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Screening and functional analysis of micro-RNAs that regulate the expression of the tumor suppression TP53 gene

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Abstract. – OBJECTIVE: With gradual increase of cancer incidence and mortality rates, the regulatory mechanism of cancer has become a hotspot. It has been known that the expression of TP53 is closely associated with the occurrence of cancer. The microRNA (miRNA)-mediated regulation of the expression of numerous proto-oncogenes has been reported. This study aimed to identify miRNAs that regulate the expression of TP53 gene.

MATERIALS AND METHODS: The sequence of TP53 gene was downloaded from NCBI and analyzed with TargetScan software to predict potential miRNAs that regulate TP53 expression. miR-122 was selected as the most potential miRNA for regulating TP53. miR-122 mimics and inhibitor were synthesized and transfected into Hela cells. The expression of TP53 mRNA was measured by qRT-PCR. The cell proliferation, migration, and invasion ability were assessed by CCK-8, scratch wounding, and transwell invasion assay, respectively.

RESULTS: Cells transfected with miR-122 mimics exhibited significantly lower TP53 expression ($p < 0.05$), but significantly increased cell proliferation and migration compared with blank control group ($p < 0.05$). Notably, cells in miR-122 mimics and control groups had similar invasion ability ($p > 0.05$). However, cells in miR-122 inhibitor group exhibited significantly higher TP53 expression ($p < 0.05$), but significantly reduced cell proliferation and invasion ability. The migration ability of cells in miR-122 inhibitor group was similar to cells in control group ($p > 0.05$).

CONCLUSIONS: The selected miR-122 effectively inhibited the expression of TP53 gene in Hela cells, and enhanced their proliferation, migration, and invasion.

Key Words:
TP53, Hela cell, Proliferation, Migration, Invasion, miR-122.

Introduction

TP53 gene is a tumor suppressor gene that regulates cell cycle, prevents mutations of normal genes, and suppresses the occurrence of cancer[1,2]. The gene is associated with the occurrence of a variety of cancers such as gastric cancer, cervical cancer, and squamous cell carcinoma[3,4]. With gradual increase of cancer incidence and mortality rates, the regulatory mechanism of cancers has become a hotspot.

A micro RNA (miRNA) is a small non-coding RNA molecule (about 22 nucleotides), which functions in RNA silencing and post-transcriptional regulation of gene expression. There are two primary regulatory mechanisms of miRNAs. While miRNAs that are fully complementary to the sequence of target genes can directly induce the degradation of mRNA of the target genes, miRNAs partially complementary to target gene sequences bind to the 5`UTR, 3`UTR, and CDS region of target gene mRNAs, and inhibit the gene expression at the post-transcriptional level[5,6]. A miRNA might regulate the expression of multiple proteins[7], and the expression of a protein might be regulated by multiple miRNAs. Studies[8-10] have suggested that miRNAs also play important regulatory roles in a variety of diseases such as cancers. A few miRNAs have been discovered as biomarkers in the early diagnosis of cancer or even therapeutic targets in gene therapy. Therefore, researches on the regulatory role of miRNAs on genes that are associated with diseases cannot only shed lights on the molecular mechanism underling the occurrence of diseases, but also provide potential targets for efficient gene therapy[11-13].

In this study, we predicted miRNAs that might regulate the expression of TP53 gene,

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and selected the most potential miR-122 for *in vitro* evaluation of its regulatory role on TP53 expression. We will provide a basis for further investigation on the molecular mechanism of miRNA-mediated regulation of TP53 gene and targeted gene therapy.

Material and Methods

Reagents and Cells

The Hela cell line was purchased from Sunbio (Shanghai, China). Cell culture medium Roswell Park Memorial Institute-1640 (RPMI-1640), trypsin, fetal bovine serum (FBS), and phosphate buffer saline (PBS) were purchased from Gibco (Waltham, MA, USA). Cell culture dishes were purchased from Corning (Corning, NY, USA). The penicillin-streptomycin double-resistance and CCK-8 assay kits were purchased from Beyotime Biotech (Nantong, China). Transwell chambers, matrigel, and crystal violet were purchased from BD Biosciences (San Jose, CA, USA). Lipofectamine 2000 transfection reagent was purchased from Invitrogen (Carlsbad, CA, USA). TRIzol RNA extraction kit and reverse transcription kit were purchased from TaKaRa (Otsu, Shiga, Japan). The reagents for quantitative Real-time PCR were purchased from Promega (Madison, WI, USA). miRNA mimics and inhibitor, and primers were synthesized by Shanghai Invitrogen (Shanghai, China).

Prediction of miRNAs Regulating TP53 Gene Expression

The sequence of TP53 gene (KR709867) was downloaded from NCBI database, and analyzed by online TargetScan software (www.targetscan.org/cgi-bin/targetscan) to predict potential miRNAs (Table I) that might regulate the expression of TP53 gene based on relevant parameters including position in the UTR, seed match, total contextual score, and probability of conserved targeting (Pct). miR-122 was selected as the most potential miRNA for TP53. miR-122 mimics and inhibitor were synthesized by Shanghai Invitrogen and used in subsequent experiments.

Cell Culture and Transfection

Aliquots of Hela cells were inoculated into 60-mm culture dishes containing RPMI-1640 culture medium with 10% FBS at a density of 1×10^5/dish, and cultured in a 37°C incubator with 5% CO_2. Cells at 70% confluent were transfected respectively with 2 ug of miR-122 mimics and 2 ug of miR-122 inhibitor using Lipofectamine 2000 according to the manufacturer's instructions. After 24 h of incubation, cells were collected for the quantification of TP53 mRNA expression.

Table I. The summary of predicte miRNAs that regulate the expression of TP53 gene.

miRNA	Position in the UTR	Seed match	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	Pct
hsa-miR-149-5p	91-97	7mer-m8	-0.13	85	-0.13	3.371	N/A
hsa-miR-331-3p	94-100	7mer-m8	-0.14	86	-0.14	2.876	N/A
hsa-miR-122-5p	112-118	7mer-1A	-0.06	66	-0.06	4.327	N/A
hsa-miR-151a-3p	142-148	7mer-m8	-0.16	79	-0.16	3.782	N/A
hsa-miR-3529-5p	144-150	7mer-m8	-0.27	94	-0.27	3.466	N/A
hsa-miR-379-5p	144-150	7mer-m8	-0.25	93	-0.25	3.466	N/A
hsa-miR-4500	146-152	7mer-m8	-0.21	77	-0.21	4.337	N/A
hsa-let-7g-5p	146-152	7mer-m8	-0.19	73	-0.19	4.337	N/A
hsa-let-7a-5p	146-152	7mer-m8	-0.19	73	-0.19	4.337	N/A
hsa-let-7f-5p	146-152	7mer-m8	-0.19	73	-0.19	4.337	N/A
hsa-let-7c-5p	146-152	7mer-m8	-0.19	73	-0.19	4.337	N/A
hsa-let-7i-5p	146-152	7mer-m8	-0.19	73	-0.19	4.337	N/A
hsa-miR-98-5p	146-152	7mer-m8	-0.19	73	-0.19	4.337	N/A
hsa-let-7e-5p	146-152	7mer-m8	-0.19	73	-0.19	4.337	N/A
hsa-let-7b-5p	146-152	7mer-m8	-0.19	73	-0.19	4.337	N/A
hsa-miR-4458	146-152	7mer-m8	-0.19	71	-0.19	4.337	N/A
hsa-let-7d-5p	146-152	7mer-m8	-0.18	69	-0.18	4.337	N/A
hsa-miR-652-3p	154-160	7mer-1A	-0.29	88	-0.29	3.705	N/A

Pct = probability of conserved targeting.

Quantitative Reverse Transcription PCR (qRT-PCR)

Cells in each group were collected and total RNA was extracted using RNA extraction kit according to the manufacturer's instructions. RNA concentration was determined by a spectrometer in which the absorption value at a wavelength of 260 nm was measured. Total RNA was reversely transcribed into cDNA. The level of TP53 and the housekeeping NAPDH mRNA was measured by qRT-PCR using cDNA as template and Real-time PCR kit following the manufacturer's instructions. The reaction conditions were as follows: 95°C for 2 min, followed by 40 cycles of 95°C 10 s, 55°C 15 s, and 72°C 10 s. After amplification, melting curve analysis was performed: 95°C 1 min, 60°C 30 s, and 95°C 30 s. The sequences of primers: TP53 forward primer: 5'-AGTAGTGGTAATCTACT-3', reverse primer: 5'-GGTCCCTCGTGATTCG-3', NAPDH forward primer; 5'-CGGAGTCAACGGATTTGGTCGTAT-3', and reverse primer: 5'-AGCCTTCTCCATGGTGG-3'. The experiment was repeated three times. The relative expression level of TP53 was calculated as the ratio of mean TP53 mRNA to mean NAPDH mRNA.

CCK-8 Assay of Cell Proliferation

Cells in all groups were collected at 6, 12, 24, and 36 h after transfection, and inoculated into 96-well plates at a density of 1×10^4 cells/100 ul. Five wells were prepared for each sample. A total of 100 ul of medium was added into each well, and 10 ul of CCK8 solution was added into each well after 12 h incubation. Cells were incubated for an additional 3 h, and the optical density of each well was detected using a spectrophotometer at the wavelength of 450 nm. The maximum and minimum OD450 were removed, and the mean OD450 of the remaining three wells for each sample was calculated. The cell proliferation and inhibition rate were calculated.

Scratch Wounding Assay

Cell migration ability was assessed by the scratch wounding assay. Cells in all groups were seeded into 6-well plates at a density of 5×10^5 cells/well, and incubated in a 37°C incubator with 5% CO_2 for 24 h. The center of cell monolayer was scraped using a sterile pipette tip and washed 3 times with PBS to create a straight, cell-free zone (gap) of constant width. Cells were placed into serum-free media. Wound closure was monitored and photographed at 0 h and 18 h with a Nikon inverted microscope. Images were analyzed by Image J software. The distances between the gaps were measured after capture of six random sites in the microscope fields, and the mean migration distance in each group was calculated.

Transwell Invasion Assay

Matrigel (BD Biosciences, San Jose, CA, USA) was spread evenly on the microfilm of the upper transwell chamber. Aliquots of 200 µl of cell suspension in all groups (1×10^5 cells/ml) were digested and added into the upper chamber of transwell. DMEM medium containing 10% FBS (1000 µl) was added into the lower chamber. After 24 h culture, transmembrane cells were washed, fixed and stained with 0.1% crystal violet. The transmembrane cells were counted under an inverted microscopy at a magnitude of 100. Mean values were obtained from three randomly selected visual fields for each well.

Statistical Analysis

All data were expressed as mean ± standard deviation (SD). Statistical analyses were performed using SPSS 11.0 (SPSS Inc., Chicago, IL, USA). The difference among groups was analyzed by ANOVA with Newman-Keuls multiple comparison post-hoc test. $p < 0.05$ was considered statistically significant.

Results

Comparison of TP53 mRNA Expression

The relative TP53 mRNA expression in different groups was quantified by qRT-PCR. As shown in Figure 1, TP53 mRNA expression in the miR-122 mimics group was significantly lower than that in the control group ($p < 0.05$),

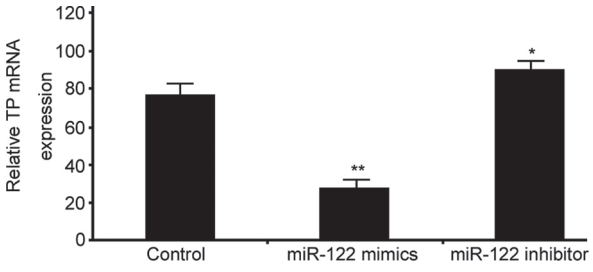

Figure 1. qRT-PCR analyses of relative TP53 mRNA expression in different groups. **, $p < 0.01$ compared with control group. *, $p < 0.05$ compared with control group.

whereas its expression in the miR-122 inhibitor group was significantly higher than that in the control group ($p < 0.05$).

Comparison of Cell Proliferation

The cell proliferation in different groups was compared by CCK-8 assay. As shown in Figure 2, the OD450 value in the miR-122 mimics group was significantly higher compared with the control group ($p < 0.05$ or 0.01 at 24 and 36 h, respectively), suggesting an increase in cell proliferation after transfection of miR-122 mimics. The OD450 value in the miR-122 inhibitor group was slightly lower compared with the control group ($p < 0.05$).

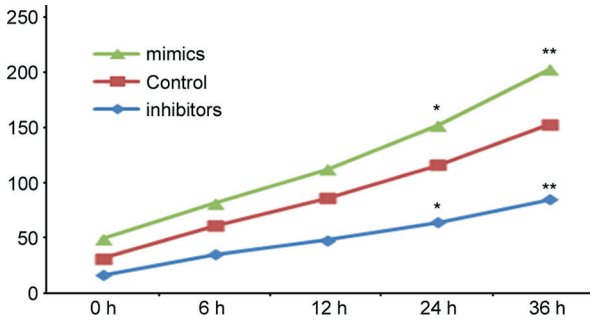

Figure 2. CCK-8 assay of cell proliferation in different groups. **, $p < 0.01$ compared with control group. *, $p < 0.05$ compared with control group.

Comparison of Cell Migration

Cell migration ability in different groups was assessed by Scratch wounding assay. As shown in Figure 3, the mean migration distance in the miR-122 mimics group (32 ± 6.85 um) was significantly longer compared with the control group (4.32 ± 3.13 um, $p < 0.05$), suggesting cell migration ability was increased after transfection of miR-122 mimics. Interestingly, the cell migration in the miR-122 inhibitor group (6.23 ± 2.13 um) was comparable to that in the control group ($p > 0.05$), indicating that miR-122 inhibitor transfection does not affect the migration ability of cells.

Comparison of Cell Invasion

The cell invasion ability in different groups was compared by transwell invasion assay. As shown in Figure 4, the number of transmembrane cells in the miR-122 mimics group was similar to

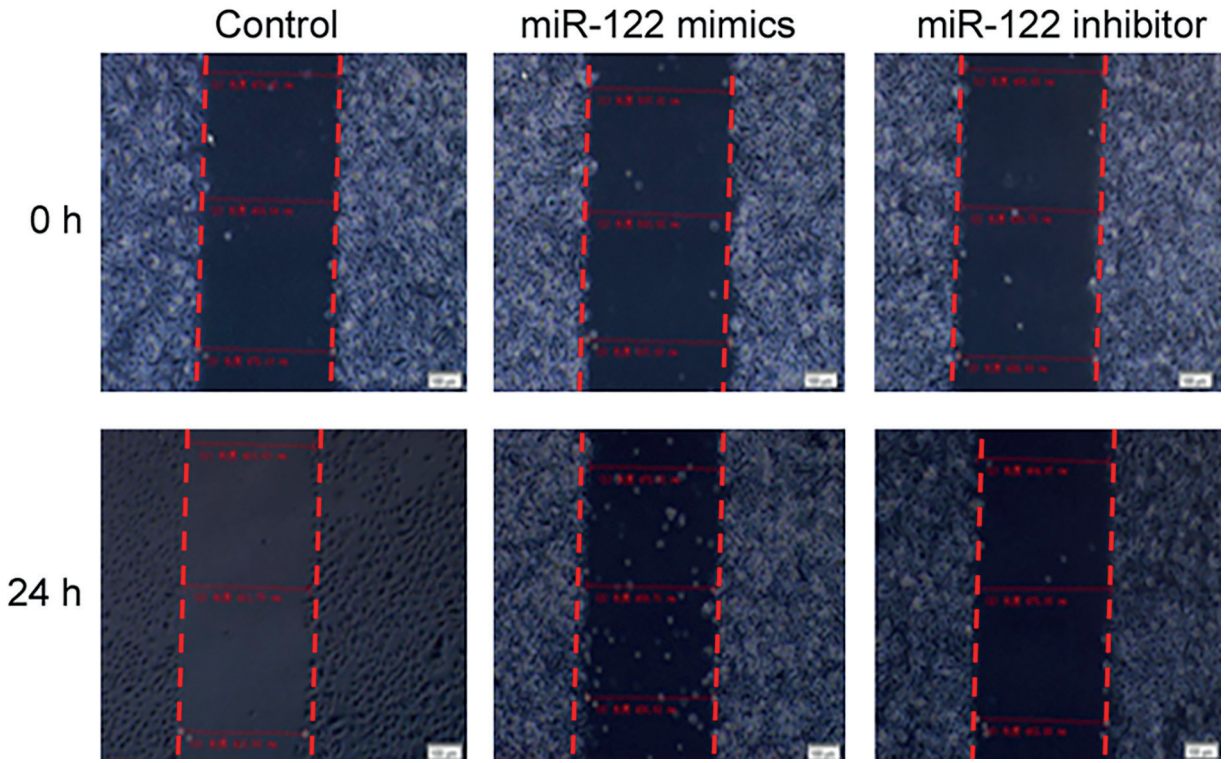

Figure 3. Scatch wounding assay showing the cell migration in different groups.

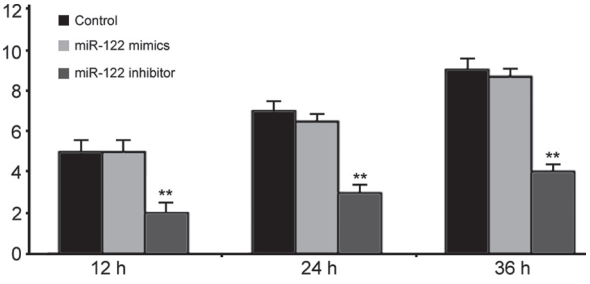

Figure 4. Comparison of the number of transmembrane cells in different groups by transwell invasion assay. **, $p < 0.01$ compared with control group.

that in the control group ($p > 0.05$), whereas the number of transmembrane cells in the miR-122 inhibitor group was significantly lower than that in the other two groups ($p < 0.05$), suggesting a decreased cell invasion ability after miR-122 inhibitor transfection.

Discussion

Cancer is a group of diseases involving abnormal, malignant cell proliferation with the potential to invade other parts of the body[14,15]. The degree of malignancy of cancer is closely associated with the cell ability of proliferation, migration, and invasion. Most tumor suppressor genes regulate a certain stage of cell cycle, and prevent the occurrence of cancer. The abnormity of the expression of cancer-related genes, especially tumor suppressor genes, may directly induce cancerous proliferation of cells[16,17]. Recently, tumor suppressor genes have become one of the research hotspots, although only a few genes have been identified so far such as transcription factors (Rb, p53), negative regulatory transcription factor (WT), cyclin-dependent kinase inhibitor, signaling pathway inhibitor, DNA repair factors, and components of signal pathways that are related to the development and stem cell proliferation[18,19]. As a major tumor suppressor gene, TP53 regulates cell cycle, and the synthesis and metabolism of functional factors, and is involved in the repair of DNA damages[12,13]. TP53 gene mutation occurs in several cancers. It has been known that the mutated TP53 gene not only loses its tumor suppression function, but also promotes the occurrence and development of cancer[20], suggesting the close association of TP53 functional abnormity with cancers.

Previous investigations have found that miRNAs play important regulatory roles in various diseases including cancer. Some miRNAs have been known as biomarkers in the early diagnosis of cancer or even therapeutic targets in gene therapy[19]. It is, therefore, essential to study the regulatory role of miRNA on the expression of disease-related genes in order to elucidate the molecular mechanism of the pathogenesis of diseases as well as provide valuable information on the development of targeted gene therapy.

In this work, miRNAs that might regulate the expression of TP53 gene were predicted by bioinformatics methods. miR-122 was selected as the most potential miRNA for regulating TP53 expression, and its regulatory role on TP53 was evaluated *in vitro*. It was shown that cells transfected with miR-122 mimics exhibited significantly lower TP53 expression ($p < 0.05$), but significantly increased cell proliferation and migration compared with blank control group ($p < 0.05$). Cells in the miR-122 mimics and control groups had similar invasion ability ($p > 0.05$). Cells in the miR-122 inhibitor group exhibited significantly higher TP53 expression ($p < 0.05$), but significantly reduced cell proliferation and invasion ability. The migration ability of cells in the miR-122 inhibitor group was similar to that in the control group ($p > 0.05$), which might be due to the involvement of a large number of genes in the regulation of tumor cell proliferation, migration, and invasion. It has been known that miRNAs such as miR-203 and 34a are indirectly involved in the regulation of TP53 through p53 signaling pathway[21,22]. To our best knowledge, we investigated the regulatory role of miR-122 on TP53 expression for the first time.

Conclusions

We showed that the selected miR-122 effectively inhibited the expression of TP53 gene in Hela cells, and enhanced cell proliferation, migration, and invasion. Our study will not only provide a basis for further investigations on the molecular mechanism of miRNA-mediated regulation of TP53 expression, but also shed lights on the development of tumor gene therapy targeting TP53.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Allo G, Bernardini MQ, Wu RC, Shih Ie M, Kalloger S, Pollett A, Gilks CB, Clarke BA. ARID1A loss correlates with mismatch repair deficiency and intact p53 expression in high-grade endometrial carcinomas. Mod Pathol 2014; 27: 255-261.
2) Bellini MF, Cadamuro AC, Succi M, Proenca MA, Silva AE. Alterations of the TP53 gene in gastric and esophageal carcinogenesis. J Biomed Biotechnol 2012; 2012: 891961.
3) Agarwal A, Durairajanayagam D, Halabi J, Peng J, Vazquez-Levin M. Proteomics, oxidative stress and male infertility. Reprod Biomed Online 2014; 29: 32-58.
4) Dufau ML, Tsai-Morris CH. Gonadotropin-regulated testicular helicase (GRTH/DDX25): an essential regulator of spermatogenesis. Trends Endocrinol Metab 2007; 18: 314-320.
5) Lasko P. The DEAD-box helicase Vasa: evidence for a multiplicity of functions in RNA processes and developmental biology. Biochim Biophys Acta 2013; 1829: 810-816.
6) Rosner A, Moiseeva E, Rinkevich Y, Lapidot Z, Rinkevich B. Vasa and the germ line lineage in a colonial urochordate. Dev Biol 2009; 331: 113-128.
7) Pek JW, Kai T. A role for vasa in regulating mitotic chromosome condensation in Drosophila. Curr Biol 2011; 21: 39-44.
8) Ferraz MA, Zabaglia LM, Pereira WN, Orcini WA, de Labio RW, Caleman Neto A, Wisnieski F, Calcagno DQ, Santos LC, Assumpcao PP, Burbano RR, Artigiani R, Smith Mde A, Payao SL, Rasmussen LT. Downregulated expression of E-cadherin and TP53 in patients with gastric diseases: the involvement of H. pylori infection and its virulence markers. J Gastrointest Cancer 2016; 47: 20-26.
9) Katoh M. Cardio-miRNAs and onco-miRNAs: circulating miRNA-based diagnostics for non-cancerous and cancerous diseases. Front Cell Dev Biol 2014; 2: 61.
10) Kim JG, Lee SJ, Chae YS, Kang BW, Lee YJ, Oh SY, Kim MC, Kim KH, Kim SJ. Association between phosphorylated AMP-activated protein kinase and MAPK3/1 expression and prognosis for patients with gastric cancer. Oncology 2013; 85: 78-85.
11) Krstic M, Stojnev S, Jovanovic L, Marjanovic G. KLF4 expression and apoptosis-related markers in gastric cancer. J BUON 2013; 18: 695-702.
12) Leal MF, Calcagno DQ, Khayat AS, Silva TC, Muniz JA, Assumpcao PP, de Arruda Cardoso Smith M, Burbano RR. hTERT and TP53 deregulation in intestinal-type gastric carcinogenesis in non-human primates. Clin Exp Med 2013; 13: 221-224.
13) Lee HE, Han N, Kim MA, Lee HS, Yang HK, Lee BL, Kim WH. DNA damage response-related proteins in gastric cancer: ATM, Chk2 and p53 expression and their prognostic value. Pathobiology 2014; 81: 25-35.
14) Mafficini A, Amato E, Fassan M, Simbolo M, Antonello D, Vicentini C, Scardoni M, Bersani S, Gottardi M, Rusev B, Malpeli G, Corbo V, Barbi S, Sikora KO, Lawlor RT, Tortora G, Scarpa A. Reporting tumor molecular heterogeneity in histopathological diagnosis. PLoS One 2014; 9: e104979.
15) Park H, Cho SY, Kim H, Na D, Han JY, Chae J, Park C, Park OK, Min S, Kang J, Choi B, Min J, Kwon JY, Suh YS, Kong SH, Lee HJ, Liu ET, Kim JI, Kim S, Yang HK, Lee C. Genomic alterations in BCL2L1 and DLC1 contribute to drug sensitivity in gastric cancer. Proc Natl Acad Sci U S A 2015; 112: 12492-12497.
16) Sehdev V, Katsha A, Arras J, Peng D, Soutto M, Ecsedy J, Zaika A, Belkhiri A, El-Rifai W. HDM2 regulation by AURKA promotes cell survival in gastric cancer. Clin Cancer Res 2014; 20: 76-86.
17) Tanaka T, Fujimura A, Ichimura K, Yanai H, Sato Y, Takata K, Okada H, Kawano S, Tanabe S, Yoshino T. Clinicopathological characteristics of human epidermal growth factor receptor 2-positive Barrett's adenocarcinoma. World J Gastroenterol 2012; 18: 6263-6268.
18) Zhang HQ, He B, Fang N, Lu S, Liao YQ, Wan YY. Autophagy inhibition sensitizes cisplatin cytotoxicity in human gastric cancer cell line SGC7901. Asian Pac J Cancer Prev 2013; 14: 4685-4688.
19) Chang VY, Federman N, Martinez-Agosto J, Tatishchev SF, Nelson SF. Whole exome sequencing of pediatric gastric adenocarcinoma reveals an atypical presentation of Li-Fraumeni syndrome. Pediatr Blood Cancer 2013; 60: 570-574.
20) Xiang B, Mi YY, Li TF, Liu PF. Updated meta-analysis of the TP53 Arg72Pro polymorphism and gastric cancer risk. Asian Pac J Cancer Prev 2012; 13: 1787-1791.
21) Garzon R, Volinia S, Liu CG, Fernandez-Cymering C, Palumbo T, Pichiorri F, Fabbri M, Coombes K, Alder H, Nakamura T, Flomenberg N, Marcucci G, Calin GA, Kornblau SM, Kantarjian H, Bloomfield CD, Andreeff M, Croce CM. MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia. Blood 2008; 111: 3183-3189.
22) Nakamura T, Canaani E, Croce CM. Oncogenic All1 fusion proteins target Drosha-mediated microRNA processing. Proc Natl Acad Sci U S A 2007; 104: 10980-10985.