의학박사 학위논문

Identification and characterization of a putative tumor suppressor gene ANXA10 in gastric carcinoma

위암에서 암 억제 유전자로 추정되는 ANXA10의 동정 및 특성 구명

2013년 8월

서울대학교 대학원

의학과 병리학전공

김 진

위암에서 암 억제 유전자로 추정되는 ANXA10의 동정 및 특성 구명

지도 교수 김 우 호

이 논문을 의학박사 학위논문으로 제출함

2013 년 4 월

서울대학교 대학원

의학과 병리학 전공

김 진

김 진의 의학박사 학위논문을 인준함

2013 년 7 월

위 원 장 _____ (인)

부위원장 _____ (인)

위 원 _____ (인)

위 원 _____ (인)

위 원 _____ (인)

Identification and characterization of a putative tumor suppressor gene ANXA10 in gastric carcinoma

by

Jin Kim

A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medical Science (Pathology) at Seoul National University College of Medicine

July 2013

Approved by Thesis Committee:

Professor _____ **Chairman**

Professor _____ **Vice chairman**

Professor _____

Professor _____

Professor _____

ABSTRACT

Genetic alterations are observed as important event in gastric carcinogenesis. In the present study, array based comparative genomic hybridization (CGH) was used to screen DNA copy number change in gastric cancer cell lines and the results obtained were compared with oligonucleotide microarray data. Novel genes that are related to human gastric cancer were identified using genome-wide screening. DNA loss of the Annexin A10 (ANXA10) locus in chromosome 4q33 was found in several gastric cancer cell lines and these cell lines showed decreased *ANXA10* expression by array based CGH and oligonucleotide microarray analysis. ANXA10 is a member of annexin family, and its function is not known clearly. To investigate whether *ANXA10* might be a putative tumor suppressor gene, we performed functional analysis using small interfering RNA or expressional cDNA transfection in gastric cancer cell lines and demonstrated that *ANXA10* inhibits gastric cancer cell growth. Furthermore, ANXA10 expression at the protein level was found to be reduced in 289/585 (49.4%) primary gastric cancer tissues. By univariate survival analysis, lack of ANXA10 expression was associated with poor survival (P = 0.016). Quantitative real-time PCR analysis validated loss of DNA at the *ANXA10* locus in gastric carcinomas with reduced ANXA10 expression. These results suggest

that inactivation by chromosomal loss of the *ANXA10* leads to a loss of ANXA10 function, which may be a factor in gastric carcinogenesis.

Keywords : annexins, tumor suppressor genes, down-regulation, DNA copy number, stomach neoplasms

Student Number : 2002-23724

CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Gastric carcinoma is the second leading cause of cancer death worldwide (1-3), but the genetic changes associated with gastric carcinoma development and progression are poorly understood. Furthermore, the developments of new diagnostic, preventive and treatment modalities require a deeper understanding of the multistep mechanisms underlying gastric carcinogenesis. A literature search revealed relatively little data on chromosome alterations in gastric carcinoma (1, 3, 4).

Tumor suppressor genes are mainly inactivated by mutations, allelic imbalances (LOH or deletion) and loss of expression due to epigenetic mechanisms (5-8). Cytogenetic and molecular biologic studies have identified chromosomal abnormalities including partial deletions of specific loci in human cancers (7-9). Moreover, allelotyping studies of solid tumors involving loss of heterozygosity (LOH) determinations and cytogenetic techniques, such as, fluorescence in situ hybridization (FISH) and comparative genomic hybridization (CGH), have shown genomic alterations in gastric cancer at specific regions, such as, 1p, 5q, 7p, 12q, 13q, 17p, 18q (1-3), and copy number losses in these regions are believed to be associated with the presence of tumor suppressor genes (1, 3). These suggestions indicate that complicated genetic alterations are an integral part of

gastric carcinogenesis. Furthermore, allelotype studies have shown that certain genomic regions are frequently deleted in tumors, and that frequent losses of heterozygosity at particular chromosomal regions are the hallmark of a tumor suppressor gene (5, 8, 9). Therefore, the identification of consistent regions of chromosomal deletion in tumor DNA may indicate the locations of tumor suppressor genes. Bacterial artificial chromosome (BAC) based comparative genomic hybridization (CGH) has been used widely to screen for genome-wide DNA copy number changes in tumors (10, 11).

The development of oligonucleotide microarray technology has facilitated the analysis of expression levels of thousands of genes in a single experiment. This technology is a powerful tool for analyzing genes the expression of various tumors (12). Recent expression microarray studies of gastric cancer indicate that chromosomal imbalances have a significant impact on global gene expression patterns and plays a major role in carcinogenesis (1, 12). The parallel analysis of cytogenetic and transcriptional profiling data has revealed that changes in DNA copy number can have noticeable effects on gene expression (10, 12, 13).

However, the merits of combined array based CGH and the oligonucleotide microarray technique for the genome-wide screening of deleted regions has not performed in gastric carcinoma. To identify tumor suppressor genes that may be silenced by DNA loss in gastric cancer, we integrated the high solution array based CGH and

oligonucleotide microarray results. Our data demonstrate that array based CGH combined with gene expression profiling provides a rationale for discovering tumor suppressor genes in regions of chromosomal loss.

Annexins are a family of calcium and phospholipid binding proteins, and to date, 13 members of the annexin family have been found in eukaryotic cells. Moreover, individual members of this family have a unique amino terminal domain that shows limited sequence similarity with those of other family members (14-17). However, despite the availability of detailed structural information, the biological functions of the annexins have not been clearly defined. The annexins have been implicated in a wide range of molecular and cellular process, such as, signal transduction, inflammation, blood coagulation, exocytosis, immunosuppression, tissue growth and differentiation (18, 19). Moreover, recently, the annexins were suggested to have complex functions, and alterations in annexin protein levels have been associated with tumor progression. In various cancers, the specificities of annexins differ with regard to their potential values as molecular markers, and furthermore, they are believed to participate in carcinogenesis, i.e., ANXA-1, -2, -8 have been reported to be upregulated and ANXA-6, and -7 to be down-regulated in various carcinomas (5, 18, 20). In particular, ANXA10 is a relatively new addition to the annexin family, and *ANXA10* down-regulation has been correlated with the presence of a p53 mutation, vascular invasion and

poor prognosis in hepatocellular carcinoma (20).

These previous findings provide new locus for exploring a candidate tumor suppressor of gastric carcinoma. In the present study, we identified a putative tumor suppressor gene *ANXA10* (4q33) by screening a panel of gastric cancer cell lines and characterized its potential functions on the tumorigenesis of gastric carcinoma.

MATERIALS AND METHODS

Cell lines

Ten gastric cancer cell lines, SNU1, 5, 16, 216, 484, 601, 620, 638, 668 and 719, were used. All were obtained from the Korean Cell Line Bank (http://cellbank.snu.ac.kr, Seoul, Korea) and maintained in RPMI-1640 (JBI, Seoul, Korea). A human embryonic kidney cell line (HEK293 cell line) was obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM (JBI). All culture media contained 10% fetal bovine serum (HyClone, Logan, UT), 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma Chemicals, St. Louis, MO). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Array-based comparative genomic hybridization (array based CGH)

Chromosomal copy number changes in the 10 gastric carcinoma cell lines were detected by array based CGH using the GenomArray (Macrogen, Seoul, Korea), according to the manufacturer's instructions. Each array was composed of 1,440 BAC clones printed in triplicate at a resolution of 2.3 Mb.

Genomic DNA was isolated from gastric carcinoma cell lines by standard phenol chloroform extraction. The normal male DNA from

blood lymphocytes (Promega, Madison, WI) was used as a reference. For hybridization, normal DNA (reference) and cancer cell line DNA (test) was labeled with Cy5-dCTP and Cy3-dCTP, respectively, using a random prime labeling kit (Invitrogen, Carlsbad, CA). The arrays were scanned using an Array Scanner (Macrogen) and images were quantified using the Array Analysis program (Macrogen). This software automatically identified targets on the array, analyzed set of Cy3/Cy5 ratios on all targets and calculated representative fluorescence ratios. For each target, ratios of test DNA versus normal reference DNA amounts were calculated. According to the manufacture's instructions and our own validation based on comparisons of normal test DNA and normal reference DNA, we defined fluorescence cut-off ratio thresholds between normal and aberrant DNA copy numbers. A $\log_2$ ratio ≥ 0.25 was scored as a DNA gain and a $\log_2$ ratio of ≤ -0.25 as a DNA loss. In addition, high-level amplification was defined as a $\log_2$ ratio ≥ 1 and homozygous deletion was defined as a $\log_2$ ratio ≤ -0.8.

Oligonucleotide microarray analysis

For RNA expression analysis, the total RNAs of the ten gastric cancer cell lines were applied to Affymetrix U133A 2.0 GeneChip microarrays (Affymetrix, Santa Clara, CA). Target preparation and microarray processing procedures were performed as described in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix). Single- and double-stranded cDNA were synthesized from 10 μg of total RNA using

SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and T7-$(dT)_{24}$ primer (Metabion, Germany). Biotin-labeled cRNA was prepared using the BioArray High Yield RNA Transcript labeling kit (Enzo Life Sciences, Farmingdale, NY) at 37°C for 6 hours. Approximately 15 μg of cRNA was fragmented at 94°C for 35 min, and then hybridized to a U133A oligonucleotide microarray containing 22,277 oligonucleotides at 45°C for 16 hours. Following hybridization, standard washing and staining procedures were carried out on an Affymetrix Fluidics Station, according to the manufacturer's instructions. Probes were then scanned using a laser scanner, and signal intensities (adjusted for background and noise) were calculated using Microarray Suite Software 5.0 (Affymetrix). Microarray Analysis Suite software used Wilcoxon's test to generate detected signals (present, absent, or marginal) and used these to determine statistically whether a transcript was expressed.

Reverse transcription-PCR

Total RNA was prepared from the 10 gastric cancer cell lines using TRIZOL reagent (Invitrogen, Carlsbad, CA). To generate cDNA, total RNA (1 μg) was reverse transcribed using MMLV-reverse transcriptase (Bioneer, Seoul, Korea) and random primers (Bioneer). cDNA synthesis was performed at 65°C for 10 min and then at 37°C for 1 hour. PCR amplification was performed over 30-35 cycles of

denaturation at 95°C for 30 sec, annealing at 38-45°C for 30 sec, extension at 72°C for 40 sec, followed by a final extension at 72°C for 10 min in a thermal cycler. Sequence-specific oligonucleotide primers for the five genes are listed in Table 1.

PCR products were electrophoresed through 2% agarose gel, stained with ethidium bromide, and visualized using UV light.

Western blot analysis

Cellular protein extracts from gastric cancer cell lines were prepared by dissociation in lysis buffer (iNtRON Biotechnology, Seongnam, Korea), and protein levels were determined using BCA protein assay kits (Pierce, Rockford, IL). The goat anti-ANXA10 (Imgenex, San Diego, CA), rabbit anti-HPGD (Novus, Littleton, CO), goat anti-MTAP (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-PLAGL1 (Santa Cruz Biotechnology), goat anti-ULBP2 (R&D systems, Minneapolis, MN) and mouse anti-α-tubulin (Sigma Chemicals, St. Louis, MO) were used as primary antibodies. After overnight incubation at 4°C and washing with TBS containing 0.1% Tween-20, blots were incubated for 1 hour at room temperature with secondary antibodies. After washing blots, antigen-antibody complexes were visualized using ECL kits (Pierce, Woburn, MA).

Fluorescence in situ hybridization (FISH)

FISH analysis was performed to confirm copy number changes of 4q33 identified by array based CGH. Metaphase and interphase cell preparations from the gastric cancer cell lines and normal embryonic kidney cell line were used for the FISH analysis. Cell line slides were covered with 10 μl of dual hybridization mixture containing a pair of target (4q33, red signal) and control (4p13, green signal) probes, which were labeled with different fluorochromes (Macrogen, Seoul, Korea). Chromosome painting probes were labeled with MacProbe^{TM} solution (Macrogen) and used to evaluate 4q33 loss. Slides and probes were denatured at 75°C for 5 min and hybridized for 1 day. Post-hybridization washes were performed, according to the manufacturer's instructions. Signals in 100 interphase cells were counted. Deletion was defined when the FISH ratio target region/control region of the chromosome concerned was lower than or equal to 0.5.

Mutation analysis

Exons 1-12 of ANXA10 were amplified using intronic primers flanking each exon. Genomic DNA from 10 gastric cancer cell lines was subjected to PCR amplification and analyzed by directly sequencing PCR products. Primer sequences for PCR and sequencings are listed in Table 2. All PCR products were purified and sequenced using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Small interfering RNA (siRNA) transfection

Double strand siRNA for ANXA10 and a nonspecific scrambled siRNA were obtained commercially (Dharmacon Research, Lafayette, CO). Cells were seeded in 60 mm culture dishes at 2 X 10^5 cells the day before transfection. Five nM aliquots of ANXA10 siRNA or scrambled siRNA were then mixed with 100 μl of serum-free culture medium and 20 μl of Hiperfect Transfection Reagent (Qiagen, Valencia, CA). ANXA10 expression and cell growth were determined by RT-PCR and by performing proliferation assays 24 hours after transfection.

Construction of full-length human ANXA10

Full-length cDNA encoding ANXA10 was obtained from the 21C Frontier Human Gene Bank (Korean BioInformation Center, Daejeon, Korea). Cells were seeded at 2 X 10^6 cells/60 mm dish and transfected with 8 μg of ANXA10 expressional cDNA construct (pcDNA3.1-ANXA10) or control vector (pcDNA3.1) using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Twenty-four hours after transfection, cells were subject to RT-PCR or proliferation assays. A successful transfection resulted in *β*-galactosidase expression in pcDNA3.1 that could be easily assayed using a *β*-Gal staining kit (Invitrogen). Efficiency of transfection in our experiments was more than 30%.

Proliferation assay

To determine the effects of siRNA or expressional cDNA construct treatment on gastric cancer cell growth, cells were detached from 60 mm culture dishes by trypsinization and counted. Cells were then transfected with siRNA or an expressional cDNA construct of ANXA10, as described above, and seeded at 5 X 10^3 cells/well on 96 well plates and incubated for one day at 37°C. After adding 10 μl of Cell Counting Kit-8 reagent (Dojindo, Tokyo) and incubating for 2 hours, absorbance was measured at 450 nm using a spectrophotometer (Thermo Labsystems, Beverly, MA). All assays were performed in triplicate and more than three times.

Apoptosis assay

Apoptosis was quantified using an annexin V-fluorescein isothiocyanate apoptosis detection kit (BD biosciences, San Diego, CA) according to the manufacturer's instructions. Briefly, transfected cells were collected through trypsinization followed by washing twice with cold phosphate-buffered saline. One hundred thousand cells in 100 μl of the binding buffer and 5 μl of FITC-conjugated annexin V/propidium iodide double staining reagents were transferred into a 5 ml culture tube and incubated for 15 min at room temperature in the dark. After 400 μl of binding buffer were added into each tube, flow cytometric analysis for cell apoptosis was determined with a

FACScalibur (Beckton Dickinson, San Diego, CA). All experiments were performed twice.

Wound-healing assay

Cell mobility was assessed using the wound healing assay (in vitro scratch assay). Transfected cells were grown to confluence in 35 mm dishes and wounded using a sterile tip. After incubation for 24 hours, cells were photographed under a phase-contrast microscope. All experiments were performed in triplicate.

Soft agar colony formation assay

Cells were cultured in soft agar and transfected as described above. At 24 hours post-transfection, cells (5 X 10^3) were suspended in 2X DMEM containing 0.7% agar and layered on 2X DMEM containing 1% agar in 35 mm Petri dishes for 14 days. Numbers of colonies present in overlayers were determined by photographing and manual counting.

Patient samples

Surgically resected consecutive gastric cancer tissues (n = 585) obtained during surgery at Seoul National University Hospital were retrieved. Clinical outcomes were followed from the date of surgery to the date of death or for up to 6 years postoperatively. The follow-up period ranged from 1 to 72 months (mean, 48 months). This work was

approved by IRB of Seoul National University Hospital.

Quantitative Real-Time PCR (Q-PCR)

ANXA10 DNA amounts were determined using an iCycler real-time PCR detection system (BIO-RAD, Hercules, CA). Quantitative real-time PCR was carried out on 10 gastric cancer cell lines. Only 93 gastric carcinoma tissues were available for Q-PCR because we selected the cases with sufficient amount of available normal and tumor paraffin blocks, and the cases in which cancer cells occupied more than 70% of the tumor area.

An iCycler real-time PCR detector was used with TaqMan probes (BIO-RAD). Primer and TaqMan probe sequences are listed in Table 1. PCR reactions were carried out in triplicate in a total volume of 20 μl consisting of 2X Premix Taq (Takara, Tokyo), 100 nM TaqMan probe, 100 nM of forward and reverse primers, and 100 ng of test DNA. Each analysis included a control tube without template DNA. After PCR amplification, a melting curve was generated for all PCR products to check PCR reaction specificities.

All control experiments demonstrated that primers and probes designed for *ANXA10* loci were suitable for the comparative C_T method ($\Delta\Delta C_T$ method), which was used to quantify DNA copy numbers and to discriminate between two and one allele copy, as described previously (8, 21). This method was used to determine target sequence (*ANXA10*) copy number in tumor DNA sample relative to the normal

DNA sample and relative to the reference sequence (GAPDH). The absence of nonspecific amplification was confirmed by analyzing the PCR amplification products by 2% agarose gel electrophoresis and staining with ethidium bromide.

Immunohistochemistry (IHC)

To investigate ANXA10 protein expression in cancer tissues, we immunostained tissue array slides, which represented 585 gastric and 320 other cancer cases collected from Seoul National University College of Medicine. Sections (4 μm) were cut from each tissue array block, deparaffinized with xylene and dehydrated using ethanol. After antigen retrieval with 10 mM sodium citrate buffer, endogenous peroxidase activity was quenched with 0.6% H_2O_2. Normal serum was used to block non-specific protein binding. Immunohistochemical staining for ANXA10 (Imgenex, San Diego, CA) was performed using a standard avidin-biotin immunoperoxidase complex method (Vectastatin Elite ABC peroxidase kit: Vector Laboratories, Burlingame, CA) after microwave antigen retrieval. The ANXA10 immunostaining interpretation was as follows; no nuclear staining or nuclear staining in <10% of tumor cells (score 0); faint/barely perceptible partial nuclear staining in >10% of tumor cells (score 1+); moderate to strong nuclear staining in >10% of tumor cells (score 2+). Scores 0 and 1+ were considered reduced expression for ANXA10, and score 2+ was considered retained expression.

Statistical analyses

ANXA10 expression status was analyzed using Pearson's chi-square test or Fisher's exact test (2-sided). Survival curves were estimated using the Kaplan-Meier product-limit method, and the significances of differences between the survival curves were determined using the log-rank test. Results were considered statistically significant when *p* values were <0.05. All statistical calculations were performed using SPSS11.0 software (SPSS, Chicago, IL).

Table 1. Primers and probes used for RT-PCR and Q-PCR analysis

Gene	Sequence (5'-3')	Product size (bp)
ANXA10		
RT-PCR	S : GCAATTCATGACTTTGGTTT	111 bp
	AS : TTTTCCATATCGCTCTTTGT	
Q-PCR	S : GCTCAGGATGCAATGGTAAC	124 bp
	AS : CTTCCCATAGGACCTGGAAC	
	P : TCTGATGCTACTTCTCTGCTGGCT	
HPGD		
RT-PCR	S : CTGTCTGCTAACTCCAGACC	100 bp
	AS : GGGCAAGATATGACAACATT	
MTAP		
RT-PCR	S : GAACATCTGGGCTTTGAA	104 bp
	AS : CAATAATGACAATATCGCCG	
PLAGL1		
RT-PCR	S : GTATTGTGCCAATCTGTCCT	232 bp
	AS : ATTTGGAGAAATCCAAACCT	
ULBP2		
RT-PCR	S : GAGAATTACACACCCAAGGA	249 bp
	AS : CCATCCTATACAGTCTCCCA	
β-ACTIN		
RT-PCR	S : ACACTGTGCCCATCTACGAGG	621 bp
	AS : AGGGGCCGGACTCGTCATACT	
GAPDH		
Q-PCR	S : AAGACCTTGGGCTGGGACTG	146 bp
	AS : GAAGATGCGGCTGACTGTCG	
	P : CTCCCGCTTCGCTCTCTGCTCCT	

S, Sense primer; AS, Anti-sense primer; P, Fluorogenic probe.

Table 2. Primers used for Mutation analysis

ANXA10	Sequence (5'-3')	Product size (bp)
Exon 1	S : TGCAATAATTCTGCCTACTCACC	662 bp
	AS : TTGCCTCTGTGGGATAATACG	
Exon 2	S : CTCATTGATTTTCCCACCAGG	177 bp
	AS : CCCACAGTATAGTCTTAAAACAAGG	
Exon 3	S : TCAGGGATAATGCGTGCATAC	442 bp
	AS : TTTGGGGAAAGAACTTGTGG	
Exon 4	S : CATGCAGGGGTGAGATCAAA	570 bp
	AS : GGGGCTGGCTCAGTAAGTTT	
Exon 5	S : GCCTGCCTTCATCCTAAACC	541 bp
	AS : GGGTAAGAGGTGAAATGCCC	
Exon 6	S : CAGAATTCTTTGGCAGACGG	304 bp
	AS : GCAATGACATATACTTATTTGCTTAGA	
Exon 7	S : CCAGTGCTGCAAAGAAAAGG	341 bp
	AS : ACTCCAGCCCATTTTAACCC	
Exon 8	S : ACCTGGGTTAAAATGGGCTG	608 bp
	AS : GGAATGTCAAACAAAGGGCA	
Exon 9	S : AAGTTTGCTGCTGACATAAGCC	522 bp
	AS : TCAATCAGAGCCACTCAGCAT	
Exon 10	S : GACCAAGTGAAAATCTGCATGAC	411 bp
	AS : CCAGTTTTTCTCACTTGGGTGT	
Exon 11	S : CGGTAAGGGAGAGGGAGAAG	482 bp
	AS : GCACCTGCCGATGTTCTAAA	
Exon 12	S : TGGTCCATGGCTCTTTCATAGT	568 bp
	AS : GGAGTCTCAAAAATTCCCCAA	

S, Sense primer; AS, Anti-sense primer.

RESULTS

Frequencies of genetic alterations as determined by array based CGH

To identify genomic areas possibly involved in gastric cancer carcinogenesis, we screened DNA copy number changes in whole genomes using array based CGH in 10 gastric cancer cell lines. Quantitative aberrations, such as, chromosomal gains or losses were detected in all 10 gastric cancer cell lines. In our array based CGH studies, the $\log_2$ ratio thresholds for homozygous loss and deletion were -0.25 and -0.8, respectively. A total of 1,440 array BAC clones were applied for the purpose of data analysis. The most frequent aberrations in terms of copy number gains were encountered at the following locations : 7p, 7q, 8q, 17q, 20q, and the most frequently detected losses occurred at 3p, 4p, 4q, 9q, 18q (Table 3). Using array based CGH, we found that an average of 251 clones were gained or lost in the gastric cancer cell lines, but homozygous deletions were rare (Table 4).

Chromosomal deletion was detected in eight cell lines, and the SNU1 cell line contained most deletions. An example of a chromosomal copy number profile is shown in Figure 1A. In the present study, we focused on remarkable chromosomal losses and homozygous deletions, which are likely to occur in tumor suppressor gene regions. Deletion of

chromosomal 4q region was observed in SNU1 (Figure 1B), and loss of this region was observed in 6 gastric cancer cell lines.

Table 3. The most frequent chromosomal abnormalities in 10 gastric cancer cell lines

Genetic alteration	Chromosome location	Number of cell lines
Copy number gains		
	7p15.3	8
	7p14.3	8
	7p13	8
	7q11.21	7
	7q21.3	8
	7q22.1	7
	8q24.21	6
	8q24.22	6
	17q21.32	5
	17q24.3	5
	20q11.23	6
	20q13.13	7
	20q13.2	6
Copy number losses		
	3p22.3	6
	3p14.2	6
	3p12.3	6
	4p15.32	5
	4p15.2	5
	4q33	6
	4q34.3	8
	4q35.2	7
	9q21.13	6
	9q33.1	6
	18q21.33	6
	18q22.1	6
	18q23	7

Table 4. Copy number alterations in gastric cancer cell lines

Gastric cancer cell lines	Copy number changes	Copy number gains	Copy number losses	Homozygous deletions
SNU 1	94	16	78	5
SNU 5	268	90	178	4
SNU 16	368	175	193	3
SNU 216	234	192	42	0
SNU 484	280	118	162	1
SNU 601	232	159	73	4
SNU 620	250	123	127	0
SNU 638	121	64	57	3
SNU 668	483	194	289	5
SNU 719	182	151	31	4
Average	251.2	128.2	123	2.9

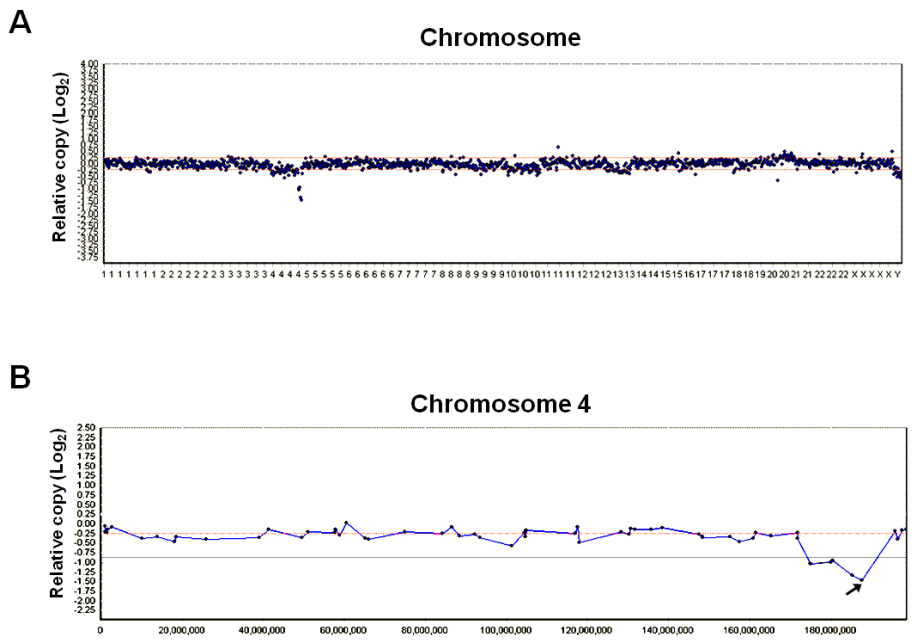


Figure 1. An example of array based comparative genomic hybridization copy number in SNU1 gastric cancer cell lines.

(A) Whole genome profile showing the gains and losses. The clones ordered from the 1p on the left to the Xq on the right. Each dot represents the signal ratio for an indivisual BAC clone. Red lines are scale bars indicating $\log_2$ signal intensity ratios of +0.25 and -0.25. (B) Genome profile of a chromosome 4 demonstrates the deletion on 4p33 position. Physical positions (bp) are indicated in X axis.

Correlation of array based CGH results and oligonucleotide microarray data

To identify genes involved in gastric carcinogenesis, we attempted to integrate oligonucleotide microarray and array based CGH data in 10 gastric cancer cell lines. The gene expression levels were determined using Affymetrix U133A oligonucleotide microarrays containing 22,277 probe sets. Using our method, we found that genomic deletions are relatively rare in gastric cancer cell lines. Of the 1,440 BAC clones printed on the arrays only 29 showed genomic deletion in at least one gastric cancer cell line. We initially identified 5 genes (*ANXA10, HPGD, PLAGL1, ULBP2* and *MTAP*) (Table 5) that showed a correlation between mRNA down-regulation and homozygous deletion in at least one gastric cancer cell line. The down-regulated mRNA expressions of these 5 genes were confirmed by RT-PCR. These 5 genes were down-regulated in 2 or more gastric cancer cell lines (Figure 2A). In particular, the *ANXA10* gene was down-regulated in the SNU1, SNU16, SNU216 and SNU668 cell lines and all of them showed chromosomal loss (Figures 3A and 3B).

To examine gene expression at the protein level, western blot analysis was performed for these five genes in the ten gastric cancer cell lines (Figure 2B). The down-regulations of these five proteins found to be correlated with a loss of mRNA expression in the same cancer cell lines. As expected, western blot analysis revealed the loss

of ANXA10 protein expression in SNU16, SNU216 and SNU668 (Figure 3B).

Oligonucleotide microarray assays showed that *ANXA10* was not expressed in 4 of the 10 cancer cell lines (SNU1, 216, 484 and 668) (Figure 3C). In particular, *ANXA10* mRNA was not expressed in cell lines showing reduced amounts of *ANXA10* DNA (SNU1 and 216) (Figures 3A and 3C). When we performed IHC analysis in the 10 gastric cancer cell lines, ANXA10 expression was reduced in 6 (SNU1, 16, 216, 484, 668 and 719) cell lines. Because of the correlation between western blot, RT-PCR and IHC results, we decided to further characterize *ANXA10* in gastric carcinoma.

Table 5. Suggested tumor suppressor genes by comparing the data of array-CGH and oligonucleotide microarray

Chromosome Locus		Cell line	Candidate gene
Band	Position[a]		
4q33	chr4:169,709,597-169,804,783	SNU1	ANXA10
4q34.1	chr4:176,107,271-176,139,501	SNU1	HPGD
6q24.2	chr6:144,242,007-144,310,111	SNU5	PLAGL1
6q25.1	chr6:150,294,127-150,301,357	SNU5	ULBP2
9p21.3	chr9:21,792,635-21,855,967	SNU16	MTAP

a, Based on UCSC Genome Browser on Human July 2003 Assembly.

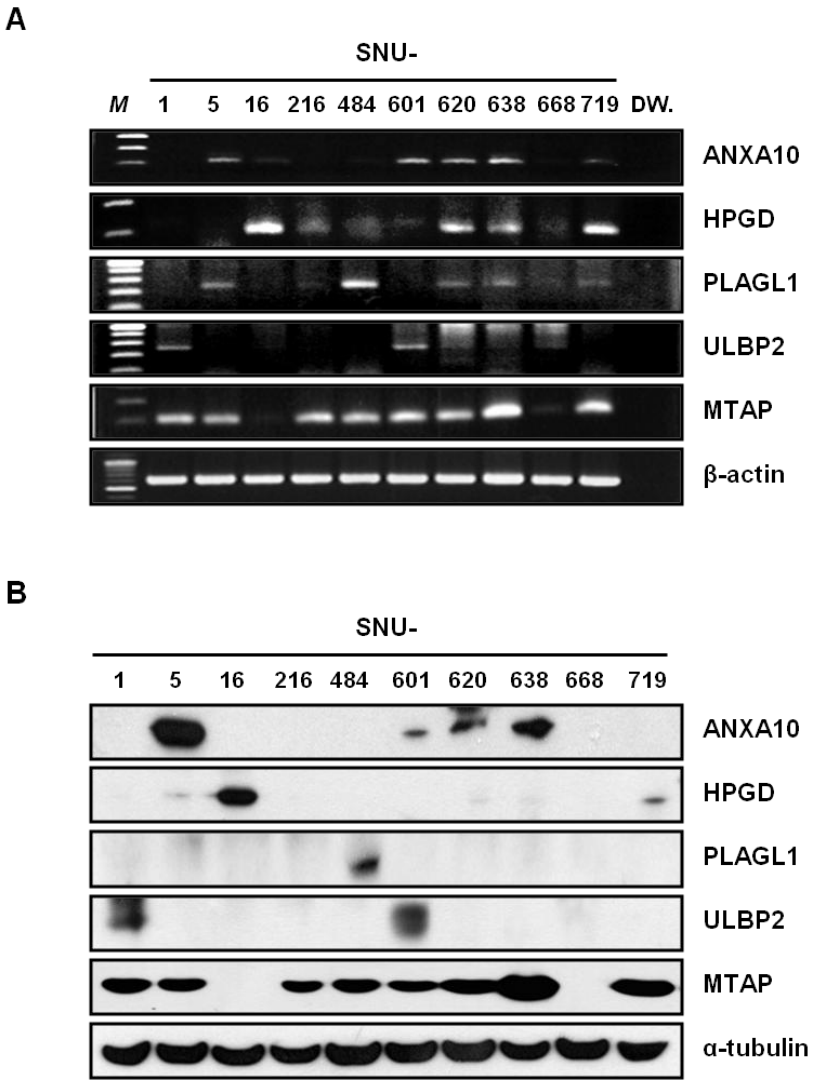
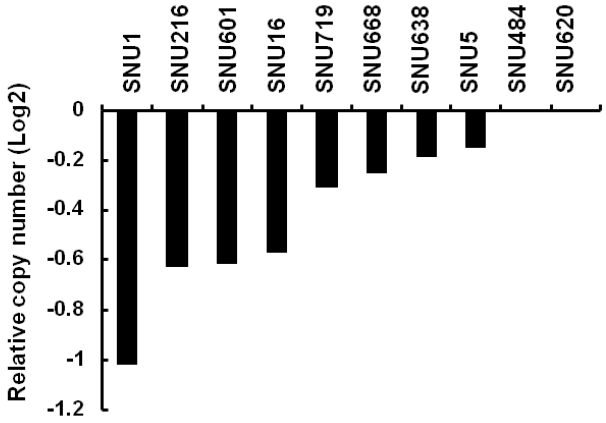


Figure 2. Expression of candidate tumor suppressor genes in gastric cancer cell lines.

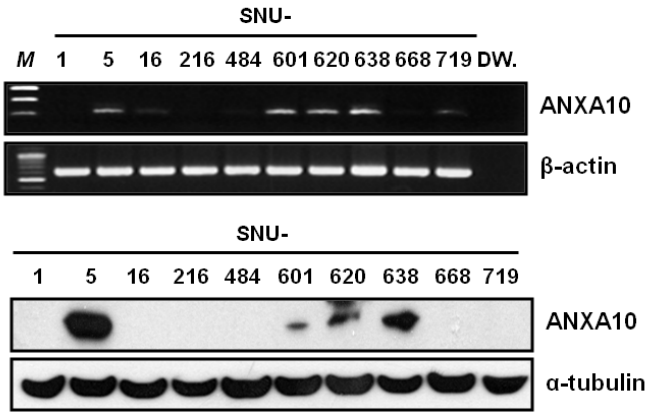
(A) RT-PCR analysis shown representative genes that were absent in at least two cell lines among ten gastric cancer cell lines. DW was used as negative control and β-actin expression was used to quantitative control. (B) Western blot analysis of candidate tumor

suppressor genes in gastric cancer cell lines. α-tubulin was used as a control.

A


SNU1
SNU216
SNU601
SNU16
SNU719
SNU668
SNU638
SNU5
SNU484
SNU620
Relative copy number (Log2)
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2

B


SNU-
M 1 5 16 216 484 601 620 638 668 719 DW.
ANXA10
β-actin
SNU-
1 5 16 216 484 601 620 638 668 719
ANXA10
α-tubulin

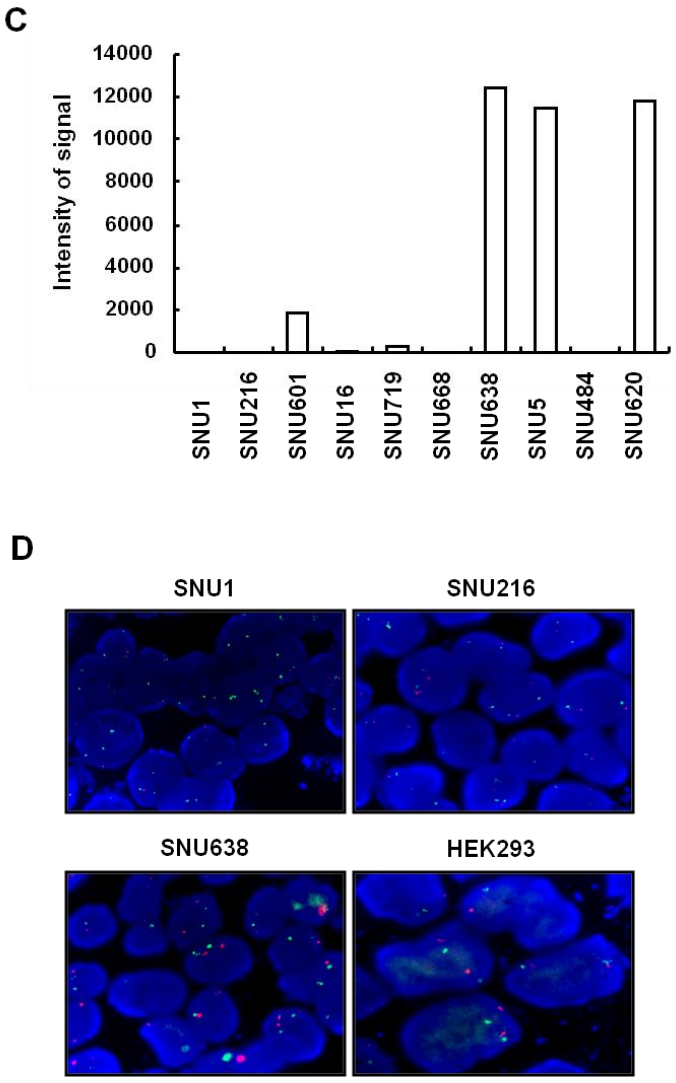


Figure 3. Expression and DNA copy number change of *ANXA10* in gastric cancer cell lines.

(A) In array based CGH analysis, *ANXA10* showed decreased DNA amount in 6 of 10 gastric cancer cell lines. Among them, SNU1 showed the most significant loss. (B) Top, RT-PCR analysis showed absence of *ANXA10* expression in 4 of 10 gastric cancer cell lines. DW

was used as a negative control and β-actin was used as an internal standard. Bottom, Western blot analysis of ANXA10 in gastric cancer cell lines. Normalization for protein loading was achieved by anti-α-tubulin antibody. (C) Signal intensity of *ANXA10* on Affymetrix GeneChip. *ANXA10* expression is absent in 4 of 10 gastric cancer cell lines, including SNU1 and SNU216. (D) Interphase FISH analysis for *ANXA10* gene with BAC clones containing human DNA sequences from 4q33 (target probe, red signal) and 4p13 (control probe, green signal) showed the deletion of *ANXA10* (ratio less than 0.5) in SNU1. SNU216 and SNU638 showed slight loss of red signal compared to green signal. The 293 cell line did not show loss of red signal. The nuclei were counterstained with DAPI.

FISH analysis for ANXA10 loss

Fluorescence in situ hybridization (FISH) analysis was performed to confirm copy number changes at 4q33 (*ANXA10*) identified in the array based CGH study. FISH analysis using individual probes was performed on selected gastric cancer cell lines and on the HEK293 cell line. A dual color FISH with the *ANXA10* gene probe (red) and chromosome 4p13 probe (green) was used to evaluate 4q33 loss in gastric cancer cell lines and in the HEK293 cell line (positive control). In each case, at least 100 cells were analyzed in the interphase chromosomes. Loss of *ANXA10* was detected in SNU1 (0.24), SNU216 (0.48) and SNU638 (0.72) (Figure 3D). To verify our results, metaphase FISH was performed and deletion of ANXA10 was confirmed in the SNU1 cell line (Figure 4). These results confirmed the validity of the array based CGH study.

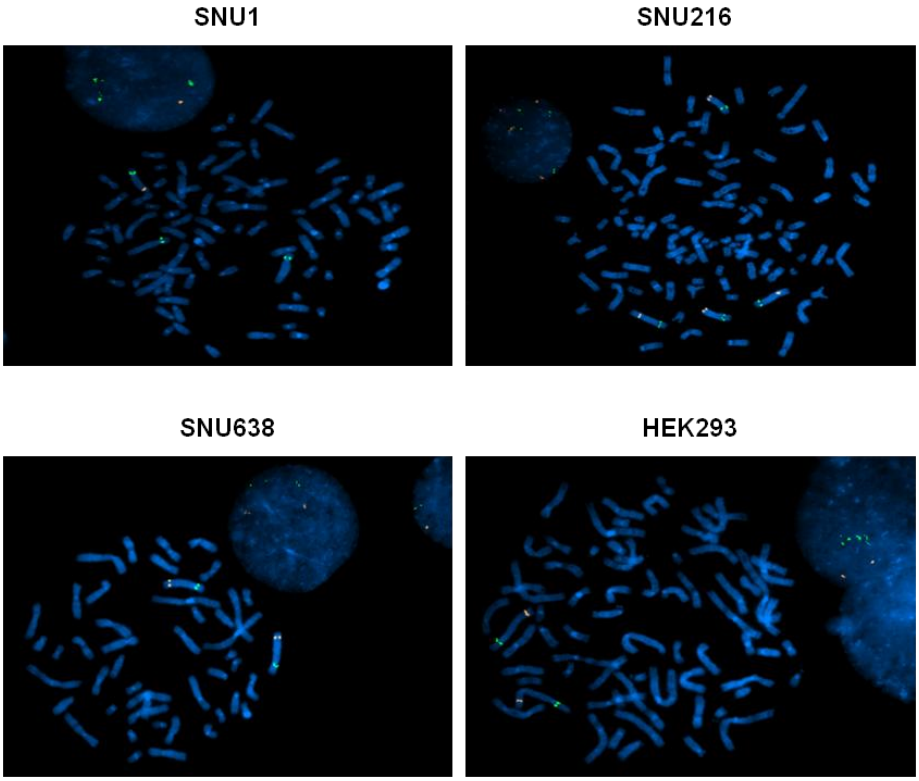


Figure 4. DNA copy number change of *ANXA10* in gastric cancer cell lines.

Metaphase FISH analysis for *ANXA10* gene with BAC clones containing human DNA sequences from 4q33 (target probe, red signal) and 4p13 (control probe, green signal) showed the deletion of *ANXA10* in SNU1. SNU216, SNU638 and 293 cell line did not show loss of red signal. The nuclei were counterstained with DAPI.

Mutation analysis of the ANXA10 gene

To investigate whether sequence alterations in *ANXA10* are responsible for its down-regulation in gastric cancer cell lines, we performed mutation analysis for all 12 exons in the 10 gastric cancer cell lines. Mutations were not found in all cell lines, but three previously known SNPs (single nucleotide polymorphisms) were found in SNU719 cells (one mis-sense mutation in exon 4, two silent mutations in exon 6 and 7; Figure 5).

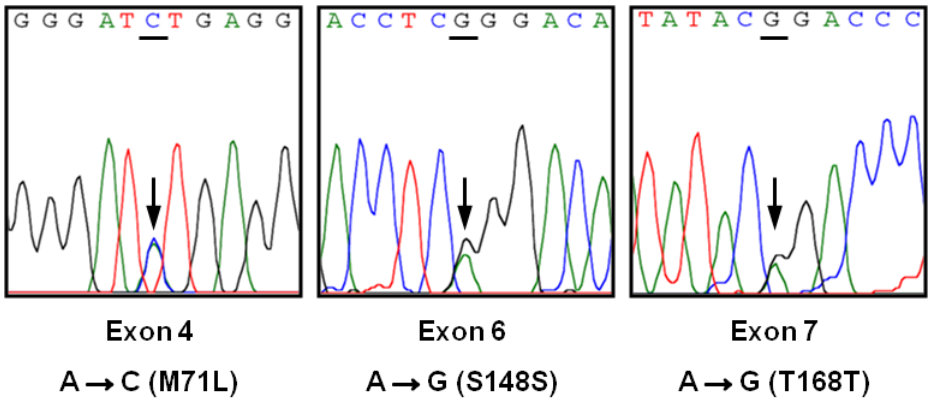


Figure 5. Mutation screening of *ANXA10* in gastric cancer cell lines.

Three representative chromatograms of DNA sequence changes located in SNU719 cell line by direct sequencing analysis. Arrows indicate the substituted nucleotides. The single base substitution was transversed from A to C at codon 71, from A to G at codon 148, and from A to G at codon 168 in exon 4, 6 and 7, respectively. All of them were known single nucleotide polymorphisms.

ANXA10 inhibits cell proliferation, clonogenicity and migration

Because tumor suppressor genes cause tumor growth suppression, we examined whether *ANXA10* can suppress gastric cancer cell line growth. SNU638 cells, which express *ANXA10*, were transfected with siRNA targeting *ANXA10*. The efficiency of this siRNA mediated *ANXA10* suppression in gastric cancer cells was determined by measuring mRNA expressions of *ANXA10* by RT-PCR. It was found that the mRNA expressions of *ANXA10* in siRNA-treated SNU638 cells were significantly lower than those of scrambled siRNA transfected cells. Proliferation assays showed that the growth rates of *ANXA10* siRNA transfected SNU638 cells were significantly higher than those of scrambled siRNA transfected cells (Figure 6A, left; Figure 7A), indicating that ANXA10 down-regulation benefits gastric cancer cell growth.

Next, SNU216 cells, which did not express *ANXA10*, were transfected with a control plasmid (pcDNA3.1) or with an *ANXA10* expression plasmid (pcDNA3.1-*ANXA10*). The over-expression of *ANXA10* in pcDNA3.1-*ANXA10* transfected cells was demonstrated by RT-PCR, and the production of ANXA10 was observed at 24 hours after transfection. It was also noted that SNU216 cell proliferation was inhibited by pcDNA3.1-*ANXA10* transfection (Figure 6A, right; Figure 7B). To determine whether over-expression of ANXA10 had any effect

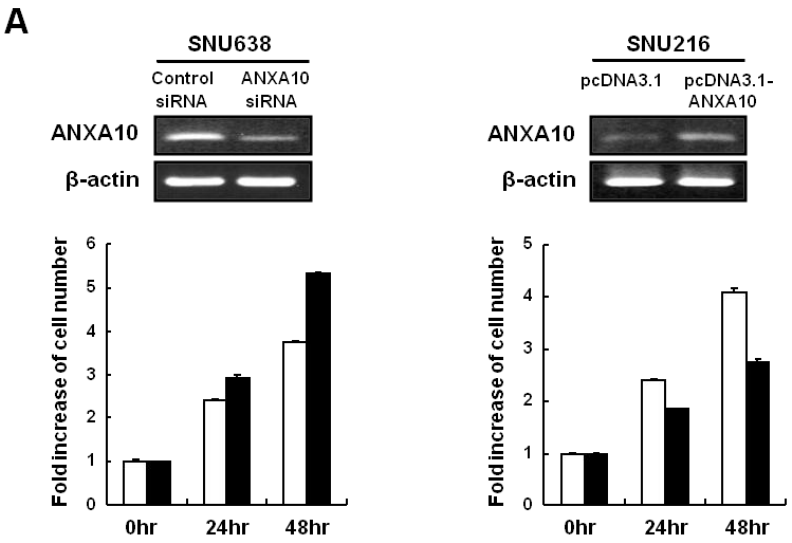
on the apoptosis, annexin V/PI dual staining was performed on the cells using flow cytometry. A slight increase in the percentage of apoptotic cells were observed in SNU216 cells transfected with pcDNA3.1-*ANXA10* compared to control cells (Figure 8).

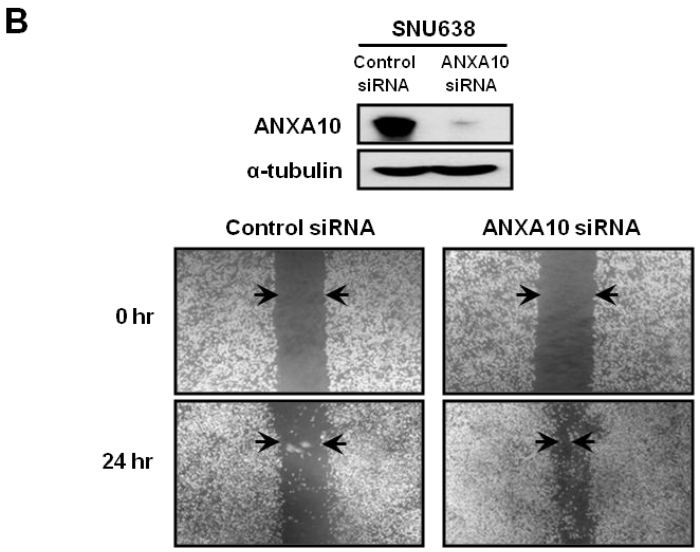
The effect of *ANXA10* on SNU638 cell motility was assessed using wound healing assay. The rate of migration was measured by quantifying the distance that the SNU638 cells (as indicated by arrows) moved from the edge of the scratch toward the center of the scratch (Figure 6B). *ANXA10* siRNA transfected cells spread along wound edges faster than control transfected cells, indicating that *ANXA10* might inhibit cell migration. Western blot analysis confirmed that *ANXA10* siRNA remarkably suppressed ANXA10 expression compared to control siRNA.

To elucidate the biological role of *ANXA10* during early carcinogenesis, we examined the physiologic effects of *ANXA10* on cell proliferation and clonogenicity using the HEK293 cells. Specifically, we investigated whether the restoration or suppression of *ANXA10* expression regulates the HEK293 cell growth. Cell proliferation assays were performed on expression plasmid cDNA or siRNA transfected HEK293 cells (Figures 7C and 7D). Cells transfected with pcDNA3.1-*ANXA10* grew slowly than control vector transfected cells, while the HEK293 cells transfected with *ANXA10* siRNA showed increased proliferation (Figure 6C).

We next compared the colony formation efficiencies of the HEK293

cells using an expression plasmid pcDNA3.1-*ANXA10* or siRNA against *ANXA10* on soft-agar. As western blot analysis indicated, we observed significantly reduced ANXA10 protein expression in siRNA-treated HEK293 cells. In contrast, transfection of the expression plasmid pcDNA3.1-ANXA10 revealed ANXA10 protein up-regulation. Two weeks after transfection, numbers of colonies of the HEK293 cells were increased by *ANXA10* siRNA and decreased by expression plasmid cDNA transfection (Figure 6D). Collectively, these data suggest that *ANXA10* can act as a negative regulator of cell growth and that its inactivation plays a significant role in gastric carcinogenesis.


A
SNU638
Control siRNA
ANXA10 siRNA
ANXA10
β-actin
Fold increase of cell number
0hr
24hr
48hr
SNU216
pcDNA3.1
pcDNA3.1-ANXA10
ANXA10
β-actin
Fold increase of cell number
0hr
24hr
48hr


B
SNU638
Control siRNA
ANXA10 siRNA
ANXA10
α-tubulin
Control siRNA
ANXA10 siRNA
0 hr
24 hr

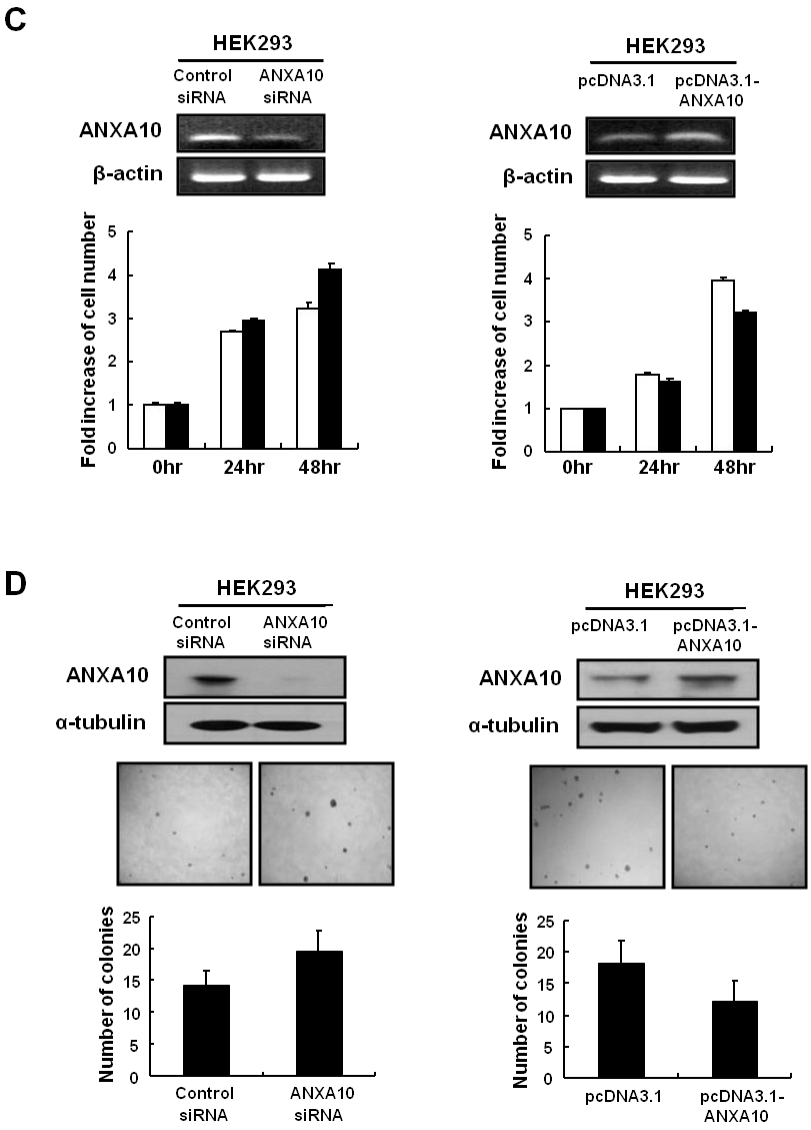


Figure 6. Effect of *ANXA10* expression on the growth of gastric cancer cells and epithelial cells.

(A) Left, Inhibition of *ANXA10* expression by siRNA promoted cell growth in SNU638 gastric cancer cell line (open box, control siRNA; closed box, *ANXA10* siRNA). Down-regulation of *ANXA10* by siRNA

was proved with RT-PCR. Right, Over-expression of *ANXA10* inhibits cell growth in SNU216 gastric cancer cell line. RT-PCR detected up-regulation of mRNA expression in pcDNA3.1-*ANXA10* transfected SNU216 cells (open box, pcDNA3.1; closed box, pcDNA3.1-*ANXA10*). (B) Wound healing assay after siRNA on SNU638 cell line. siRNA transfected cells migrated and repopulated more efficiently compared to the control cells. Arrows indicate the wound edge. The silencing effect of siRNA against ANXA10 was proved by western blot analysis, using anti-α-tubulin as a control. (C) Modulation of *ANXA10* expression in the HEK293 cells with expressional cDNA construct or siRNA. Cell growth was promoted in *ANXA10* siRNA transfected cell line, while cell growth was inhibited in *ANXA10* over-expressed cell line. (D) The HEK293 cells transfected with expressional cDNA construct or siRNA of *ANXA10* were analyzed for their ability to form colonies in soft agar. Western blot analysis indicated that ANXA10 expression was reduced by siRNA and increased by expressional cDNA construct transfection. Cells transfected with expression vector significantly decreased colony formation, while cells treated with *ANXA10* siRNA transfection increased colony formation. Data are presented as a mean ± SD and were determined from two separate experiments performed in triplicate.

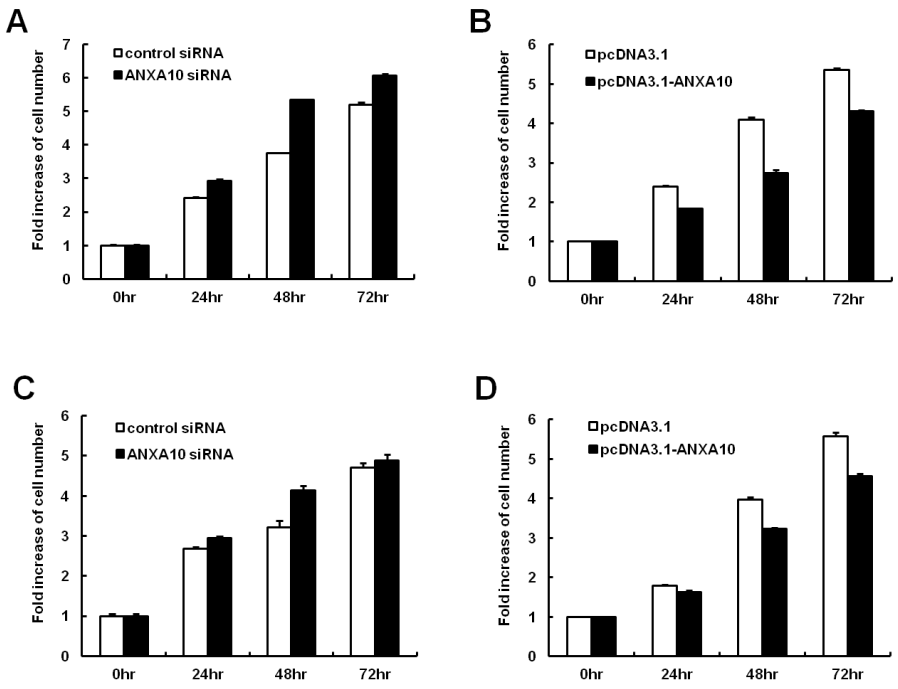


Figure 7. Effect of *ANXA10* expression on the growth of gastric cancer cell lines and epithelial cell line.

(A) Inhibition of *ANXA10* expression by siRNA promotes SNU638 cell growth. (B) Over-expression of *ANXA10* in SNU216 cells inhibits cell growth. (C) Induction of cell growth was found in *ANXA10* siRNA transfected the HEK293 cells. (D) Over-expression of *ANXA10* in the HEK293 cells inhibits cell growth.

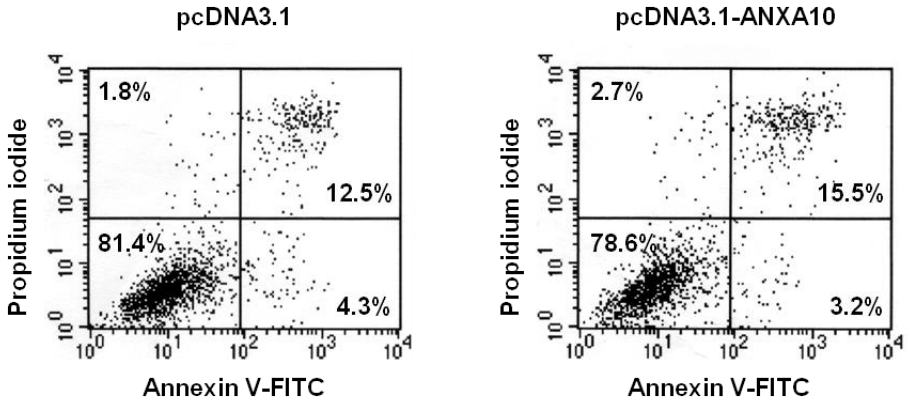


Figure 8. Quantification of apoptosis by flow cytometry.

The cells were transfected with a control plasmid (pcDNA3.1) or with an *ANXA10* expression plasmid (pcDNA3.1-ANXA10), respectively. Apoptosis was analyzed with FITC-annexin V and PI double staining. Horizontal and vertical axes present annexin V and PI detection.The lower left quadrant represents unstained viable cells, the lower right quadrant represents single stained early apoptotic cells, and the upper right represents double stained late apoptotic or already dead cells. The percentage of apoptotic cells were slightly increased in SNU216 cells transfected with pcDNA3.1-*ANXA10* compared to control cells.

Quantitation of ANXA10 DNA by Q-PCR

We investigated whether gastric carcinoma samples showed *ANXA10* DNA copy number losses. Initially we investigated the copy number statuses of the ANXA10 gene in the 10 gastric cancer cell lines by Q-PCR (Figure 9). The results obtained revealed that cell lines showing chromosomal loss in array based CGH (i.e., SNU1, SNU216, SNU601 and SNU16) had low *ANXA10* DNA contents by Q-PCR. Q-PCR of gastric cancer tissue samples revealed that 51 of the 93 cases showed reduced *ANXA10* DNA contents as compared with normal mucosa. The other 42 cases showed no evidence of reduction.

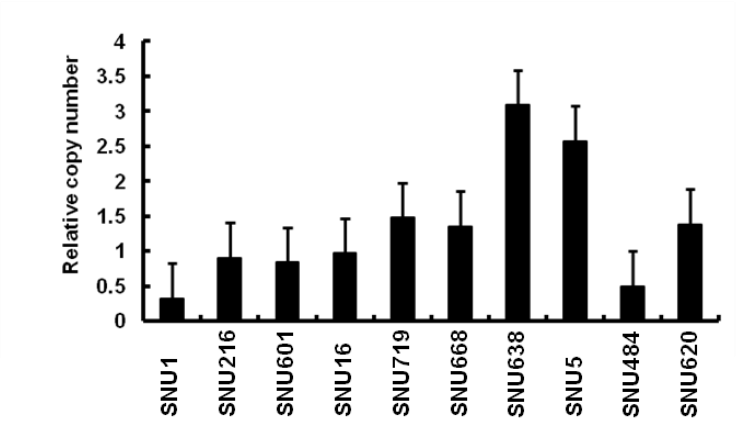


Figure 9. DNA copy number variations of *ANXA10* in gastric cancer cell lines.

Q-PCR analysis for *ANXA10* gene revealed copy number losses in 5 cancer cell lines, including SNU1 showing homozygous deletion.

ANXA10 protein expression in gastric carcinomas

To further assess the significance of ANXA10 protein expression in gastric carcinoma, we performed immunohistochemistry in 585 patients using poly-clonal ANXA10 antibody, and characterized the clinicopathologic features of tumors with reduced ANXA10 levels. Whereas ANXA10 was observed mainly in the nucleus of normal gastric epithelium, weak or negative nuclear staining results were obtained for 49.4% of the 585 gastric carcinomas (Figures 10A-10C; Table 6). When we analyzed the associations between ANXA10 down-regulation and clinicopathological parameters, loss of ANXA10 was found to be correlated with a T stage (P = 0.011), advanced gastric carcinoma (P = 0.001) and lymph node metastasis (P = 0.001) (Table 6). In addition, Kaplan-Meier survival curves revealed that patients with a low level of ANXA10 protein had an unfavorable prognosis (P = 0.016) (Figure 10D). However, there was no significant survival difference in the multivariate analysis between the subjects with ANXA10 positive gastric carcinomas and those with ANXA10 negative gastric carcinomas (data not shown). We expanded expression analysis to other primary human cancers, and ANXA10 was found to be remarkably reduced in most cancer specimens (Table 7).

Table 8 demonstrates the correlation between ANXA10 protein expression and alterations in DNA quantities. Q-PCR showed *ANXA10* losses in 51 of 93 cases (54.8%). Among 51 cases, 34 cases (66.7%) represented reduced ANXA10 protein expression on

immunohistochemistry (*P* = 0.001). Interestingly, DNA loss in the *ANXA10* region was more frequent in ANXA10 negative cases (34/47, 72.3%) as compared with ANXA10 positive cases (17/46, 36.9%). The results therefore support that the DNA loss at the *ANXA10* locus may be responsible for ANXA10 down-regulation in gastric carcinoma.

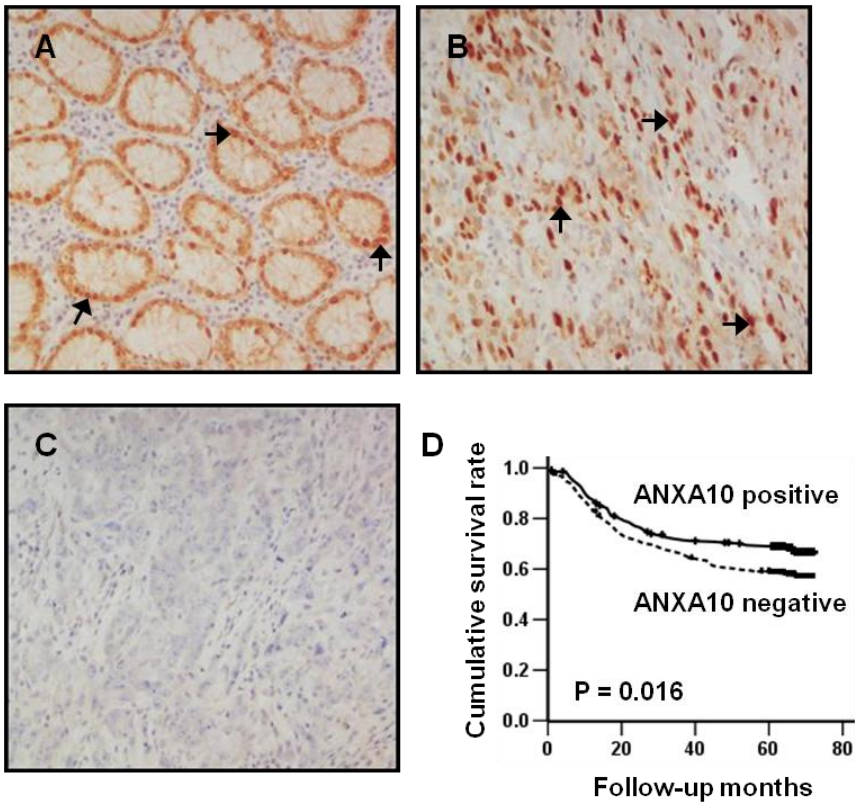


Figure 10. Expression and clinical outcomes of ANXA10 in gastric carcinomas.

(A) Strong nuclear staining for ANXA10 in normal gastric glandular epithelium tissue. (B) Positive nuclear staining of ANXA10 in gastric carcinoma cells. Arrows indicate ANXA10 positive cells. (C) Gastric carcinoma tissue with loss of ANXA10 expression. Original magnification, X200. (D) Kaplan-Meier curves of disease specific survival according to the expression of ANXA10 in patients with gastric cancer. Survival curves demonstrates that the ANXA10 negative carcinomas have an unfavorable prognosis compared to the ANXA10

positive carcinomas (P = 0.016).

Table 6. Correlation of ANXA10 protein expression with clinicopathological features in gastric carcinoma

	ANXA10 expression		
	Negative (n = 289)	Positive (n = 296)	*P* value
Sex			N.S
Male	201	188	
Female	88	108	
WHO classification			0.016
W/D	21	23	
M/D	89	87	
P/D	132	121	
Mucinous	23	13	
Histologic type			N.S
Intestinal	114	105	
Diffuse	157	167	
Mixed	18	24	
pTNM stage			0.011
I	68	105	
II	160	129	
III	59	60	
IV	2	2	
Tumor invasion			0.001
EGC	68	105	
AGC	221	191	
Lymph node metastasis			0.001
Absent	96	137	
Present	193	159	
Distant metastasis			N.S
Absent	260	275	
Present	22	21	

N.S, not significant; EGC, early gastric carcinoma; AGC, advanced gastric carcinoma; W/D, Well differentiated; M/D, Moderately differentiated; P/D, Poorly differentiated.

Table 7. Frequent loss of ANXA10 expression in various cancers by immunohistochemistry

Type of cancer	Loss of ANXA10 expression
Endometrium (n = 12)	100%
Kidney (n = 13)	92.3%
Larynx (n = 15)	80.0%
Adrenal gland (n = 14)	71.4%
Ovary (n = 38)	71.0%
Uterine cervix (n = 26)	69.2%
Thyroid (n = 15)	66.7%
Testis (n = 11)	63.6%
Melanoma (n = 10)	60.0%
Lung (n = 33)	57.6%
Breast (n = 14)	57.1%
Lymphoma (n = 18)	55.6%
Salivary gland (n = 15)	53.3%
Stomach (n = 585)	49.4%
Soft tissue (n = 25)	48.0%
Bone (n = 16)	43.8%
Colon (n = 17)	35.3%
Liver (n = 13)	30.8%
Urinary bladder (n = 15)	26.7%
Total (n = 905)	59.6%

Table 8. Comparison of ANXA10 status between Q-PCR and IHC

Immunohistochemistry	Quantitative real-time PCR result		
	Less than normal mucosa	Same or greater than normal mucosa	*P* value
Reduced expression	34	13	0.001
Retained expression	17	29	
Total	51	42	

DISCUSSION

Homozygous deletions in cancer cell lines and human cancers have helped defining candidate tumor suppressor genes within deleted regions (22, 23). Cytogenetic studies have reported chromosome 4q deletions and frequent occurrence of allelic losses in various cancers, including bladder, breast, esophagus, head and neck and liver cancers. These findings indicated that the long arm of chromosome 4 probably contains one or more tumor suppressor genes such as hCDC4, CARF and PRDM5 (24-27).

Annexins are commonly down-regulated in cancers and this has been suggested to be indicative of a possible tumor suppressing effect (5, 18). *ANXA10,* a member of the annexin family is also down-regulated in this manner and has been proposed to be a potential gene therapy target in hepatocellular carcinoma, although its functional role has not been determined (14, 20, 28). In this study, we carried out genome-wide screening to identify putative tumor suppressor genes in gastric cancer cell lines using array based CGH and oligonucleotide microarray analyses, and the discovery of homozygous loss at 4q33, the location of *ANXA10*, which has not been previously documented in gastric cancer, prompted us to examine whether *ANXA10* is involved in gastric carcinogenesis.

Gastric carcinogenesis is a complex and multifactorial process, and is

associated with a multiplicity of etiologies and genetic alterations (1, 29, 30). Chromosomal instability in gastric carcinoma appears to be essentially associated with genetic damage, as has been shown by conventional CGH studies, which revealed a variety of chromosomal abnormalities (3, 4, 29, 31). In the present study, we analyzed chromosomal aberrations in gastric cancer cell lines using an array based CGH method that can provide quantitative data on chromosomal regions, and therefore, greatly facilitate cancer-associated gene identification. The primary biological value of high-resolution array based CGH is its ability to detect small amplifications and deletions, which in many cases, appear to harbor specific oncogenes and tumor suppressor genes (7, 10, 32).

Using the higher resolution array based CGH method described, we identified a minimal deletion region at 4q33 in one of ten gastric cancer cell lines. However, in four of these gastric cancer cell lines, ANXA10 was significantly reduced or absent at the mRNA level by RT-PCR and oligonucleotide microarray analysis. Moreover, most of these cell lines displayed chromosomal loss at 4q33, which suggested that this down-regulation may have been caused by loss of DNA. In addition, we also found that *ANXA10* was down-regulated in gastric cancer cell lines by western blotting. Furthermore, we found that the incidence of *ANXA10* copy number losses was 54.8% in 93 cases of gastric primary tumors using Q-PCR. Among these cases, copy number losses were more frequent in ANXA10 negative cases (34/51, 66.7%) compare to the

ANXA10 positive cases (17/51, 33.3%) (P = 0.001). These data suggest that the down-regulation of ANXA10 expression is significantly associated with chromosomal loss in gastric carcinoma. Thus, it appears reasonable to suspect that *ANXA10* is a candidate tumor suppressor gene in gastric carcinoma.

The combination of copy number changes and gene expression analysis followed by a functionally-guided approach represents a powerful means of identifying genes using genome-wide array based studies (10, 12, 13, 33). In the present study, we utilized combined array based CGH, FISH and Q-PCR analyses to gastric cancer cell lines to investigate copy number alterations in chromosomal regions. Frequent allelic loss in a certain chromosomal regions provides strong evidence of the existence of tumor suppressor genes (2, 5, 8, 9, 34). In the present study, the findings obtained using these modalities support the notion that *ANXA10* is involved in gastric tumor suppression.

Tumor suppressor gene expressional loss appears to be a biomarker of tumor proliferation and progression (5, 30, 35, 36). Our functional assays demonstrated that *ANXA10* knockdown promotes gastric cancer cell growth and migration, whereas the over-expression of *ANXA10* was found to inhibit gastric cancer cell growth according to our proliferation and wound healing assay results. To identify the target gene that regulated cell growth, we silenced or induced *ANXA10* in a normal epithelial cell line (HEK293), an established premalignant model system for studying the mechanistic bases of the multistep

pathogenesis of carcinomas. In the present study, over-expression of *ANXA10* was found to inhibit cell growth, whereas silencing of ANXA10 with siRNA promoted colony formation and migration.

Observed reductions in tumor suppressor gene expression may be due to the loss of one allele or promoter region hypermethylation (7, 23, 30). Although promoter hypermethylation is responsible for reducing the expressions of many tumor suppressor genes (12, 30, 35, 37), *ANXA10* does not contain CpG islands in its promoter region or coding sequence, which implies that hypermethylation is unlikely to contribute to its down-regulation.

Microsatellite instability (MSI) has been used to identify regions on chromosomes that may contain putative tumor suppressor genes (38, 39). To examine the etiological association of genetic instability in gastric carcinogenesis, we investigated the frequency of MSI as well as the association between MSI and expression of ANXA10 protein in 90 gastric carcinomas. However, incidence of MSI did not correlated with ANXA10 expression.

To evaluate the clinical significance of ANXA10 expression, we used an immunostaining method to analyze a large numbers of gastric carcinoma specimens, and found that ANXA10 protein expression was substantially attenuated in 49.4% of 585 primary gastric carcinoma samples. Several annexin family, such as ANXA-1, -2, -3, -6, -7, -11 and -13, have been linked to exocytotic processes (5, 14, 28). Although it is not clearly demonstrated, there is enough reason to

suspect its involvement in the Ca^{2+} regulated exocytosis. In this study, we demonstrated a correlation between ANXA10 expression and the differentiation of gastric carcinoma by histological examination. ANXA10 expresssion was reduced in mucinous adenocarcinoma suggesting that ANXA10 loss may reflect the loss of exocytotic function during the dedifferentiation process in gastric carcinogenesis. Furthermore, low ANXA10 expression was also found to be associated with a poor prognosis. When we examine the expression of ANXA10 in various primary human cancers, ANXA10 was remarkably reduced in most of the cancer specimens. These findings suggest that the *ANXA10* gene is a tumor suppressor in gastric carcinoma, and that it may play a similar role in other carcinomas.

The short isoform of ANXA10 has been identified and suggested to have a role during hepatocellular carcinoma progression (20, 40). In our experiment, short isoform was not detected by RT-PCR in all 10 gastric carcinoma cell lines (data not shown).

In conclusion, our results demonstrate the feasibility of correlating DNA copy number with gene expression to identify a putative tumor suppressor gene and found that *ANXA10* is a gastric carcinoma associated tumor suppressor gene. Therefore, we suggest that the study of *ANXA10* action in gastric cancer cells and gastric carcinoma specimens has great potential importance for not only understanding human gastric cancer progression, but also for development of novel diagnostic approaches.

REFERENCES

1. Hasegawa S, Furukawa Y, Li M, Satoh S, Kato T, Watanabe T, et al. Genome-wide analysis of gene expression in intestinal-type gastric cancers using a complementary DNA microarray representing 23,040 genes. Cancer Res. 2002 Dec 1;62(23):7012-7.
2. Kakinuma N, Kohu K, Sato M, Yamada T, Nakajima M, Akiyama T, et al. Candidate regions of tumor suppressor locus on chromosome 9q31.1 in gastric cancer. Int J Cancer. 2004 Mar;109(1):71-5.
3. Sakakura C, Mori T, Sakabe T, Ariyama Y, Shinomiya T, Date K, et al. Gains, losses, and amplifications of genomic materials in primary gastric cancers analyzed by comparative genomic hybridization. Genes Chromosomes Cancer. 1999 Apr;24(4):299-305.
4. Kang JU, Kang JJ, Kwon KC, Park JW, Jeong TE, Noh SM, et al. Genetic alterations in primary gastric carcinomas correlated with clinicopathological variables by array comparative genomic hybridization. J Korean Med Sci. 2006 Aug;21(4):656-65.
5. Srivastava M, Bubendorf L, Srikantan V, Fossom L, Nolan L, Glasman M, et al. ANX7, a candidate tumor suppressor gene for prostate cancer. Proc Natl Acad Sci U S A. 2001 Apr 10;98(8):4575-80. Epub 2001 Apr 3; PMID: 11287641

6. Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, et al. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res. 1998 Nov 15;58(22):5248-57.
7. Nakaya K, Yamagata HD, Arita N, Nakashiro KI, Nose M, Miki T, et al. Identification of homozygous deletions of tumor suppressor gene FAT in oral cancer using CGH-array. Oncogene. 2007 Aug 9;26(36):5300-8. Epub 2007 Feb 26; PMID: 17325662
8. Senchenko VN, Liu J, Loginov W, Bazov I, Angeloni D, Seryogin Y, et al. Discovery of frequent homozygous deletions in chromosome 3p21.3 LUCA and AP20 regions in renal, lung and breast carcinomas. Oncogene. 2004 Jul 29;23(34):5719-28.
9. Wang SI, Parsons R, Ittmann M. Homozygous deletion of the PTEN tumor suppressor gene in a subset of prostate adenocarcinomas. Clin Cancer Res. 1998 Mar;4(3):811-5.
10. Patil MA, Gütgemann I, Zhang J, Ho C, Cheung ST, Ginzinger D, et al. Array-based comparative genomic hybridization reveals recurrent chromosomal aberrations and Jab1 as a potential target for 8q gain in hepatocellular carcinoma. Carcinogenesis. 2005 Dec;26(12):2050-7. Epub 2005 Jul 6; PMID: 16000397
11. Veltman JA, Fridlyand J, Pejavar S, Olshen AB, Korkola JE, DeVries S, et al. Array-based comparative genomic hybridization

for genome-wide screening of DNA copy number in bladder tumors. Cancer Res. 2003 Jun 1;63(11):2872-80.

12. Mestre-Escorihuela C, Rubio-Moscardo F, Richter JA, Siebert R, Climent J, Fresquet V, et al. Homozygous deletions localize novel tumor suppressor genes in B-cell lymphomas. Blood. 2007 Jan 1;109(1):271-80. Epub 2006 Sep 7; PMID: 16960149
13. Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F, et al. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science. 1992 Oct 30;258(5083):818-21.
14. Gerke V, Moss SE. Annexins: from structure to function. Physiol Rev. 2002 Apr; 82(2):331-71.
15. Leighton X, Srikantan V, Pollard HB, Sukumar S, Srivastava M. Significant allelic loss of ANX7region (10q21) in hormone receptor negative breast carcinomas. Cancer Lett. 2004 Jul 16;210(2):239-44.
16. Moss SE, Morgan RO. The annexins. Genome Biol. 2004;5(4):219. Epub 2004 Mar 31; PMID: 15059252
17. Tatenhorst L, Rescher U, Gerke V, Paulus W. Knockdown of annexin 2 decreases migration of human glioma cells in vitro. Neuropathol Appl Neurobiol. 2006 Jun;32(3):271-7.
18. Garcia Pedrero JM, Fernandez MP, Morgan RO, Herrero Zapatero A, Gonzalez MV, Suarez Nieto C, et al. Annexin A1 down-regulation in head and neck cancer is associated with epithelial

differentiation status. Am J Pathol. 2004 Jan;164(1):73-9.

19. Hsiang CH, Tunoda T, Whang YE, Tyson DR, Ornstein DK. The impact of altered annexin I protein levels on apoptosis and signal transduction pathways in prostate cancer cells. Prostate. 2006 Sep 15;66(13):1413-24.
20. Liu SH, Lin CY, Peng SY, Jeng YM, Pan HW, Lai PL, et al. Down-regulation of annexin A10 in hepatocellular carcinoma is associated with vascular invasion, early recurrence, and poor prognosis in synergy with p53 mutation. Am J Pathol. 2002 May;160(5):1831-7.
21. Senchenko V, Liu J, Braga E, Mazurenko N, Loginov W, Seryogin Y, et al. Deletion mapping using quantitative real-time PCR identifies two distinct 3p21.3 regions affected in most cervical carcinomas. Oncogene. 2003 May 15;22(19):2984-92.
22. Sato N, Fukui T, Taniguchi T, Yokoyama T, Kondo M, Nagasaka T, et al. RhoB is frequently downregulated in non-small-cell lung cancer and resides in the 2p24 homozygous deletion region of a lung cancer cell line. Int J Cancer. 2007 Feb 1;120(3):543-51.
23. Takada H, Imoto I, Tsuda H, Nakanishi Y, Ichikura T, Mochizuki H, et al. ADAM23, a possible tumor suppressor gene, is frequently silenced in gastric cancers by homozygous deletion or aberrant promoter hypermethylation. Oncogene. 2005 Dec 1;24(54):8051-60.
24. Deng Q, Huang S. PRDM5 is silenced in human cancers and has

growth suppressive activities. Oncogene. 2004 Jun 17;23(28):4903-10.

25. Piao Z, Park C, Park JH, Kim H. Deletion mapping of chromosome 4q in hepatocellular carcinoma. Int J Cancer. 1998 Aug 21;79(4):356-60.
26. Shivapurkar N, Sood S, Wistuba II, Virmani AK, Maitra A, Milchgrub S, et al. Multiple regions of chromosome 4 demonstrating allelic losses in breast carcinomas. Cancer Res. 1999 Aug 1;59(15):3576-80.
27. Sterian A, Kan T, Berki AT, Mori Y, Olaru A, Schulmann K, et al. Mutational and LOH analyses of the chromosome 4q region in esophageal adenocarcinoma. Oncology. 2006;70(3):168-72. Epub 2006 Jul 7; PMID: 16837775
28. Schlaepfer DD, Haigler HT. Expression of annexins as a function of cellular growth state. J Cell Biol. 1990 Jul;111(1):229-38.
29. Kim YH, Kim NG, Lim JG, Park C, Kim H. Chromosomal alterations in paired gastric adenomas and carcinomas. Am J Pathol. 2001 Feb;158(2):655-62.
30. Takada H, Imoto I, Tsuda H, Nakanishi Y, Sakakura C, Mitsufuji S, et al. Genomic loss and epigenetic silencing of very-low-density lipoprotein receptor involved in gastric carcinogenesis. Oncogene. 2006 Oct 19;25(49):6554-62. Epub 2006 May 22; PMID: 16715143
31. Tay ST, Leong SH, Yu K, Aggarwal A, Tan SY, Lee CH, et al. A combined comparative genomic hybridization and expression

microarray analysis of gastric cancer reveals novel molecular subtypes. Cancer Res. 2003 Jun 15;63(12):3309-16.

32. Hui AB, Or YY, Takano H, Tsang RK, To KF, Guan XY, et al. Array-based comparative genomic hybridization analysis identified cyclin D1 as a target oncogene at 11q13.3 in nasopharyngeal carcinoma. Cancer Res. 2005 Sep 15;65(18):8125-33.
33. Kawaguchi K, Honda M, Yamashita T, Shirota Y, Kaneko S. Differential gene alteration among hepatoma cell lines demonstrated by cDNA microarray-based comparative genomic hybridization. Biochem Biophys Res Commun. 2005 Apr 1;329(1):370-80.
34. Araki D, Uzawa K, Watanabe T, Shiiba M, Miyakawa A, Yokoe H, et al. Frequent allelic losses on the short arm of chromosome 1 and decreased expression of the p73 gene at 1p36.3 in squamous cell carcinoma of the oral cavity. Int J Oncol. 2002 Feb;20(2):355-60.
35. Ying J, Li H, Seng TJ, Langford C, Srivastava G, Tsao SW, et al. Functional epigenetics identifies a protocadherin PCDH10 as a candidate tumor suppressor for nasopharyngeal, esophageal and multiple other carcinomas with frequent methylation. Oncogene. 2006 Feb 16;25(7):1070-80.
36. Wu X, Jia HL, Wang YF, Ren N, Ye QH, Sun HC, et al. HTPAP gene on chromosome 8p is a candidate metastasis suppressor for human hepatocellular carcinoma. Oncogene. 2006 Mar

16;25(12):1832-40.

37. Carvalho R, Kayademir T, Soares P, Canedo P, Sousa S, Oliveira C, et al. Loss of heterozygosity and promoter methylation, but not mutation, may underlie loss of TFF1 in gastric carcinoma. Lab Invest. 2002 Oct;82(10):1319-26.
38. Hoeve MA, Ferreira Mota SC, Schuuring E, de Leeuw WJ, Chott A, Meijerink JP, et al. Frequent allelic imbalance but infrequent microsatellite instability in gastric lymphoma. Leukemia. 1999 Nov;13(11):1804-11
39. Woerner SM, Kloor M, Schwitalle Y, Youmans H, Doeberitz Mv, Gebert J, et al. The putative tumor suppressor AIM2 is frequently affected by different genetic alterations in microsatellite unstable colon cancers. Genes Chromosomes Cancer. 2007 Dec;46(12):1080-9.
40. Peng SY, Ou YH, Chen WJ, Li HY, Liu SH, Pan HW, et al. Aberrant expressions of annexin A10 short isoform, osteopontin and alpha-fetoprotein at chromosome 4q cooperatively contribute to progression and poor prognosis of hepatocellular carcinoma. Int J Oncol. 2005 Apr;26(4):1053-61.

국문 초록

위암발생에 있어 유전적 변화는 중요한 현상으로 관찰되어 왔다. 본 연구에서는 위암 세포주에서 DNA 복제 수 변화를 조사하기 위해 배열에 기반한 비교 유전체 부합법을 이용하였고 그 결과를 올리고 핵산 미세배열법 결과와 비교하였다. 전장 유전체 조사를 통해 인간 위암과 관련된 새로운 유전자들을 찾아내었다. 이중 Annexin A10은 annexin 집단의 한 구성요소이나 아직 그 기능이 많이 알려져 있지 않다. 배열에 기반한 비교 유전체 부합법과 올리고 핵산 미세배열법 분석을 통해 몇 개의 위암 세포주에서 ANXA10이 위치한 염색체 4q33에서 DNA 손실이 관찰되었고 이 세포주들은 ANXA10의 발현도 감소되어 있었다. ANXA10이 암 억제 유전자로 작용할 수 있을지 조사하기 위해 우리는 작은 간섭 RNA 또는 발현되는 cDNA를 위암 세포주들에 형질주입하여 기능을 분석하였고 ANXA10이 위암 세포의 성장을 억제시키는 것을 증명하였다. 또한, 원발성 위암 조직 중 289/585 (49.4%)는 ANXA10 단백질 발현이 감소된 것을 발견하였다. 단변량 생존분석 결과 ANXA10 발현의 감소는 좋지 않은 생존률과 서로 연관성을 보였다. 정량적인 실시간 중합효소 연쇄반응 분석을 통해 ANXA10 발현이 감소한 위암 조직들은 ANXA10 위치에서의 DNA 손실이 되었음을 확인하였다. 이러한 결과들로 보아 염색체 손실로 인한 ANXA10의 발현 감소가

ANXA10의 기능 상실을 유도하고, 이는 아마도 위암발생에 작용하는 한 요소임을 시사한다.

주요어 : 아넥신, 암 억제 유전자, 하향 조절, DNA 복제 수, 위 종양

학 번 : 2002-23724