이학박사 학위논문

Gastric carcinogenesis of miR-222/221 transgenic mouse

마이크로RNA- 222/221 형질전환 마우스의 위암 발생에 대한 연구

2012년 6월

서울대학교 대학원

의과대학 협동과정 종양생물학 전공

유 지 은

A thesis of the Degree of Doctor of Philosophy

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The Department of cancer biology,

Seoul National University

College of Medicine

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by

Jieun Yu

A thesis submitted to the Department of cancer biology in partial fulfillment of the requirement of the Degree of Doctor of Philosophy in cancer biology at Seoul National University College of Medicine

June 2012

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Professor _____ **Vice chairman**

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이 논문을 이학박사 학위논문으로 제출함

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ABSTRACT

MicroRNAs (miRNAs) are small RNAs that regulate various cellular functions such as development, cell proliferation, apoptosis and tumorigenesis. miRNA expression profiling of human tumors has identified different signatures associated with various tissue type, diagnosis, progression, prognosis, staging, and response to treatments. Functionally, miRNAs have been shown to reduce the levels of their target transcripts as well as the proteins encoded by these transcripts. Some miRNAs function as oncogenes, while others act as tumor suppressors. Aberrant miRNA expression has also been frequently reported in various tumors, indicating that there is a close correlation between miRNAs and human malignancy. Recently, there is increasing interest in the relation between gastric cancer and miRNA due to the high incidence in Asian countries.

miR-222/221 which was increased in gastric tumor tissue compared to

normal gastric tissues was chosen in a previous study and identified the oncogenic effect of miR-222/221 in gastric cancer with functional study and xenograft model. In this study, miR-222/221 overexpressed transgenic mice were generated to confirm the gastric carcinogenesis effect of miR-222/221 by using gastric carcinogen. Sixty-five transgenic mice and 53 wild type mice were used to compare tumorigenic effects in gastric cancer. At six weeks of age, wild type and transgenic mice were given drinking water containing MNU (*N* - Nitroso – *N* - Methylurea) for five alternating weeks to promote gastric cancer occurrence. At 36 weeks of age, mice were sacrificed and histologic analysis was performed. When all four frequently found pre-cancerous stages and carcinoma are included, the frequency of pre-cancerous stage was significantly higher in transgenic mice compared to wild type mice (P=0.010). Hyperplasia was observed in 3.77% of the wild type mice and in 18.46% of the transgenic mice (P=0.014). However, the frequency of carcinoma (P=0.383), microcarcinoma (P=1.000), adenoma

(P=0.395) and microscopic dysplasia (P=0.920) was not statistically significant different between groups. These results showed that the development of hyperplasia, which is considered as one of gastric precancerous lesions in mouse, was associated with miR-222/221 expression.

Keywords:
microRNA, , microRNA function, transgenic mouse, gastric cancer, pre-cancerous lesions, MNU (*N* - Nitroso – *N* - Methylurea)

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INTRODUCTION

microRNA and cancer

miRNAs are small non-coding RNAs of ~22 nucleotides in length that function as post-transcriptional regulators by base-pairing with the complementary sites at the 3' untranslated region (3'UTR) of mRNA. Since microRNA (miRNA) was identified, there has been an increasing number of studies of small regulators (1). miRNAs can control the stability of target mRNA and the efficiency of translation (2). The latest version of the miRBase database (Release 18) includes 1,921 miRNAs from human (3). Bioinformatic analyses predict that miRNAs may regulate almost all of the human protein-coding genes (4). Each miRNA is predicted to suppress over hundreds of target genes by target prediction programs. In other words, each target mRNA can be repressed by different miRNAs. Since the importance of miRNAs was magnified, many efforts have been made to identify miRNA target genes for individual miRNA using computational and experimental approaches.

Until now, various functions of miRNAs were suggested, ranging from development to life span of many diseases, especially for cancer (5). Many targets of miRNAs are also related to human diseases especially for cancer.

For instance, cell cycle regulators, tumor suppressor genes, oncogenes, growth factors, and various genes regulating cell growth are targets of miRNAs that relate to cancer. These findings suggest that aberrant alterations of miRNA levels are linked to human malignancy (6- 7). Recent studies have shown that by regulating the expression of oncogenes and tumor suppressor genes, miRNAs may have critical roles in maintaining cellular homeostasis in practice. Based on the broad scope of the target genes regulated by miRNAs, it is important to find the role of various miRNAs and incorporate these functions into cellular signaling pathway and apply to treatment of diseases.

microRNAs and gastric cancer

Gastric cancer is the second most common cause of death from cancer worldwide and in many Asian countries, such as China, Japan, and Korea, and is responsible for ~10% of tumor-related deaths worldwide (8-9). Particularly, it received international attention because high incidences and mortality rates are observed in Eastern Asia (10). Although the level of cell cycle regulator has known to be abnormal in gastric cancer, the molecular pathology of gastric cancer is not yet completely known (11). Because of the importance of gastric cancer in Eastern Asia, many groups have studied the relation between microRNAs and human gastric malignancy. Differential

expression of microRNA in Gastric cancer has been shown recently (12). Furthermore, functional studies of microRNAs in gastric cancer have been reported by many groups (13-16). In a previous study, our group investigated human miRNA profiles in gastric carcinoma compared to matched normal gastric tissue using miRNA microarray method (17). Then miR-222/221 cluster, one of the most up-regulated, was selected as a candidate oncogene miRNA in gastric carcinoma. Several targets for miR-222/221 cluster were predicted and we narrowed down the candidate genes and p27Kip1 was selected. The result was confirmed in several in vitro studies and xenograft model experiment. From this finding, it is proposed that miR-222/221 overexpressed transgenic mice would promote gastric carcinogenesis by suppressing the target genes of miR-222/221, p27, CDK inhibitor, and it would help cancer cells to overcome the cell-cycle checkpoint (17).

In the present study, miR-222/221 transgenic mouse was generated to provide further evidence supporting the capacity of gastric cancer development of miR-222/221 by inhibiting the target gene, p27. *N*-methyl-*N*-nitrosourea (MNU), an alkylating agent that induces adenomas and adenocarcinomas in the stomach, was given to mice to elucidate the mode of gastric carcinogenesis in mice (18-20). The goals of the present study were:

(i) to clarify the role of miR-222/221 in gastric carcinogenesis not only in vitro study but also in vivo model by using MNU, carcinogen; (ii) to construct miR-222/221 transgenic mice and observe the phenotype of TG mice; and (iii) to validate the suppressive effect of miR-222/221 on the target gene, p27.

MATERIALS AND METHODS

Animals

All mice were housed in an air-conditioned room under adequate temperature (20.7~22.8℃) and humidity (41.6~59.9%) control with a 12-hr dark/light cycle, and free access to water and food (Lab diet 5002. Indiana, USA). All animal experiments were done after receiving approval from the Institutional Animal Care and Use Committee (IACUC) of Clinical Research Institute at Seoul National University Hospital (SNU-070509-4), and the National Research Council (NRC) guidelines for the care and use of laboratory animals were followed (revised in 1996).

Transgenic mouse

C57/BL6J mice were used to generate transgenic mice. To produce miR-222/221 transgenic mice, miR-222/221 was first amplified with primer (5'-GGA TCC TCA CTC AGT CAG TAT CTG TTG GAT AAG-3' / 5'- CGA GAA ATC ATT CAT TGC TGA GGT GAT-3'). It was cloned into pGEM-T easy vector and subsequently transferred into the pcDNA3 vector at BamH1 and Xho1 site. The nucleotide sequences of the plasmid were confirmed by sequencing, and then the plasmid was injected into fertilized eggs from C57BL/6J mice. To screen transmission test, the primers were 5'-CGC TAT

TAC CAT GGT GAT GCG-3’ and 5’- CTA CTG AGC CAT TGA GGG TAC-3’ and their offspring were divided into transgenic mice and wild type mice by RT-PCR screening.

Genetic typing and PCR

Genomic DNA was extracted from the tail of each mouse. Mouse screening was done using LaboPass Tissue Mini DNA Purification Kit (Cosmo Genetech, Seoul, Korea) according to the manufacturer's protocol. The extracted DNA was then subjected to PCR. The following primers were used: pcDNA3 - Forward: 5'- CGC TAT TAC CAT GGT GAT GCG - 3', miR- Reverse: 5'- CTA CTG AGC CAT TGA GGG TAC - 3'.

MNU treatment

MNU (Sigma Chemical Co, USA) was dissolved in distilled water at a concentration of 240ppm and contained in drinking water in light-shielded bottles and freshly prepared thrice per week. At six weeks of age, each group was given drinking water containing MNU on alternating weeks for a total of 10 weeks of exposure according to the protocol described in other reports (21-22). After MNU administration was finished, all the drinking water was switched to autoclaved distilled water.

Experimental design

The experiment design is shown in Figure 1. A total of 53 wild type mice and a total of 65 transgenic mice were used. At 6 weeks of age, each group was treated with MNU for five alternating weeks. The animals were carefully autopsied at the time of their death, at 36 weeks of age, under anesthesia. At the end of the experiment, all mice were fasted for 24h but had access to water. Prior to sacrifice, blood samples were collected from vena cava for blood chemistry test. The stomachs and other organs were carefully examined macroscopically. After pathologic observation of stomach and intestine, immunohistochemistry and validation study were performed.

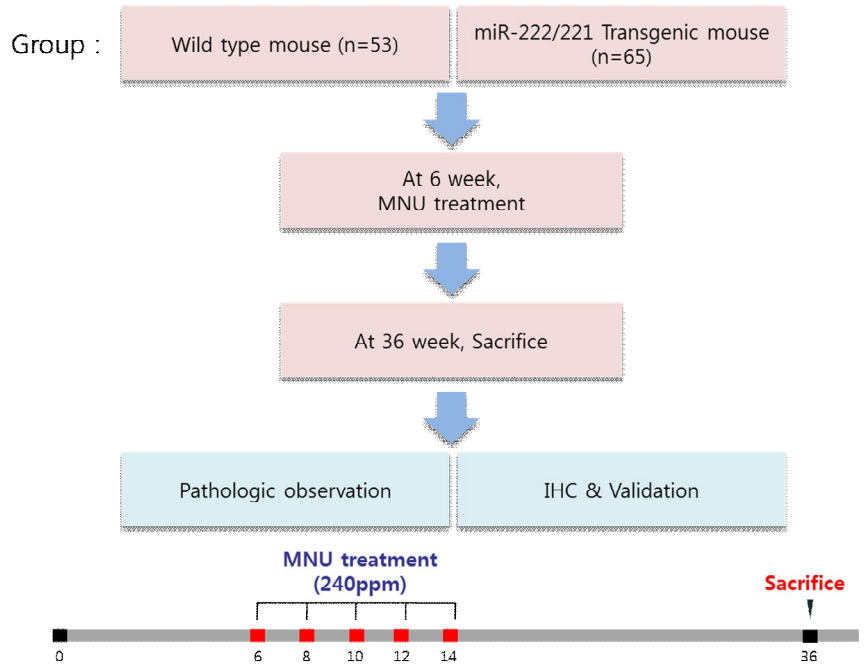


Figure 1. Protocol for the study

Fifty-three wild type mice and 65 transgenic mice were used. At six week, they were given MNU that was mixed in their drinking water at concentrations of 240ppm for a total of five cycles of one-week regimens with a one-week pause. After completion of MNU administration, they were given autoclaved distilled water. After pathologic observation of stomach and intestine, immunohistochemistry and validation study were conducted. and intestine, immunohistochemistry and validation study were conducted.

Tissue preparation

All mice underwent a thorough postmortem examination at the time of sacrifice. The stomach was tied with silk thread at esophagus and duodenum, and then removed. Thereafter, PBS was injected into the stomach and 10 minutes later, the stomach was excised along the greater curvature with scissors and fixed with a pin. Half of the excised stomach was frozen in liquid nitrogen for molecular biological experiment and the remaining stomach was fixed for 24 hours, processed by standard methods, embedded in paraffin, sectioned at 5- μm, and stained with Hematoxylin and Eosin (H&E). The intestine was removed from the abdomen and tied with silk thread at duodenum and the edge of the rectum was then injected with 10% neutral buffered formaldehyde solution. 24 hours later, the knots were untied, the intestine was excised longitudinally with scissors, and the contents were carefully removed. Next, the segment was rolled up longitudinally, with the mucosa outwards, using a forceps (17). Finally, the intestine was placed in a fixative for paraffin-wax embedding, and sectioned. The 5-μm thick sections were stained with H&E.

Northern blot analysis

Total RNA was extracted from mouse tissues by using TRIzol reagent (Invitrogen, Carlsbad, USA). Total RNA (30 μg) and was loaded on a 12.5%

urea- polyacrylamide gel. The resolved RNA was transferred to a Zeta-Probe GT blotting membrane (Bio-Rad, Hercules, CA) by capillary method and fixed by ultraviolet crosslinking. Oligonucleotides complementary to each miRNA were endlabeled at the 5′ end with 32P-γ-ATP using T4 polynucleotide kinase (Takara, Japan) and used as probes. Prehybridization and hybridization were carried out using ExpressHyb Hybridization Solution (Clontech) according to the manufacturer's instruction. The sequences of the antisense oligos are complimentary to mature miR-222 and miR-221. The sequences of the probes are: 5'-GAAACCCAGCAGACAATGTAGCT-3' (miRNA-221), 5'-GAGACC CAGTAGCCAGATGTAGCT-3' (miRNA-222). Membranes were prehybridized for 30min at 37℃ using hybridization solution in hybridization tubes. Subsequently, the probes were added into tubes and incubated for 1hr at 37℃. After hybridization, the membranes were washed twice for 30 min using buffer 1 (0.05% SDS, 2x SSC) at room temperature and then washed twice for 15 min using buffer 2 (10% SDS, 20x SSC) at room temperature. BAS 2500 Image Analysis System (Fujifilm) and Multi Gauge V3.0 software (Fujifilm) were used for quantitation. Ethidium bromide staining of rRNA was used for RNA loading control.

Cell culture

The human gastric cancer cell lines, SNU-638, and AGS obtained from Korean cell line bank were cultured in RPMI1640 (WelGENE) and supplemented with 10% FBS (WelGENE). These cells were maintained at 37℃ under an atmosphere of 5% CO_2-95% air. Transfection to AGS cells was performed one day after seeding using Lipofectamin 2000 (Invitrogen, Carlsbad, USA).

Quantitative Real-time PCR

Total 1 μg of total RNA was used for single-stranded cDNA using the SuperScript First Strand cDNA synthesis (Life Technologies Inc., Rockville, MD). RNA quantifications were conducted by real-time PCR using the comparative Ct method with the Bio-Rad iCycler Real-Time PCR system. Real-time PCR reaction was conducted by using the SyBR green 2X master mix (Stratagene). Thermocycling was performed in a final volume of 20 μl containing; 1 μl cDNA, 10 pmol of each primer, 2x SyBR green 2X master mix and carried out as follows; initial denaturation at 95℃ for 10 min, 40 cycles of 94℃ for 30 sec, 57℃ for 30 sec, and 72℃ for 30 sec. All reactions were triplicate repeated to ensure the reproducibility of the results. Primers for PCR amplification were 5'- AGT CAG CGC AAG TGG AAT TT -3' (forward) and 5'- AGT AGA ACT CGG GCA AGC TG -3' (reverse)

for the amplification of mouse p27 mRNA. 5'-ACACTCCAGCTGGGAGCTACATTGTCTGCT-3' (forward) 5'- CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG GAA ACC CA -3' (reverse) for the amplification of mature miR-221 mRNA. 5'- ACA CTC CAG CTG GGA GCT ACA TCT GGC TAC TG -3' (forward) 5'- CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG GAG ACC CA -3' (reverse) for the amplification of mature miR-222 mRNA. 5'- TGG TGT CGT GGA GTC G -3' for the amplification of URP. All reactions were performed three times with triplicate, and GAPDH amplification kit was used for normalization (Bio-Rad Laboratories).

Western blot analysis

The proteins were extracted from SNU638, AGS cell and stomach tissue samples obtained from mice were dissolved in lysis buffer containing 25mmol/L HEPES (pH7.5), 150mmol/L NaCl, 1% Triton-X 100, 5mmol/L EDTA, 10% glycerol, and protease inhibitor cocktails (Roche Applied Science). For immunoblotting, lysates were allowed remain on ice for 30 minites and then centrifuged at x13,200 rpm for 30 minutes at 4℃. 50 μg of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to polyvinylidene difluoride membrane. Anti-p27 antibodies (1:200, BD Biosciences), anti-

mouse IgG (1:1000, Cell signaling) were used for western blot experiments. Normalization was done using mouse monoclonal antitubulin antibody (1:1000, Sigma,). The bands were visualized using the Enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Pierce) system after extensive washing of the membranes.

Immunohistochemistry

Formalin-fixed, paraffin-embedded, 4-μm sections were dewaxed in xylene, rehydrated through graded alcohol, and placed in an endogenous peroxide block for 15 minutes. Sections were washed in water, antigen retrieved, and then placed in citrate buffer. Nonreactive staining was blocked by treating sections with 1% horse serum in Tris-buffered saline (pH 6.0) for 3 minutes. Anti-CD31 (1:80, goat polyclonal, Santacruz), anti-ki67 (1:200, rat monoclonal, DAKO), anti-cleaved caspase-3 (1:100, rabbit monoclonal, Cell signaling), anti-p27 (1:500, rabbit polyclonal, Santacruz), anti-p57 (1:300, rabbit monoclonal, Epitomics) were then applied, and antibody binding was detected using an avidin-biotin-peroxidase complex (Universal Elite ABC Kit; Vectastain, Burlingame, CA) for 10 minutes. Diaminobenzidine tetrahydrochloride solution (Kit HK153-5K; Biogenex, San Ramon, CA) was then used as a chromogen.

RESULTS

Previously, in order to identify miRNAs involved in gastric carcinogenesis, I and my colleagues carried out miRNA expression profiling in a gastric cancer patient tissue (17). Several miRNAs were found to be enriched in tumor tissues compared to their normal counterparts (data not shown). Other groups also reported enhanced expression of these miRNAs in various tumors including stomach, colon, and prostate cancer (24-25). Among overexpressed miRNAs in tumor sample, miR-222/221 cluster was selected and the result was confirmed with northern blot experiments (data not shown). This miRNA cluster was upregulated in the gastric tumor samples compared to the normal counterparts. With target prediction programs, it was expected that miR-222/221 inhibits the expression of p27, CDK2 inhibitor. Then we made xenograft mouse model by subcutaneous injection of miRNA stable cell line, to elucidate the ability of cancer promotion. The tumor size was bigger in the mice injected with microRNA stable cell line compared to the control group. This data demonstrated that the expression of p27 is inhibited by miR-222/221 through the complement binding to the 3'-UTR of p27 and it may keep cell cycle proceeding.

Phenotype of transgenic mice

In the present study, miR-222/221 overexpressed transgenic mice were

generated from Macrogen (Seoul, Korea). The gastric cancer cell line AGS was introduced with the miR-222/221-expression plasmid (Fig. 2-A). The expression test of miR-222/221 vector construct was performed with northern blot analysis (Fig. 2-B). The expression level of pre-miRNA-222/221 and mature miRNA-222/221 was overexpressed in the stable cell line transfected with miR-222/221 than the cell line transfected with pcDNA3 null vector. Ethidium bromide staining of rRNA was used for RNA loading control.

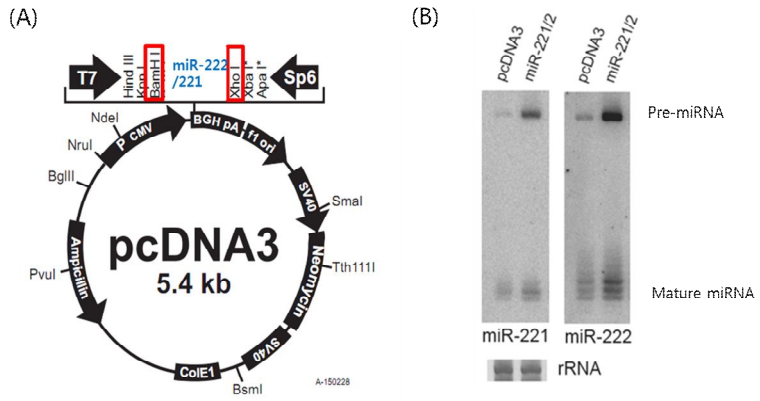


Figure 2. The expression test of miR-222/221 vector constructs

(A) miR-222/221 was inserted between BamH1 and Xho1 and miR-222/221 pcDNA3 vector was constructed. (B) The expression test of miR-222/221 gastric cancer AGS stable cell line was performed with northern blot analysis. Both pre-miRNA-222/221 and mature miRNA-222/221 were overexpressed in the stable cell line transfected with miR-222/221 compared to the cell line transfected with pcDNA3 null vector. Ethidium bromide staining of rRNA was used for RNA loading control.

To examine whether offspring contains injected foreign DNA, germ-line transmission test was conducted with PCR using CMV promoter primer and miR-222 primer (Fig. 3-A). At three weeks of age, all mice were screened by PCR. Then, at ten weeks of age, the expression level of pre miRNA-222/221 and mature miRNA-222/221 were compared between wild type mouse and transgenic mouse in nine organs (Brain, thymus, heart, lung, stomach, liver, intestine, spleen, kidney) with northern blotting (Fig. 3-B). miR-222/221 was expressed in transgenic mouse but it was not expressed in wild type mouse. The expression pattern in organs was also different. For instance, stomach, brain, lung and kidney were strongly overexpressed in transgenic mice compared to wild type mice in both pre miR-222/221 and mature miR-222/221. Ethidium bromide staining of rRNA was used for RNA loading control.

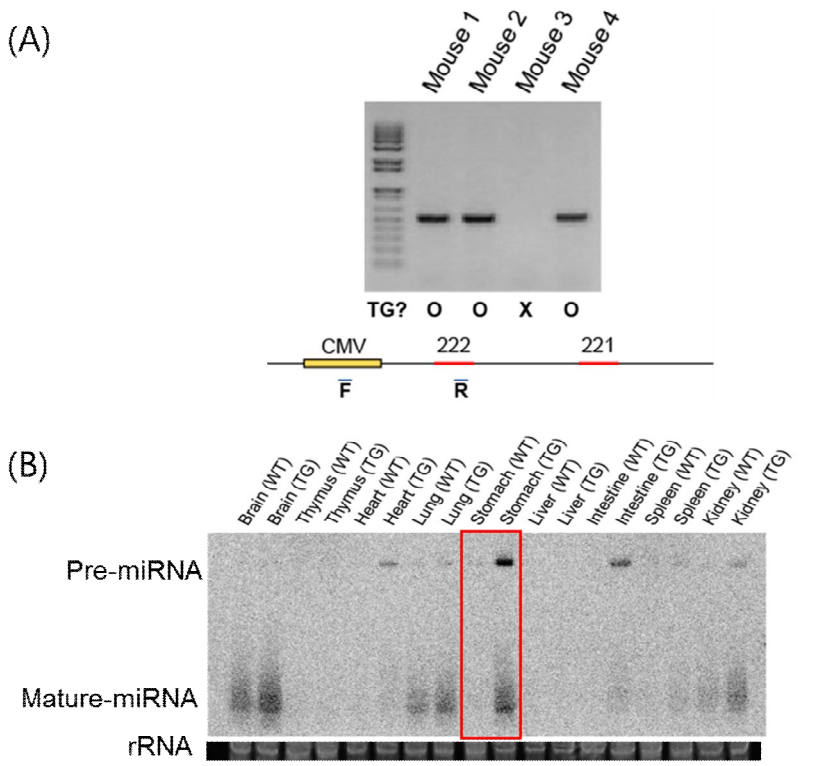


Figure 3. Selection of transgenic mouse

(A) Germ-line transmission test: To examine whether offspring contains injected foreign DNA, germ-line transmission test is conducted with PCR

(B) Expression test by northern blot analysis: the expression levels of miR-222/221 pre miRNA and mature miRNA were compared between wild type mice and transgenic mice in nine organs. Stomach, brain, lung and kidney were strongly overexpressed in transgenic mice compared to wild type mice in both pre miR-222/221 and mature miR-222/221. Ethidium bromide staining of rRNA was used for RNA loading control.

To observe the phenotype of miR-222/221 transgenic mouse, 56 weeks old age transgenic mice and wild type mice were selected (Table 1).

Before sacrifice, blood samples were obtained directly from the vena cava. The stomachs and other organs were carefully examined macroscopically. From this result, organ weight, blood chemistry test was investigated. When we observed the organ weight between the two groups, they were not any different but filled with fat. When we compared body weight with male mice, average body weight of transgenic mice were about 20% heavier than wild type mice. WBC (white blood cell) count was 1.8 fold lower in transgenic mice in blood chemistry test. No significant difference in p27 between transgenic mice and wild type mice was found. From the pathologic result, no cancer or cancer related lesions were found until 56 weeks.

Table 1. Organ weight and blood chemistry test of miR-222/221 transgenic mice

(A)

Type	Male	Female	Age(week)
TG mouse	6	3	56
WT mouse	6	3	56

(B)

Type	Body weight	Spleen	Liver	Kidney-L	Kidney-R	Adrenal(L)	Adrenal(R)	Testis(L)	Testis(R)	Thymus	Heart	Lung	Brain
WT-M	32.95	0.0578	0.8953	0.1306	0.1392	0.0016	0.0016	0.0776	0.0803	0.0366	0.2831	0.1342	0.4234
TG-M	39.6	0.0556	0.8943	0.1333	0.1330	0.0016	0.0013	0.0744	0.0766	0.0346	0.1160	0.1262	0.4152
WT-F	28.28	0.0598	0.0598	0.0984	0.1057	0.0023	0.0023	0.0034	0.0046	0.0401	0.1041	0.1320	0.4276
TG-F	29.10	0.0613	0.0613	0.1086	0.1111	0.0024	0.0025	0.0029	0.0033	0.0464	0.1043	0.0890	0.4279

(c)

Type	Sex	WBC (10^3/mm^3)	RBC (10^6/mm^3)	HGB (g/dl)	HCT (L%)	PLT (10^3/mm^3)	MCV	MCH (H pg)	MCHC (H g/dl)	Neutrophils (%)	Eosinophils (%)	Basophils (%)	Lymphocytes (%)	Monocytes (%)
WT	Male	2.36	7.36	11.2	30.0	678	40.8	15.3	37.4	6.7	0.1	0.2	88.2	3.9
TG	Male	1.30	7.82	11.5	32.6	665	41.7	14.8	35.5	5.7	0.0	0.2	90.3	2.9

(A) At 56 weeks old, nine mice were selected from each group and (B) organ weight and body weight were investigated. Difference of organ weight was not found but body weight was heavier in transgenic mice group. Prior to sacrifice, blood samples were collected directly from the vena cava, and blood chemistry test was conducted from 13 categories. From the pathologic results, no tumor or pre-cancerous stage lesions were found during 56 weeks and also no significant difference of p27, the target gene of miR-222/221, was found between transgenic mice and wild type mice.

To promote gastric carcinogenesis, MNU was used with drinking water for 5 alternating weeks. It has been known that MNU treatment is toxic to mice. At first, a total of 59 wild type mice and a total of 76 transgenic mice were used. 53 wild type mice and 65 wild type mice were sacrificed at 36 weeks of age. At 6 weeks of age, each group was treated with MNU for five alternating weeks. Six mice died from the MNU-administered wild type group and 11 mice died from MNU-administered transgenic mice group during the 30 weeks and were omitted from the final analysis. According to other group's reports (18-19), ten percent of mortality was found in MNU treated control group, which is consistent with mice mortality result found in this study. Total mortality for each group is shown in Table 2. Approximately 85% of transgenic mice survived until 36 weeks old.

Table 2. Mortality within 30 weeks and MNU intake of mice according to group

Group	Total No. of mice	No. of mice finally evaluated	Dead No. of mice (%)
Wild type mouse	59	53	6 (10.1)
Transgenic mouse	76	65	11 (14.4)

Because of the toxicity of MNU, some mice died within 30 weeks before they were sacrificed. After MNU treatment with drinking water, 10.1% of mice died from wild type mice group and 14.4% of mice died from transgenic mice group.

After mice were sacrificed at 36 weeks of age, five transgenic and four wild type mice were randomly selected, and then mice stomachs were collected for the northern blot analysis (Fig. 4). The result was consistent with the data collected from PCR (data not shown). Both pre-miRNA and mature miRNA of miRNA-222/221 were overexpressed in all five transgenic mice stomach whereas the expression of miR-222/221 was not found in four wild type mice stomach. Ethidium Bromide staining of rRNA was used for RNA loading control.

Increased expression of miR-222/221 in human gastric cancer could cause malignant histology or determine important aspects of cancer biology. According to our previous results from functional study of miR-222/221 (17) and other groups, (25-26) this microRNA cluster functions as oncogene by suppressing the p27, CDK inhibitor, and it would help cancer cells to overcome the cell-cycle checkpoint.

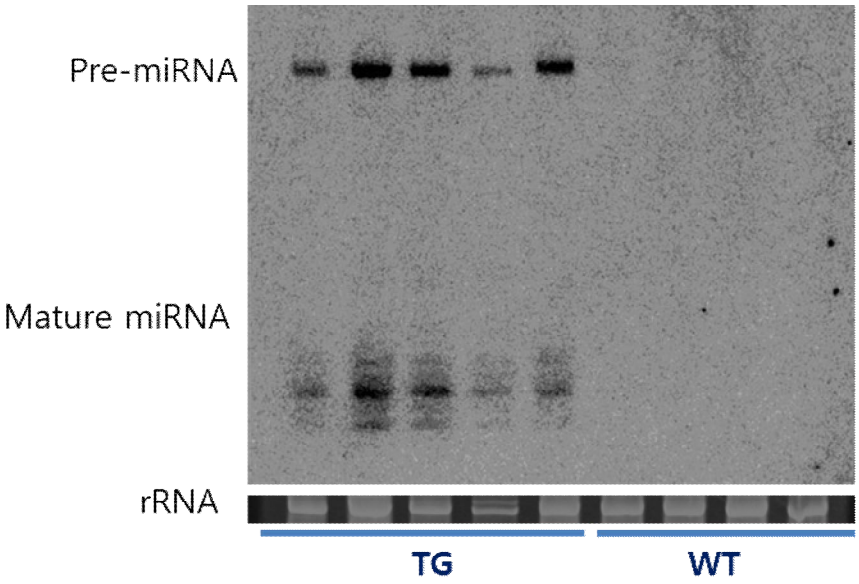


Figure 4. The verification of the expression level of miR-222/221 in mice stomach

After the mice were sacrificed at 36 weeks of age, both pre-miRNA and mature miRNA of miRNA-222/221 were overexpressed in all five transgenic mice stomach whereas the expression of miR-222/221 was not found in any of the four wild type mice. Normalization of loading RNA was used with rRNA.

Figure 5 shows that after all MNU treated mice were sacrificed, two of the mice were randomly selected from each group and organs were collected, and then expression of miR-222/221 was evaluated with northern blot analysis. This expression pattern of miR-222/221 was similar with expression test result, and it was overexpressed in the stomach of transgenic mice.

To verify mature form of miRNA is actually existed in transgenic mice, was quantitative Real-time PCR was conducted with mature miR-222 and miR-221. Each mature miR-222 and miR-221 was compared between wild type mice and transgenic mice using Real-time PCR (Fig. 6). Gastric cancer cell line, SNU-638 was used as positive control and AGS cell line was used as negative control. Expression of mature miR-222 and miR-221 was higher in transgenic mice than wild type mice even though each sample had different expression level. Protein level of p27, target gene of miR-222/221, was evaluated with western blot analysis. When the two groups were compared, the expression of p27 was higher in wild type mice than transgenic mice group (Fig. 7). This result demonstrates that miR-222/221 inhibits target mRNA of p27 and that it also affects in protein level. When MNU was treated with each group, the expression pattern of p27 in stomach was changed. Figure shows that the p27 protein was down expressed in MNU

treated wild type mice stomach than no MNU treated mice stomach and the expression level of p27 was higher in MNU treated transgenic mice stomach than no MNU treated transgenic mice stomach. However, mRNA level of p27 was not significantly different between wild type mice stomach and transgenic mice stomach.

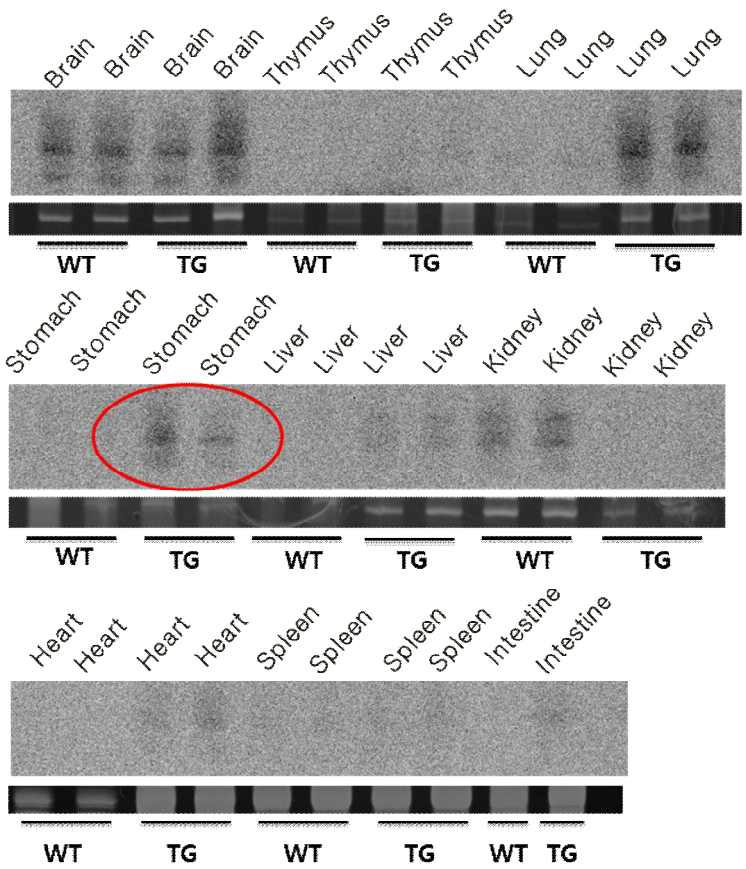


Figure 5. Expression of miR-222/221 in each organ by northern blot analysis

After all MNU treated mice were sacrificed, two of the mice were randomly selected in transgenic mice group and wild type mice group and organs were collected, and then expression of miR-222/221 was evaluated with northern blot analysis. The stomach of transgenic mice was overexpressed compared to control mice.

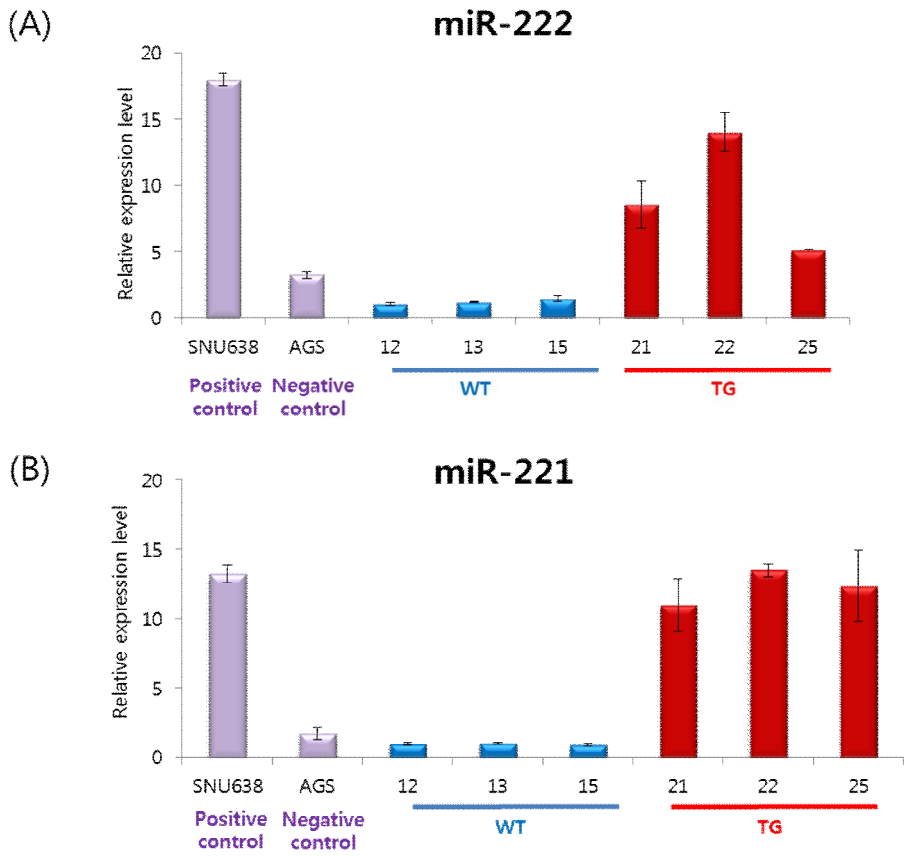


Figure 6. Real time PCR analysis for mature miRNA

(A and B) Each mature miR-222 and miR-221 was compared between wild type mice stomach and transgenic mice stomach. Gastric cancer cell line, SNU-638 was positive control and AGS cell line was negative control. Expression of mature miR-222 and miR-221 was higher in transgenic mice than wild type mice.

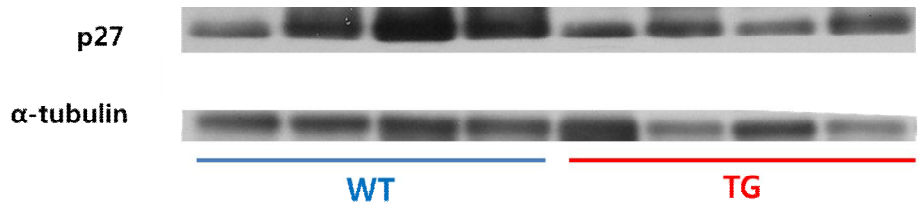


Figure 7. Western blot analysis of target gene in mice stomachs

The protein level of p27, the target gene of miR-222/221, in mice stomach was evaluated with western blot analysis. When the two groups were compared, the expression of p27 was higher in wild type mice than transgenic mice group. Normalization was used with mouse antitubulin antibody.

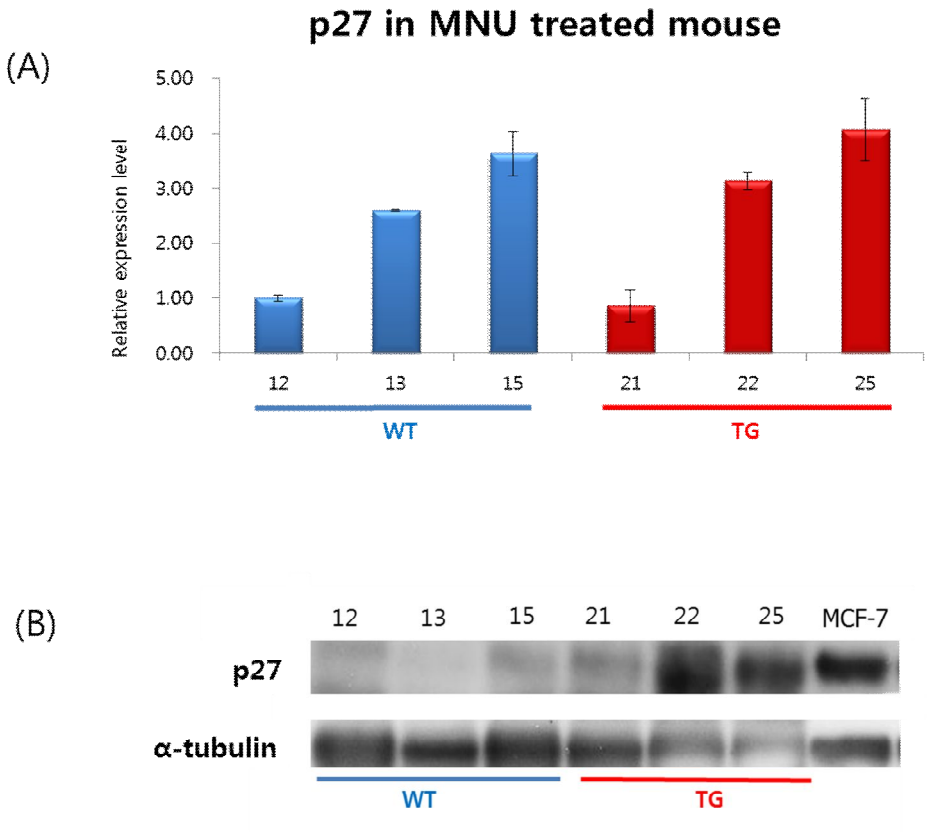


Figure 8. p27 expression in MNU treated mice stomachs

When mice were treated with MNU, the mRNA level of p27 changed and it was difficult to find any difference between two groups. The protein level of p27 was lower in wild type mice stomach compared to transgenic mice.

Histological findings

At the end of the experiment, all surviving mice were sacrificed and their stomachs and other organs were carefully examined. From the pathological finding, after the carcinogen, MNU, was treated to mice, carcinoma and other various lesions: hyperplasia, microcarcinoma in adenoma, angiosarcoma, microscopic dysplasia, adenoma in pylorus region, squamous papilloma in fundus region were found in mice stomachs. However, only flat serrated adenoma was observed from three mice intestines. From this result, four most frequently observed pre-cancerous lesions were selected and included for MNU-induced multi gastric carcinogenesis steps. Histologic appearance of representative stomach and four pre-cancerous lesions are shown in Figure 9. Table 3 shows that percent of carcinoma and pre-cancerous lesions occurrence by histologic findings. When carcinoma and four pre-cancerous lesions were included for evaluating gastric carcinogenesis step, the occurrence of pre-cancerous lesions was significantly higher in transgenic mice compared to wild type mice (P=0.010). Hyperplasia was observed in 3.77% of the wild type mice and in 18.46% of the transgenic mice (P=0.014). However, frequency of carcinoma (P=0.383), microcarcinoma (P=1.000), adenoma (P=0.395), microscopic dysplasia (P=0.920) was higher in the transgenic mice but the difference

was not statistically significant.

To test the correlation between cancer and miR-222/221 in vivo model, cancer related markers were selected for immunohistochemistry. CD31 was used for angiogenesis marker, ki67 was assessed for cell proliferation, and cleaved-caspase3 was used for apoptosis marker. Moreover, two target genes antibodies, p27 and p57, were used for comparing their expression level in both group mouse stomachs (Table 4). In accordance with our hypothesis, transgenic mice group overexpressed miR-222/221, which would repress the expression of p27 and p57, target genes of miR-222/221, compared to wild type mice group. However, there was no significant difference between two groups in immunohistochemistry. When transgenic mice group was compared with wild type mice group on cancer related markers in case of carcinoma development, the expression of CD31 (angiogenesis marker) and ki67 (proliferation marker) were highly expressed in transgenic mice than wild type mice, suggesting that more cancer related phenomenon, angiogenesis and proliferation, could be activated (Fig. 10). However, cleaved-caspase 3 (apoptosis marker) and the targets of miR-222/221, p27 and p57 were not significantly different between two groups (Fig. 11 and Fig. 12). Figure 13 shows that the expression of p27 was similar between no MNU treated wild type mouse

stomachs and transgenic mouse stomachs.

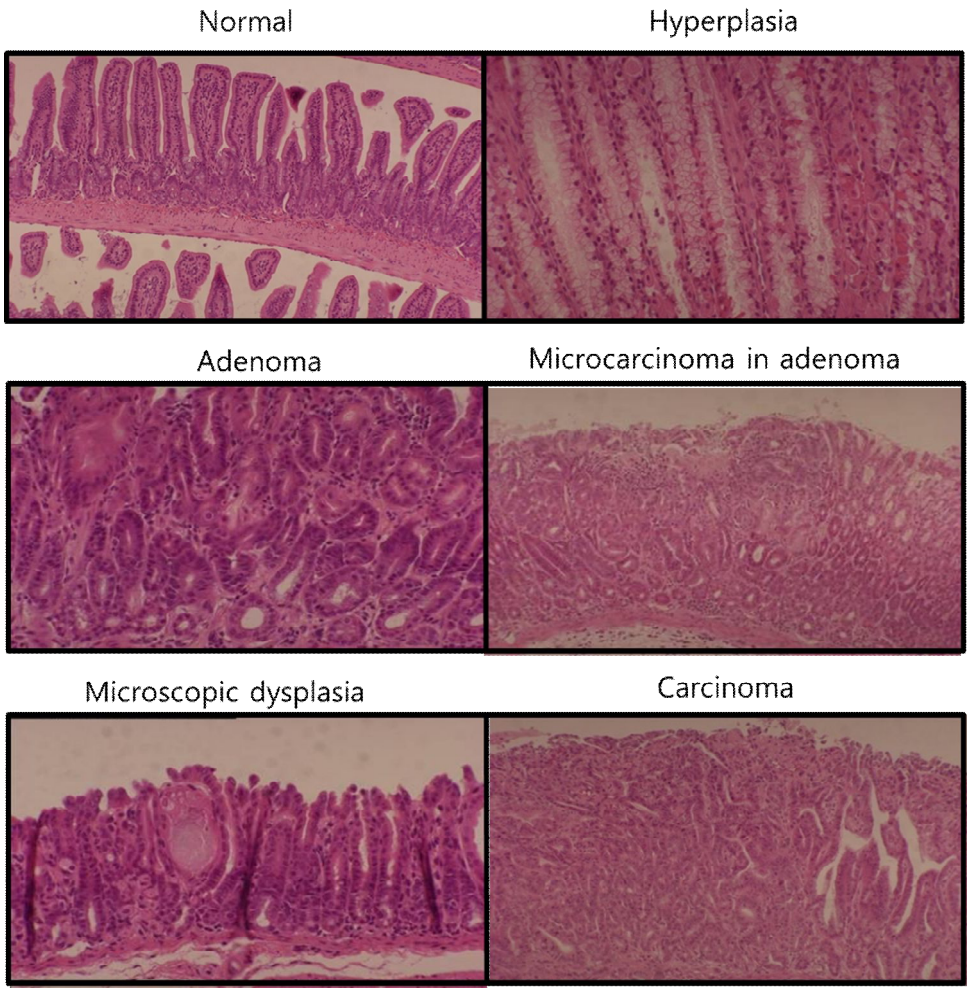


Figure 9. Histologic appearance of representative stomach and four pre-cancerous lesions

Carcinoma and four possible pre-cancerous lesions; microscopic dysplasia, hyperplasia, adenoma, microcarcinoma in adenoma were included in mouse gastric carcinogenesis classification (H&E, x200).

Table 3. Percent of carcinoma and pre-cancerous lesions occurrence by histologic findings

	WT (n=53)		TG (n=65)		P-value
Hyperplasia	2	3.8%	12	18.5%	0.014*
Adenoma	4	7.5%	8	12.3%	0.395
Microscopic dysplasia	7	13.2%	9	13.8%	0.920
Microcarcinoma in adenoma	3	5.7%	5	7.7%	0.729
Carcinoma	14	26.4%	22	33.8%	0.383
Total	27	50.9%	47	73.8%	0.010*

* Significantly different between two groups P<0.05

When MNU treated mice stomach was observed, hyperplasia was statistically significant in transgenic mice group (P=0.014). When carcinoma and four pre-cancerous lesions were included, the occurrence of pre-cancerous lesions was significantly higher in transgenic mice group compared to wild type mice (P=0.010).

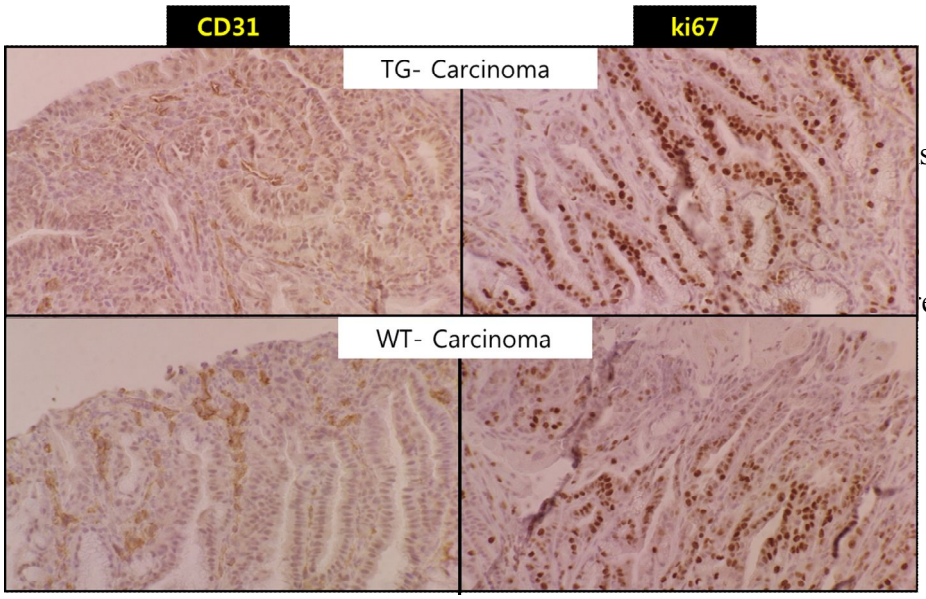
Table 4. Immunohistochemistry with cancer related markers

Group	No treatment	MNU treated mice	
		No-Carcinoma	Carcinoma
Wild type mouse	2	2	2
Transgenic mouse	2	2	2

Marker	Description
CD31	Angiogenesis marker
Ki67	Cell Proliferation marker
Cleaved-Caspase3	Apoptosis marker
p27	target gene of miR-222/221
p57	target gene of miR-222/221

To test the correlation between cancer and miR-222/221 in vivo model, cancer-related markers were selected for immunohistochemistry.

CD31 was used for angiogenesis marker, ki67 was assessed for cell proliferation and cleaved-Caspase3 was used for apoptosis marker in both group mice stomachs. Moreover, two target gene antibodies, p27 and p57, were used for comparing their expression level in the two groups.



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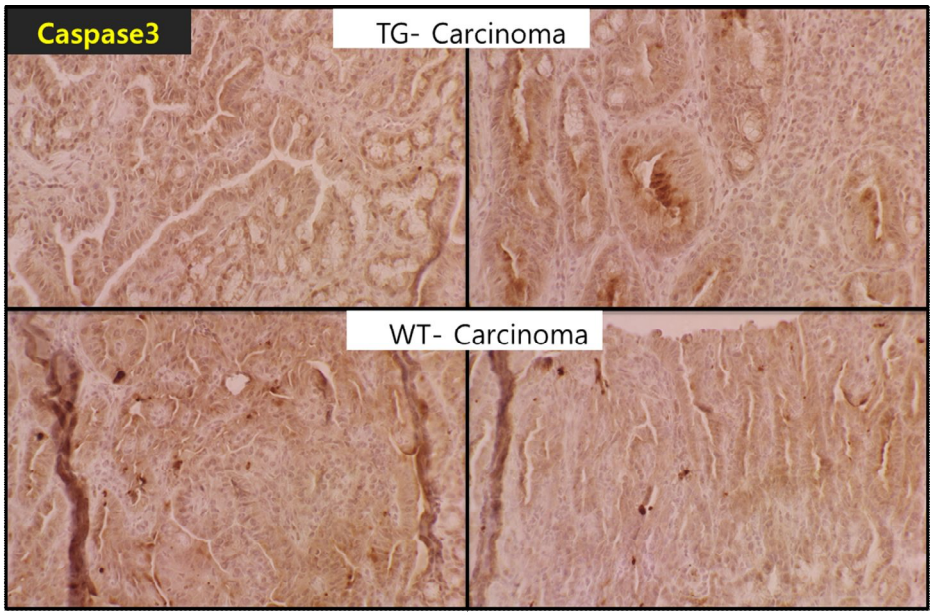

Figure 11. Caspase3 expression in MNU treated mice stomachs

Between the two groups, no significant difference was found regarding the expression of Cleaved-Caspase3.

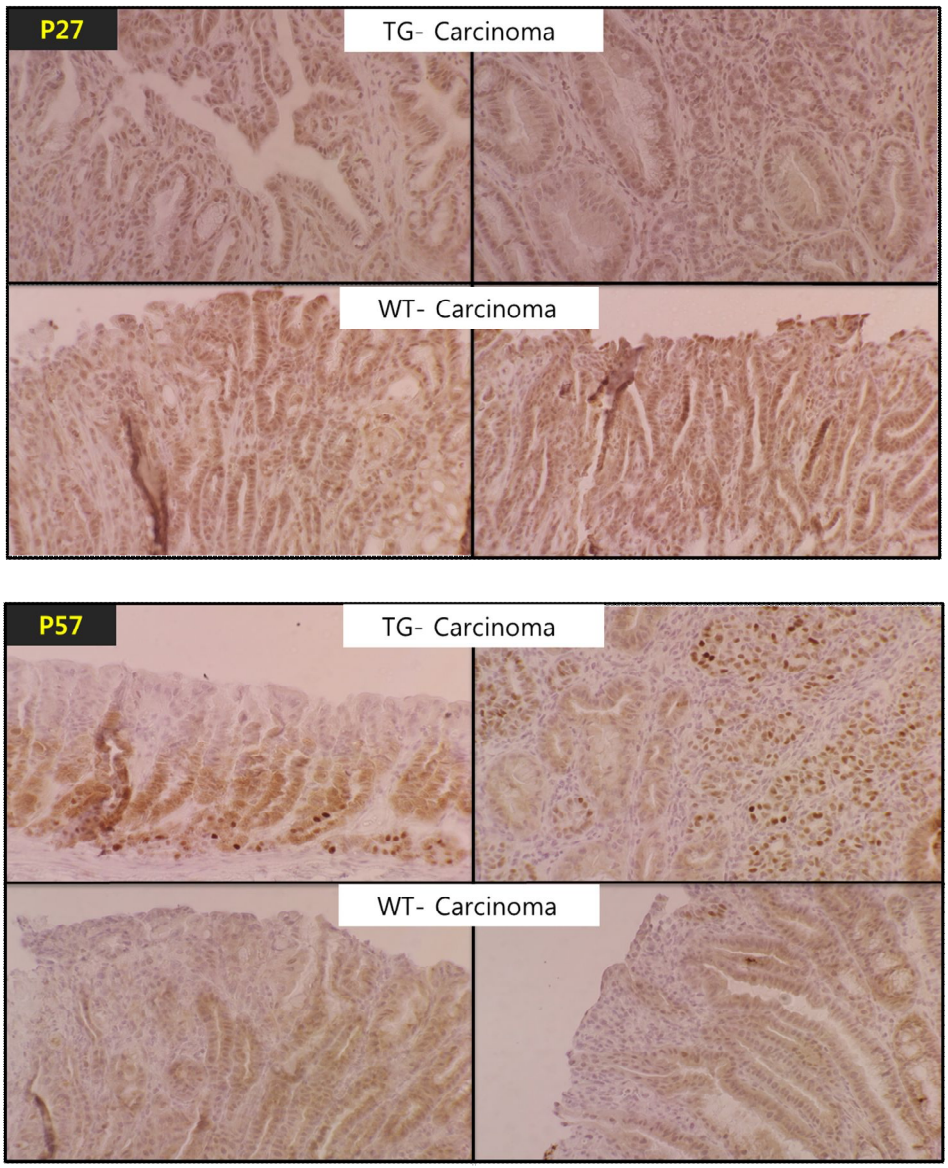

Figure 12. p27 and p57 expression in MNU treated mice stomachs

The expression level of the target genes of miR-222/221, p27 and p57, was not significantly different between the two groups in case of carcinoma.

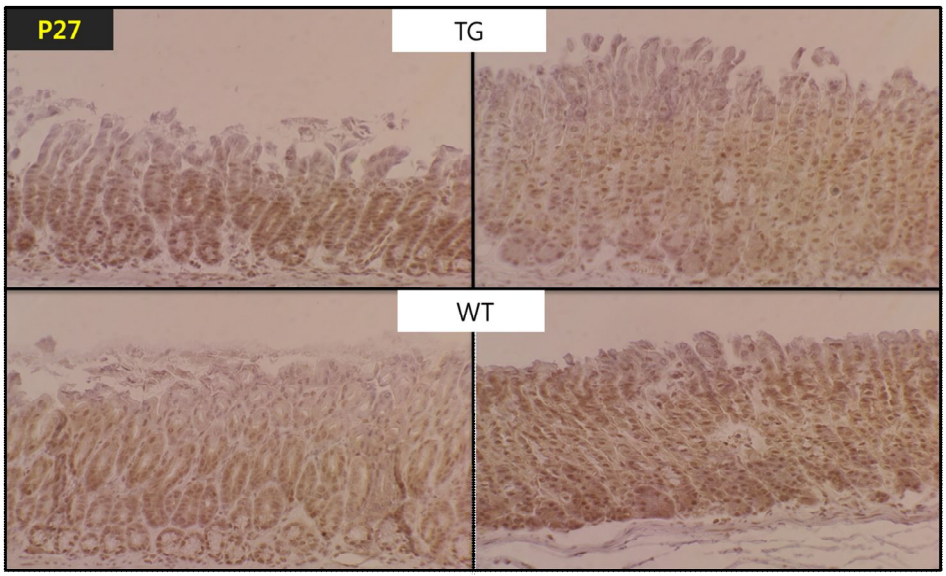

Figure 13. p27 expression in no MNU treated mice stomachs

When mice were not treated with MNU, the expression level of p27 was similar in both groups.

DISCUSSION

Despite the significant number of cancer patients, surgery is often the only treatment option because of many side effects of chemotherapy and radiation therapy (10, 27). Surgery is also limited to local treatment. For this reason, miRNA has gotten much attention due to its assumed role in carcinogenesis. The molecular pathology of gastric cancer still remains poorly understood even though gastric cancer is a major health burden throughout the world. Therefore, the study of miRNAs implicated in gastric cancer formation is urgent and may provide opportunities to develop new means for diagnosis and treatment of gastric cancer. In addition, there still remains a need to further investigate the accurate function of miRNAs in vivo. Many groups tried to generate microRNA knockout mice or transgenic mice to validate specific and effective ability of microRNA in vivo model.

Regarding the phenotype of the transgenic mice, the body weight of male transgenic mice were found to be ~20% higher than the control group whereas the body weight of female transgenic mice was almost same as control mice. The result of blood testing had unique differences in WBC and monocyte. The level of WBC and monocyte were significantly 1.8 fold and 1.3 fold lower respectively in transgenic mice than control group. WBC and monocyte are related to immune response, and therefore, aberrant low levels

of WBC and monocyte have low ability to fight virus or other pathogens. This shortage of immune response may contribute to the enhanced gastric cancer development in miR-222/221 transgenic mice. After the sacrifice, swiss roll technique was performed on all mice to detect abnormal intestine lesions. No carcinoma or cancer related lesions were found in intestine, but flat serrated adenoma was found in three transgenic mice.

When the phenotype of transgenic mice was examined at 56 week age, no aberrant lesions were observed macroscopically and the result was also the same in histological examination. This result suggested that miR-222/221 overexpressed transgenic mouse was not enough to cause gastric carcinogenesis by itself. Therefore, MNU, which is a well-known rodent carcinogenesis method, was applied to both transgenic mouse and wild type mouse to promote gastric carcinogenesis effect and experimental design was referred to other group protocols (18-21).

The MNU-induced mouse models for gastric carcinogenesis have been widely used to study carcinogenesis of the stomach (18-20). These animal models have been used not only for investigating the pathogenesis of gastric carcinogenesis but also for identifying tumor promoters and chemo preventive agents (21, 22, 28). To find and observe accurate point of carcinogenesis and cancer related lesions, before all mice were sacrificed at

36 weeks old, five MNU treated mice from each group were sacrificed at 27 weeks of age, but nearly no occurrence of tumor was found. There was no significant histological difference between transgenic mice and wild type mice. This was because the mice have been known to be relatively resistant to MNU, and it required a longer period to induce gastric carcinoma in C57BL/6J mice (19, 22, 29, 30). When the mice were treated with MNU at 36 weeks of age, MNU-induced gastric carcinogenesis steps were found.

At 36 weeks old, all mice were sacrificed and pathological examination was conducted. From various aberrant lesions, it was necessary to evaluate whether the lesions were related to cancer. When we use the alkylating agent MNU, it develops different pattern of cancer related lesions. For instance, hyperplasia is frequently observed when we use carcinogen. This pattern was already reported by many groups (18, 19, 31). The exact reason of hyperplasia occurrence is frequently detected from MNU-induced gastric carcinogenesis model have not been fully understood, but it was commonly found from other carcinogen induced gastric carcinogenesis models. The result was also similar to macroscopic findings that it was often observed that whole stomach was thickened and stiff with pre-cancerous lesions found mice. Until now, mouse gastric carcinogenesis classification has not been clearly established. Especially MNU-induced carcinogenesis pattern is

different from normal gastric carcinogenesis. This is the reason we included frequently detected four pre-cancerous lesions in addition to carcinoma to evaluate the effect of gastric carcinogenesis. Frequently observed four pre-cancerous lesions including hyperplasia, adenoma, microcarcinoma in adenoma and microscopic dysplasia were selected and included for MNU-induced multi gastric carcinogenesis steps (Fig. 14). When all of four pre-cancerous stages were included, the frequency of pre-cancerous stages was significantly higher in transgenic mice compared to wild type mice. (P=0.010) Hyperplasia was observed in 3.77% of the wild type mice and 18.46% of the transgenic mice (P=0.014).

miR-221 and miR-222 are encoded by a gene cluster on the X chromosome. They have the same seed sequence and appear to have identical target genes and similar functions. Previously, we demonstrated that miR-222/221 have a role as oncogene by suppressing CDK inhibitors (p27, p57). It has been well known that cancer is a proliferative disease of the cell cycle, and many cancers result from the deregulation of CDK regulators. Any suppression of these regulators will result in unlimited cell growth and carcinogenesis. G1 Cyclins and CDKs are the focus of cancer research because their roles in tumorigenesis have emerged significantly (32).

The p27, the target of miR-222/221, is a Cyclin-dependent Kinase inhibitor

that causes G1 arrest by inhibiting Cyclin E-CDK2 kinase activity, which is required for S phase entry and the initiation of DNA replication (33-34). Down expression of p27 is frequently detected in various tumors, such as lung (36), gastric (37), skin (38), breast (39-40), prostate (41), colon (42), and ovarian cancers (43). And it is also reported that a reduced expression of p27 has been correlated with cancer progression and poor survival rate for cancer patients (35). The pattern of p27 degradation is progressed posttranscriptionally by ubiquitin-mediated proteasome, suggesting that reduced expression of p27 is the result of enhancement of ubiquitin-mediated proteasome degradation. It has been reported that p27 is a haplo-insufficient tumor suppressor and is hypersensitive to carcinogen (44). Therefore, one possible explanation for the down expression of p27 is that p27 was down regulated under the influence of MNU in wild type mouse. In addition to the effect of miR-222/221 on p27, other miRNAs may also be involved in the regulation of p27 expression via indirect mechanisms. For example, it has been found in many studies that PTEN, a tumor suppressor gene, is one of the targets of miR-21 in various cancers (45). Also, PTEN is able to reduce the ubiquitination of p27 (35), so it is possible that the reduced PTEN levels could cause a reduction of p27 levels. Additionally, p27 could be deregulated for other reasons. The expression of p27 could be

increased by VHL, a tumor suppressor gene (46-47), and the oncogenic signal from HER2 could down regulate p27 (48-49). Lastly, c-myc also antagonizes the function of p27 (50-51). Another reason could be the expression change of p27 caused by the MNU effect. At first, miR-222/221 transgenic mouse by itself could not cause gastric carcinogenesis, so we treated the mouse with MNU, a carcinogen, to promote cancer development. After MNU treatment, the aberrant change in mRNA and protein level of p27 indicates that MNU worked as alkylating agent and affected cell cycle related genes, and p27 would also be down regulated in wild type mice. Additionally, MNU is also related to mismatch repair genes and play a critical role in G2/M checkpoint (52). Moreover, this agent has methylation ability so that it could be expected that this agent methylated the promoter of p27 and decreased the expression.

Recent studies already have revealed that miR-222/221 are overexpressed in several types of cancers and have oncogenic effect (53-58). In most cases, in vitro studies have been reported, but one group recently developed in vivo model in liver cancer. The transgenic model, which is liver specific miR-221 expressed, was developed and characterized by the appearance of spontaneous liver tumors in a fraction of male mice and a strong acceleration of tumor development in 100% of mice treated with

diethylnitrosamine (59). However, in our study, carcinoma occurrence was not statistically significant but frequently observed pre-cancerous lesions were statistically significant. The reason that this transgenic model was not able to cause dramatic carcinoma occurrence in our study was expected that we did not use tissue specific promoter for stomach and could not control transcriptionally the expression of miR-222/221 when transgenic mouse model was developed. In case of liver cancer model of miR-221 transgenic mouse, they used liver specific promoter and induced the overexpression of miR-221. Our Transgenic mice expressed miR-222/221 from all organs, so it is expected to be difficult to show a strong effect of the miR-222/221 in stomach.

Ultimately, even though observing the expression of p27 turned out to be difficult because of the changes (mRNA, protein, IHC) by MNU, we demonstrated that the gastric carcinogenesis effect of miR-222/221 with the help of MNU was more frequently detected in transgenic mice than wild type mice, although it was not a very strong effect.

In conclusion, this study reveals that MNU-induced generation of neoplastic lesions was enhanced in miR-222/221 overexpressed transgenic mice group compared to wild type mice group, possibly in association with hyperplasia and frequent cancer-related lesions.

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국문 초록

마이크로RNA는 small RNA 중의 하나로 분화, 세포증식, 세포사멸, 심지어 암 형성능과 같은 다양한 세포단계의 기능들을 조절하는 것으로 잘 알려져 있다. 인체의 암 조직에서의 마이크로RNA 발현 프로파일은 조직의 종류, 진단, 진행 정도, 예후, 암의 단계, 치료법에 대한 반응 등에 따라 발현 패턴이 특징적으로 나타내는 특성을 지닌다. 마이크로RNA가 결합하는 특정 전사체로부터 인코딩하는 단백질의 발현을 억제하거나 타겟 유전자의 전사체 자체를 분해해서 타겟 유전자의 발현을 조절하는 것으로 알려져 있다. 또한 특정 마이크로RNA는 암 발생에 기여하거나 혹은 암 억제유전자로서 기능하는 것으로 알려져 있다.

다양한 암에서 마이크로RNA의 이상적인 발현이 자주 보고됨에 따라 마이크로RNA와 인체 종양 사이에는 밀접한 관련이 있을 것으로 여겨진다. 최근에 많은 연구 그룹에서는 위암이

동아시아에서 높은 발생률을 보이기 때문에 그 중요성을 깨달아 위암과 마이크로RNA의 관계에 대해서 연구하고 있다. 이전의 실험에서 마이크로RNA 발현패턴 분석을 통해 정상 위 조직에 비해 위암 조직에서 많이 발현되는 miR-222/221을 선택하였고 여러 기능 연구들과 마우스 실험을 통해서 miR-222/221이 위암에서 암 발생 능력이 있는 것을 확인하였다.

이번 연구에서는 miR-222/221이 과발현된 형질전환 마우스를 제작하여 위암을 일으키는 발암 물질을 이용하여 형질전환 마우스의 위암 발생능에 대해서 확인하는 실험을 진행하였다.

형질전환 마우스 65마리와 일반마우스 53 마리를 이용하여 위암 발생 효과를 실험하였다. 마우스가 6주령이 되었을 때 일반 마우스와 형질전환 마우스 모두에게 위암 발생이 빠르게 하기 위해서 MNU (*N* - Nitroso – *N* - Methylurea) 를 음수에 섞어서 격주로 다섯 번을 주었고 36주령이 되었을 때 모든 마우스는 실험 종료시키고 병리적 관찰과 분자생물학적 실험이 진행되었다.

암을 비롯하여 가장 자주 관찰되었던 4가지 종류의 전암 단계를 모두 포함시켜 평가 할 때 발생률은 형질전환 마우스군에서 대조군에 비해서 유의하게 높았다. (P=0.010) Hyperplasia는 대조군에서 3.77%, 형질전환 마우스군에서는 18.4% 관찰되었고 통계적으로 유의한 차이를 나타내었다. 그러나 carcinoma (P=0.383), microcarcinoma (P=1.000), adenoma (P=0.395), microscopic dysplasia (P=0.920) 각각의 발생률은 모두 형질전환 마우스군에서 대조군에 비해서 높았으나 통계적으로 유의하지는 않았다.

이 모든 결과들을 통해 각각의 전암 단계들이 miR-222/221이 과발현된 형질전환 마우스와 관련이 있는 것은 아니지만 MNU를 이용한 위암발생 실험에 있어서 네 가지 전암 단계를 암과 함께 평가할 경우에는 통계적으로 유의하였고 전암 단계 중의 하나인 hyperplasia 또한 miR-222/221에 의하여 유의하게 위암발생에 영향을 나타내는 것을 관찰 할 수 있었다.

주요어: 마이크로 RNA, 마이크로RNA 기능, 위암, 전암 단계,

형질전환 마우스, MNU(N-Nitroso -N-Methylurea)