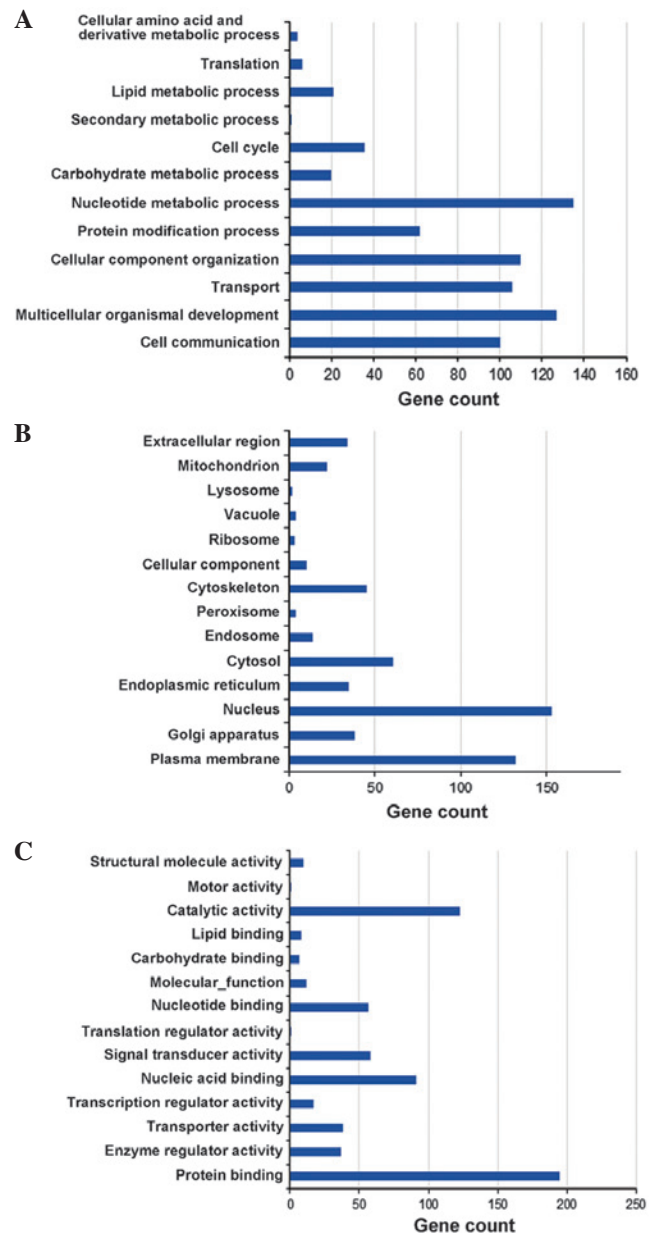


Figure 4. miR-34a-targets genes were categorized in gene ontology analysis. (A) Biological process, (B) cellular component and (C) molecular function.

30 overlap genes that were associated with the development and progression of gastric cancer and that were also potential miR-34a target genes were obtained using this integrative analysis (Table III). A network analysis was also conducted to map the overlapped genes (Fig. 6). From the current results, it appears reasonable to conclude that the *SMAD4*, *CCND1*, *MAP2K1*, *BCL2* and *NOTCH2* hub genes are potential miR-34a target genes and are also essential in the molecular mechanism of gastric cancer. The *SMAD4*, *CCND1*, *MAP2K1*, *BCL2* and *NOTCH2* genes represent the Smad signaling pathway, the cell cycle, MAPK signaling pathway, apoptosis pathway and the Notch signaling pathway, respectively.

Discussion

The present study performed a systematic review of a pooled collection of English language studies of gastric cancer-associated

Table III. Integrative analysis of miR-34a target genes and the NLP results.

Targets	Count	P-value	Gene description
<i>BCL2</i>	106	1.00x10 ⁻¹²	B-cell CLL/lymphoma 2
<i>JAG1</i>	57	7.54x10 ⁻⁹	Jagged 1
<i>CCND1</i>	54	1.02x10 ⁻⁸	Cyclin D1
<i>MET</i>	18	1.00x10 ⁻¹²	Met proto-oncogene (hepatocyte growth factor receptor)
<i>CYCS</i>	14	1.00x10 ⁻¹²	Cytochrome c, somatic
<i>SERPINE1</i>	12	1.00x10 ⁻¹¹	Serpin peptidase inhibitor, clade E, member 1
<i>SMAD4</i>	8	4.95x10 ⁻⁸	SMAD family member 4
<i>MAP2K1</i>	6	1.19x10 ⁻⁶	Mitogen-activated protein kinase kinase 1
<i>AREG</i>	4	5.40x10 ⁻⁶	Amphiregulin
<i>SATB1</i>	4	5.49x10 ⁻⁷	SATB homeobox 1
<i>PDCD4</i>	3	5.11x10 ⁻⁵	Programmed cell death 4
<i>CDK6</i>	3	6.78x10 ⁻⁴	Cyclin-dependent kinase 6
<i>GAS1</i>	2	4.45x10 ⁻⁴	Growth arrest-specific 1
<i>IGFBP3</i>	2	1.27x10 ⁻¹	Insulin-like growth factor binding protein 3
<i>IRF1</i>	2	2.54x10 ⁻²	Interferon regulatory factor 1
<i>PTPRD</i>	2	7.21x10 ⁻⁴	Protein tyrosine phosphatase, receptor type, D
<i>NR4A2</i>	1	1.32x10 ⁻¹	Nuclear receptor subfamily 4, group A, member 2
<i>NOTCH2</i>	1	1.14x10 ⁻¹	Notch homolog 2
<i>PDGFRA</i>	1	2.60x10 ⁻¹	Platelet-derived growth factor receptor, α polypeptide
<i>CDC25A</i>	1	1.53x10 ⁻¹	Cell division cycle 25 homolog A
<i>MTA2</i>	1	5.66x10 ⁻²	Metastasis associated 1 family, member 2
<i>SOX4</i>	1	5.66x10 ⁻²	SRY (sex determining region Y)-box 4
<i>MYH9</i>	1	1.84x10 ⁻¹	Myosin, heavy chain 9, non-muscle
<i>DLL1</i>	1	8.18x10 ⁻²	δ -like 1
<i>LEF1</i>	1	1.46x10 ⁻¹	Lymphoid enhancer-binding factor 1
<i>PRKD1</i>	1	1.50x10 ⁻¹	Protein kinase D1
<i>JMJD1C</i>	1	1.86x10 ⁻²	Jumonji domain-containing 1C
<i>CR2</i>	1	1.37x10 ⁻¹	Complement component receptor 2
<i>KITLG</i>	1	1.88x10 ⁻¹	KIT ligand
<i>MDM4</i>	1	1.16x10 ⁻¹	Mdm4 p53-binding protein homolog

In the NLP analysis, 1,183 genes that were associated with the carcinogenesis, progression and chemoresistance of gastric cancer were identified. The potentially functional classification of the genes was obtained from the GO analysis. The pathway analysis identified 148 pathways and 33 of these were statistically significant, including the p53 signaling pathway, the Wnt signaling pathway, the cell cycle, the MAPK signaling pathway, apoptosis, and the TGF- β signaling pathway. A number of previous studies have identified the same pathways to be involved in tumorigenesis, metastasis and chemotherapy resistance (2,11,24,25). In addition, the network and connectivity of those 1,183 genes was constructed in the present study. The highly connected hub genes are crucial to the stability of the network. PIK3CA, with the highest connectivity, had a total of 43 gene connections. A previous study demonstrated that PIK3CA is mutated frequently in a range of human tumors and that its activation is associated with a number of chemotherapeutic agents (26). The results of the present study are consistent with a previous study that analyzed lung cancer-associated genes with NLP and concluded that the gene with the highest connectivity was PIK3CA (12).

In order to obtain the miR-34a target genes in gastric cancer, 3 computational algorithms (miRanda, PicTar, and TargetScan) were used to analyze the predicted targets. From this analysis, 460 unique gene symbols targeted by miR-34a were obtained. These genes were categorized using GO, followed by pathway and network analysis in parallel with the NLP analysis. The results demonstrated that the putative target genes of miR-34a include the tumor-associated genes *CCND1*, *SMAD4*, *PRKD1*, *BCL2*, *NOTCH2* and *SATB1*, among others. A total of 98 pathways were obtained in the miR-34a targets pathway analysis, and the PI3K-Akt signaling pathway was identified as the most significant pathway. The 3 genes with the highest connectivity among all 110 hub gene obtained in the miR-34a targets-network analysis were *CCND1*, *SMAD4* and *BCL2*. The *CCND1* gene encodes Cyclin D1, a key protein required for G₁/S cell cycle transition. Mutations, amplification and overexpression of Cyclin D1 are frequently observed in a number of different types of cancer and may contribute to tumorigenesis (27). *SMAD4*, which is mutated in a variety of tumors and functions as a tumor suppressor, belongs to the Darwin family of proteins

that modulate members of the TGF- β protein superfamily (28). BCL2 is considered to be an important anti-apoptotic protein and is a member of the BCL2 family of regulator proteins.

In the subsequent integrative analysis of NLP and miR-34a targets, 30 hub genes were obtained. The results indicated that miR-34a is essential in carcinogenesis, progression and the response to chemotherapy in gastric cancer through the Smad signaling pathway, the cell cycle, the MAPK signaling pathway, the apoptosis pathway, the Notch signaling pathway and other pathways. The overlapped targeting hub genes and their pathways may become novel targets for controlling gastric cancer or reversing chemoresistance. Notably, the *PIK3CA* gene and its pathway, which had the highest connectivity in the NLP analysis, were not involved in the final integrative analysis. This is in agreement with the evidence that *PIK3CA* was the most significant hub gene in the NLP analysis of lung cancer, but it was not involved in the overlapped analysis with miR-21 (12). The possible explanation for these discrepancies may be that the computational target gene prediction methods have certain limitations in determining actual multifactorial associations.

Collectively, the present study systematically analyzed gastric cancer-associated genes and the putative targets of miR-34a by using a computational and bioinformatics approach. Although additional experiments are required to confirm these results, the systematic integration of miR-34a-targets and their potential modulators provides an efficient approach to discover novel target genes and co-regulatory networks in gastric cancer. Identification of these molecular pathways and networks controlled by miR-34a may provide unique insights into the pathogenesis of gastric cancer.

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