



ORIGINAL ARTICLE

The Effect of miR-372 on Cell Proliferation in MKN-45 cell line

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ABSTRACT

Gastric cancer (GC) is one of the most common cancers and the world's second leading cause of cancer death in human. MiR-372 acts as an oncomir and may disturb cell cycle, apoptosis, invasion, and cell proliferation in various types of cancer by its target LATS2. In this study, we increased the expression of miR-372 through transduction with lentivirus inside the GC cell line MKN-45. After selection of positive cells, total RNA was extracted and Quantitative Real-Time PCR (QRT-PCR) was performed for miR-372 and LATS2. MTT assay was used for evaluating of cell proliferation in treated and control cells. The amount of miR-372 expression significantly increased compared to the control cells (fold changes in 7, 14 and 21 days after transduction; 7.85, 50.22 and 114.68; $P=0.03$). In contrast to the control cells the level of LATS2 expression was reduced (fold changes in 7, 14 and 21 days after transduction; 0.39, 0.29 and 0.15; $P=0.016$). In addition, the proliferation of treated cells increased significantly ($P<0.05$). These results indicate that LATS2 is a target of miR-372 and it may be an important tumor suppressor in GC MKN-45 cell line.

Key words: Gastric cancer (GC), MKN-45, miR-372, LATS2

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INTRODUCTION

Gastric cancer (GC) is one of the most common cancers in the world [1] and is regarded as the world's second cause of cancer death in human [2]. MicroRNAs (miRNAs) are endogenous, noncoding and small RNAs 21-23 nucleotides in length Mature [3, 4]. miRNAs can bind to the complementary sequences in the 3'-untranslated regions (3'-UTR) of target mRNAs, resulting in post-transcriptional repression [5,6]. Some miRNAs have oncogenic or tumor suppressing effect and have been shown to be strictly related to cancers [7-9]. A number of miRNAs profiles have been identified in human tumors which seem to be associated with diagnosis, prognosis, stages of development and response to therapy [10]. Previous studies have used a miRNA profiling approach to investigate the up regulation and down regulation of the miRNA in GC [11, 12]. Since miRs are associated with different stages of GC, early detection is to identify improvements [13]. miRs are presented as biomarkers for GC and can be manipulated to achieve therapeutic effects in the future [14]. Recently, studies have revealed that miR-372 acts as an oncomir and may regulate cell cycle, apoptosis, invasion, and cell proliferation in various types of cancer by affecting its target Large Tumor Suppressor kinase 2 (LATS2) [12,15-19]. The role of LATS2 is in the cell cycle control, induction of apoptosis, some cell cycle checkpoints [20, 21, and 22]. MiR-372 is known as a biomarker which is associated with advanced stages of glioma and is considered as a factor for the progression of this cancer [23]. Yu et al. showed that miR-372 is one of the five-microRNA signatures associated with survival and cancer relapse in NSCLC (non-small cell lung cancer) patients and dysregulate LATS2 expression. This results in dysregulation of cellular processes which may ultimately lead to tumorigenesis of NSCLC [18]. In 2012, Yamashita et al reported that miR-372 is a poor prognostic factor in colorectal cancer [17]. Furthermore, in 2009 Cho et al. demonstrated that GC cell line AGS expresses miR-372 [16]. The aim of this study was to investigate the effect of induced miR-372 expression on expression level of LATS2 gene to examine whether the possible changes in expression level of LATS2 results in alteration of cell proliferation in MKN-45 cell line.

MATERIALS AND METHODS

Cell culture

The human GC cell line (MKN-45) was cultured in high-glucose Dulbecco's modified Eagle medium (DMEM; GIBCO-BRL) supplemented with 10% fetal bovine serum (FBS) (GIBCO-BRL) and 1% penicillin and streptomycin (GIBCO-BRL). This cell line was cultured in a 95% humidified incubator with 5% CO₂ at 37 °C. Media was changed every 2 days. They were detached with 0.25% trypsin-EDTA (GIBCO-BRL).

Lentivirus packaging and miR-372 transduction

Human embryonic kidney (HEK) 293T cells were cultured and grown in DMEM supplemented with 10% FBS in a 10 cm cell culture plate and at 37°C and 5% CO₂. Lentiviruses were produced by co-transfection of three plasmids into 293T cells, using calcium phosphate transfection buffer with plasmid DNA. HEK 293T cells (5×10⁶) were transfected with 10.5 µg envelope plasmid pMD2G, 21 µg packaging plasmid psPAX2 and 21 µg transfer plasmid harboring the miR-372 gene (Lenti Mira-GFP-has-miR-372 vector, cat#mh11132) and control plasmid (Lenti GFP-blank vector, cat# m001) separately. The medium containing viral particles was collected and concentrated by precipitation with PEG-8000. After titration, The MOI (number of viral particles per cancer cell) of MKN-45 cell line was calculated using different concentrations of the concentrated virus. For better transduction, a concentration of 2 µg/ml Polybrene (hexadimethrine bromide; Sigma-Aldrich, No. H9268) was added to the culture environment. 48-72h after transduction, puromycin (2 µg/ml; Sigma) was used for cell selection.

Isolation of total RNA and Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR)

Total RNA was extracted using RiboEx reagent (Gene All, Korea) from these groups of cells: transduced with Lenti Mira-GFP-has-miR-372 vector and Lenti GFP-blank vector and cells without treatment (control) in 7, 14 and 21 days after treatment. For mRNA (LATS2) quantitative analysis, 1 µg of total RNAs were reverse transcribed using oligo (dT) primer (thermo#K1621). The cDNA product was used as a template for Quantitative Real-Time PCR (QRT-PCR) with primers that have been designed with primer 3 plus online Software. LATS2 expression was normalized against housekeeping (hk) RNA, GAPDH levels. The relative expression of LATS2 (QRT-PCR) was analyzed using SYBR Green (TAKARA#RR820A). High-specificity miRNA qRT-PCR detection kit (Stratagene#600545) was used for quantification of miR-372 and relative miRNA expression levels were normalized to RNAU6B as an internal control. Gene Runner 3 was used for Forward primer design. Real-time PCR was performed on an ABI Instrument (Applied Biosystems, Foster City, CA).

MTT assay

Cell viability and cell growth was measured by MTT assay. 10,000 cells were seeded in each well in 96-well plates. 24, 48 and 72 h after transduction with miR-372 Lentivirus and control Lentivirus and without any treatment, the cells were incubated with 10 µl (5mg/ml in phosphate- buffered saline :PBS) of MTT ((3-(4,5-dimethyl diazol-2-yl) -2,5 diphenyl tetrazolium bromide); Sigma) at 37 °C for 4 h. Then the medium was removed, and the precipitated formazan was dissolved in 100 µl of dimethyl sulfoxide (DMSO). After shaking for 15 min, optical density (OD) was measured spectrophotometrically in an ELISA plate reader (Dynex MRX) at 570 nm.

Statistical Analysis

All of the experiments were repeated at least three times. The experimental data are expressed as mean ± standard deviation. Analysis of variance (ANOVA) test was used to compare values. P<0.05 was considered to indicate a statistically significant result. All data were analyzed by SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL, USA).

RESULTS

Lentivirus transduction:

48 h after transfection by the plasmids, HEK 293T cells were indicated by green fluorescence microscope. The lentivirus vector contains green fluorescent protein (GFP) the coding sequence of which is expressed by cytomegalovirus promoter. Most of the cells were GFP-positive. High levels of GFP-positive cells represented that lentivirus harboring miR-372 were packaged in a high efficiency (data not shown). The efficiency of lentivirus transduction in MKN-45 was determined by a fluorescent microscope to achieve the MOI of MKN-45. The best MOI of MKN-45 for lentivirus was 5 (Figs. 1A, 1B).

Real Time PCR:

Real-Time PCR analysis for miR-372 confirmed that the MKN-45 cell line is transduced with miR-372 lentivirus after selection with puromycin. In 7, 14 and 21 days after transduction with miR-372 lentivirus, the miR-372 fold change was 7.85, 50.22 and 114.68 compared to control cells respectively. MiR-372 expression level significantly (*P*<0.05) increased (Fig. 2A). The fold change of LATS2- mRNA in these days was 0.39, 0.29 and 0.15 and significantly (*P*<0.05) decreased (Fig. 2B).

MTT assay:

Treated cells showed more remarkable proliferation ($P < 0.05$). The MTT assay statistical analysis confirmed the significant difference between treated and control cells in 24, 48 and 72 h after treatment (Fig 3).

DISCUSSION

MiRNAs are capable of silencing gene expression and they are involved in cancers such as GC [14]. Since miRs are associated with different stages of GC, early detection is to identify improvements [13]. Cho et al reported that MiR-372 supports the proliferation and reduces apoptosis in AGS by AS-miR-372 [16]. Voorhoeve et al have demonstrated that miR-372 and miR-373 are oncogenes in testicular germ cell tumors [15]. In addition, in the study conducted on colorectal cancer it was shown that miR-372 acts by mediating LATS2 expression [17]. Our study investigated the possible role of miR-372 in GC, to evaluate the relationship between miR-372 and LATS2 in vitro. Results indicate that up-regulation of miR-372 by miR-372 GFP lentivirus transduction into MKN-45 significantly decreases LATS2 mRNA (Fig 2). Belair et al. reported that miR371-3 cluster is repressed upon *H. pylori* infection in a CagA-dependent manner. The repression of miR-372 and miR-373 is associated with the upregulation of LATS2, leading to the inhibition of cell cycle progression in *H. pylori*-infected cells [24]. We also demonstrated that miR-372 promotes cell proliferation through a LATS2 mediated pathway (Fig 3). Due to the essential role of LATS2 in the accuracy of cell division, miR-372 and miR-373 are known as important factors in tumorigenesis of some tumors that retain wt p53 [15, 25]. Importantly, these observations for different tumors suggest that miR-372 is involved in cancer development through inhibition of LATS2 expression. Our results suggest that miR-372 expression plays a pivotal role in GC. High level of miR-372 induced cell proliferation through LATS2 mediated pathway. However, the molecular details of miR-372 involvement in GC development remain unclear. Our findings in MKN-45 cell line are in line with Cho et al study which suggested that up-regulation of miR-372 may down-regulate LATS2 expression and the subsequent cellular processes such as cell proliferation. This in turn could ultimately lead to tumorigenesis or development of GC (16). Certainly, these findings require further examination to be confirmed in animal models, serum and tumor tissues of GC patients.

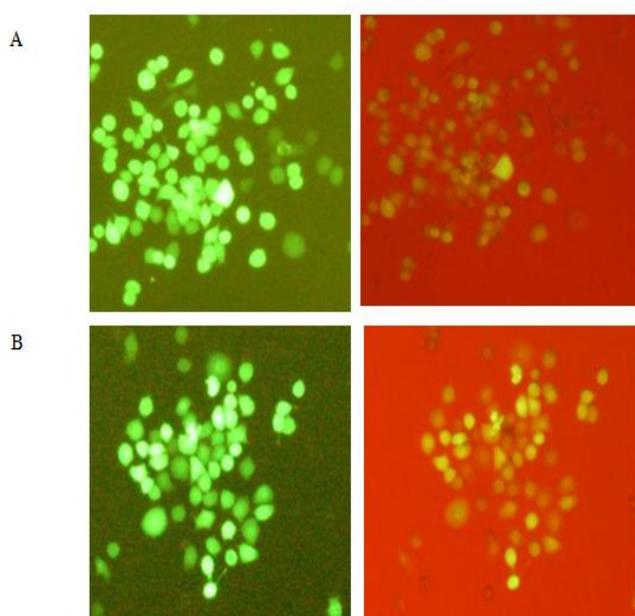
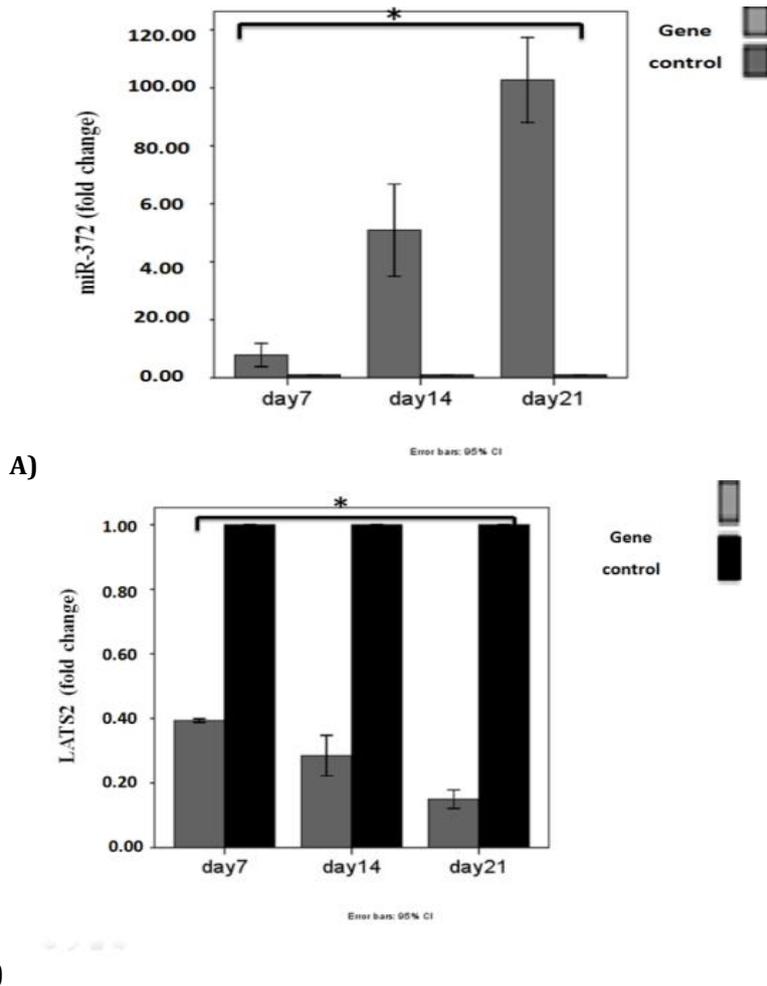


Fig 1: GFP⁺-MKN-45 cell line MiR-372 after puromycine selection: the GFP⁺-cells A) control-GFP-lentiviral vector transduced cells and B) miR-372-GFP-lentiviral vector transduced cell. The MOI of MKN-45 for lentiviral vector was 5.



Figs 2: real time PCR: Histograms show the mean values of miR-372 and LATS2 relative expression in treated and control samples, with a confidence interval as an error bar. **A)** The expression of miR-372 is significantly upregulated in miR-372 lentivirus transduced samples compared to their control counterparts ($P=0.03$). **B)** LATS2 expression: the target gene, LATS2, significantly decreased in treated cells compared to the control cells ($P=0.016$).

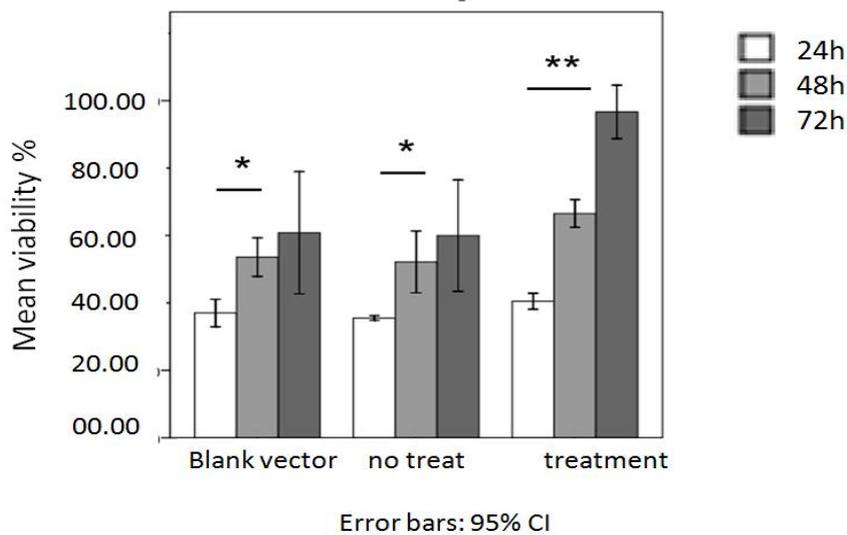


Fig 3: MTT assay; effects of ectopic miR-372 on the growth and proliferation of the gastric cancer cell line MKN-45: miR-372 induced proliferation significantly in this cell line compare control cells. The statistical analyses confirmed significant differences between treatment and control groups. For control groups, the differences were not significant. (* $P < 0.001$); (** $P < 0.05$).

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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