

GASP-1, A NEW TUMOR BIOMARKER, CONTRIBUTES TO TUMORIGENESIS
IN BREAST CANCER.

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By
XiaoyiZheng
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Dissertation Examining Committee:

Dr. Chang, Frank, Biology (Chair)

Dr. Amini, Shohreh, Biology

Dr. Tuszynski, George, Neuroscience

Dr. Marcinkiewz, Cezary, Biology

Dr. Hu, Wenhui, Neuroscience (External Reader)

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ABSTRACT

Breast cancer is the second leading cause of death in United States. Using 2D-HPLC, a novel separation technology, G-protein coupled receptor-associated sorting protein 1 (GASP-1) was identified in sera of patients with early stage cancer, while it could not be detected in sera from healthy individuals. This was the first indication that GASP-1 was positively correlated with breast cancer. However, the function of GASP-1 in breast cancer was unknown. In this study, I verified the 2D-HPLC results by quantifying the expression level of GASP-1 in sera and tissue specimens of cancer patients using specific antibodies against GASP-1. A GASP-1 specific ELISA was developed and used to quantify GASP-1 levels in cancer patient sera. Immunohistochemistry was performed to verify and localize GASP-1 expression in tumor. I also characterized the tumorigenic potential of GASP-1 and identified the signaling pathways mediated by GASP-1 in breast cancer cells *in vitro*. GASP-1 expression levels in MDA-MB-231 cells were modified by transfecting cells with anti-GASP-1 shRNA and over-expression plasmids. Stable cell lines were prepared and their tumorigenic potential was evaluated using cell proliferation, migration, and colony formation assays. These cells were analyzed for markers used to identify epithelial to mesenchymal transition (EMT) using RT-PCR and western blot. They were also analyzed for NF κ B activity, src phosphorylation, and GPR30 expression. The results showed that GASP-1 was over-expressed in sera and tissue specimens of breast cancer patients and other cancer types including brain, lung, liver and pancreatic cancer and that it correlated with early stage disease. GASP-1 positively regulated migration, and is required for cell proliferation and colony formation. GASP-1 is also necessary for the expression of EMT marker slug, increases NF κ B activity and

GPR30 expression level, while decreases the inhibitory phosphor-src Tyr 530. I conclude that GASP-1 is a nearly marker for multiple cancer types. GASP-1 promotes tumorigenesis in breast cancer, possibly through multiple cancer related signaling pathways. These findings may contribute to our understanding of the mechanism of breast cancer tumorigenesis and identify new biomarkers that can be used for diagnosis and therapy of cancer.

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TABLE OF CONTENTS

ABSTRACT.....	iii
ACKNOWLEDGEMENTS.....	v
LIST OF TABLES.....	ix
LIST OF FIGURES.....	x
CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW.....	1
1.1 Breast Cancer.....	1
1.2 G-protein Coupled Receptor Associated Sorting Protein 1 (GASP-1)...	5
1.3 Epithelial to Mesenchymal Transition (EMT).....	7
1.4 NFκB signaling pathway.....	10
1.5 Src signaling.....	11
1.6 GPR30.....	12
1.7 Competitive ELISA.....	13
1.8 Objectives and Hypothesis.....	14
CHAPTER 2: MATERIALS AND METHODS.....	15
CHAPTER 3: RESULTS.....	29
3.1 GASP-1 Epitope Has a Higher Serum Level in Cancer Patients rather than Normal Control.....	29
3.1.1 Antibody Characterization.....	29
3.1.2 Checker Board Titration and Optimal Analysis Range.....	32
3.1.3 Detect GASP-1 Epitope Level in Sera from Cancer Patients and Controls.....	36
3.2 GASP-1 Peptides Containing the Epitope Discovered by 2D-HPLC is More Highly Expressed in Cancer Cells than in Normal Cells.....	39

3.2.1 GASP-1 is Over-Expressed in Tissue Specimens of Breast Cancer Patient.....	39
3.2.2 GASP-1 is Over-expressed in Pancreatic Cancer and Brain Cancer.....	45
3.3 GASP-1 Promoted tumorigenesis in MDA-MB-231 Breast Cancer Cells.....	48
3.3.1 Establish MDA-MB-231 Cell Clones That Stably Over-/Under-Express GASP-1.....	48
3.3.2 Down-Regulation of GASP-1 Decreased the Cell Proliferation Rate in MDA-MB-231 Cells.....	52
3.3.3 Down-regulation of GASP-1 Decreases the Migration Rate in MB231 cells.....	52
3.3.4 Down-regulation of GASP-1 in MDA-MB-231 Cells Decreased the Number and the Size of Colonies in Soft Agar Assay	54
3.4 IDENTIFY THE SIGNALING PATHWAYS MEDIATED BY GASP-1 IN BREAST CANCER CELLS IN VITRO.....	59
3.4.1 Epithelial to Mesenchymal Transition (EMT).....	59
3.4.2 NF κ B signaling pathway.....	61
3.4.3 Src signaling pathway.....	64
3.4.4 G-protein coupled receptor 30 (GPR30).....	67
CHAPTER 4: GENERAL DISCUSSION AND POTENTIAL FUTURE DIRECTIONS.....	68
REFERENCES.....	74

LIST OF TABLES

Table	Page
1. Stage distribution and 5-year relative survival rate (2002-2008)	3
2. RT-PCR primers	23

LIST OF FIGURES

Figure	Page
1. Age distribution of breast cancer. Data is from National Cancer Database (NCDB)	1
2. ShRNA plasmid used to inhibit GASP-1 expression.....	19
3. pCMV-AC-IRES-GFP-puro plasmid was applied to over-express GASP-1.....	20
4. Characterize the Biotinylated Antibody.....	31
5. The strategy of developing competitive ELISA. (A) flow chart of competitive ELISA	33
6. Checker board titration is applied to find out the best antibody concentration and amount of immobilized antigenic peptide.	34
7. Determine the optimum analysis range.....	35
8. GASP-1 is over-expressed in the sera of cancer patients.....	38
9. GASP-1 is over-expressed in breast cancer cells of tissue specimens.....	42
10. A typical immunohistochemistry picture was shown.	43
11. GASP-1 did not correlate with hyperplasia and triple negative breast cancer	44
12. GASP-1 is overexpressed in pancreatic cancer and brain cancer	46
13. A typical immunohistochemistry picture is shown.....	47

14. GASP-1 was down-regulated in shGASP-1 clones and is over-expressed in pGASP-1_3 and 9 clones	50
15. GASP-1 modification was confirmed by western blot	51
16. Down-regulation of GASP-1 decreased the cell proliferation rate in MDA-MB-231 cells	53
17. GASP-1 positively regulates the migration ability of MDA-MB-231 breast cancer cells in the Boyden chamber migration assay	55
18. GASP-1 positively regulates the migration ability of MDA-MB-231 breast cancer cells in a wound healing assay	56
19. Inhibition of GASP-1 decreased the number of the colony formed in the soft agar assay	58
20. GASP-1 maintains slug mRNA, and decreased N-Cadherin mRNA level in MDA-MB-231 breast cancer cells	62
21. GASP-1 maintained slug protein expression, but N-cadherin and vimentin protein levels did not change	63
22. NFκB activity increased in GASP-1 over-expression cells	65
23. GASP-1 inversely regulate phospho-src Tyr 530, but did not change Tyr 419 level	66
24. Over-expression of GASP-1 increased GPR30 protein level	66

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Breast cancer

Breast cancer is the second leading cause of death (after lung cancer) in United States, and many new cases are reported every year. National Cancer Institute estimated the deaths resulting from breast cancer in 2012 would be 39510 cases in females and 410 deaths in males.

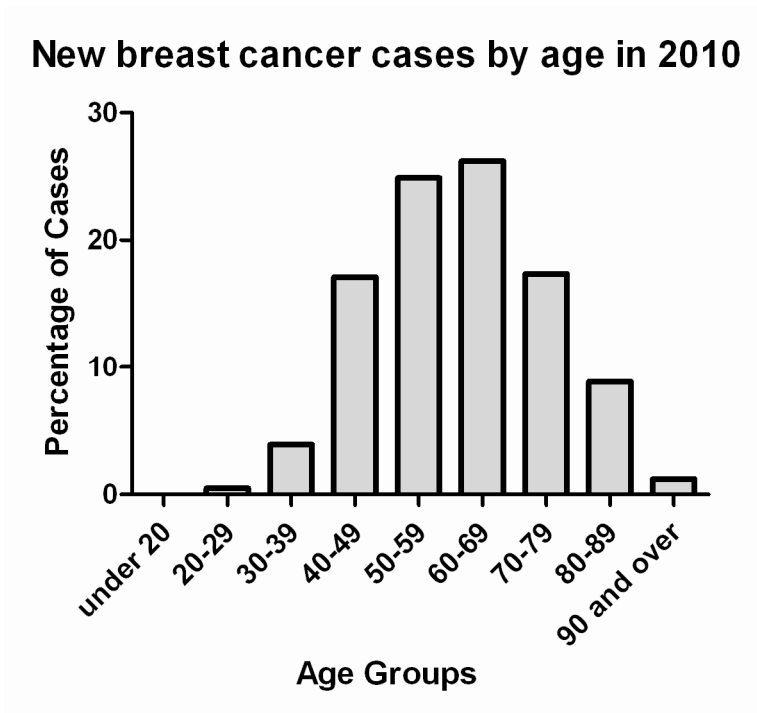


Figure 1. Age distribution of breast cancer. Data is from National Cancer Database (NCDB).

According to the cancer center statistical report by Lehigh Valley Health Network (2011), breast cancer was the most prevalent cancer treated from 2002 to 2009, comparing to colon cancer, prostate cancer, skin cancer, lung cancer, etc. In 2010, newly identified

breast cancer cases in Lehigh Valley Health Network accounted for 13% of the total newly diagnosed cases. Breast cancer mainly occurred in women. National Cancer Institute estimates 226870 new cases for women and 2190 cases for men in United States in 2012. Breast cancer happened more in older people than younger people. Among cases recorded in the National Cancer Database (NCDB) from 1370 hospitals in 2010 (Figure 1), 95% happened above age 40. Only about 4% happened below age of 39.

National Cancer Institute divided breast cancer disease progression into 5 stages (Stage 0 – stage 4), according to the tumor size and cancer cell invasion level. Stage 0 described non-invasive breast cancer, including ductal carcinoma *in situ*. There were abnormal cells in the wall of the breast duct, but these cells did not invade into the nearby breast tissue. Many doctors did not consider DCIS as breast cancer, but DCIS sometimes developed into invasive breast cancer if left untreated. In stage 1, tumors grew into a size of less than 2 cm in diameter, and cancer cells began to invade into nearby breast tissue. In Stage 2, tumors grew bigger, up to 5 cm in diameter, and cancer cells invaded into lymph nodes under the arm (axillary lymph nodes). In stage 3, tumors grew bigger, from 5 cm in diameter to any size, and cancer cells spread from axillary lymph nodes to other structures such as lymph nodes behind breastbone. Stage 4 was the metastatic breast cancer, cancer cells spread into other organs such as bones or liver. As stages progressed, cancer cells spread further and further from the original sites, and the tumor size becomes bigger and bigger. National Cancer Institute summarized the 5 year relative

survival rate of breast cancer stages at diagnosis from 2002 to 2008 (Table 1). According to this summary, 60% people were diagnosed as localized cancer, 33% were diagnosed as regional cancer. 5% were diagnosed as distant cancer. 2% were unstaged. The more invasive the cancer cells were, the lower the survival rate of the breast cancer patients was. Therefore, the early identification of breast cancer would give the patient a better chance to survive.

Table 1: Stage distribution and 5-year relative survival rate (2002-2008)

Stage at Diagnosis	Stage Distribution (%)	5-year Relative Survival (%)
Localized (confined to primary site)	60	98.4
Regional (spread to regional lymphnodes)	33	83.9
Distant (cancer has metastasized)	5	23.8
Unknown (unstaged)	2	50.7

There are two major diagnostic methods of breast cancer. The first one is mammogram, using X-ray to take the picture of breast tissue. The picture will show lumps and calcium specks resulted from cancer, precancerous cells or other reasons. Further tests are needed to determine cancer formation. The second method is clinical breast exam. The doctors will check the morphology of the breast, and feel lumps by fingers. Other tests include biopsy, ultrasound, MRI, and lab test for hormone receptors and HER2/neu receptors. Currently, most breast cancer could be diagnosed earlier than Stage 2, but only 20% of the cases could be diagnosed at stage 0. The issue is: in Stage 2, breast cancer cells

already spread into axillary lymph nodes, and the survival rate decreased. Therefore, new methods are needed to identify breast cancer when the cancer cells are confined to the primary site.

The current treatment of breast cancer includes surgery, radiation therapy, hormone therapy, chemotherapy, and targeted therapy. However, recurrence and high death rates are always a problem. A clinical research conducted for 20 years and published in 2002 claimed that radiation therapy significantly decreased the recurrence rate from 39.2% to 14.3% in women patients whose tumor specimen has disease-free margins. However, radiation therapy didn't decrease the death rate. The total death rate went up to 60%, and the death rate resulted from recurrence is about 40% ¹. The therapy of monoclonal antibody against HER2 positive breast cancer was efficient in a short period. In a clinical study of 3387 patients receiving surgery + radiation + chemotherapy, additional treatment of trastuzumab gave a 90% of disease free survival within two years compared to 80% in control group ². However, 2 year period was too short to make a judgment of drug efficiency, and this benefit cannot apply to HER2 negative breast cancer. Hormone treatment applies to hormone receptor positive breast cancer. In a clinical study of more than 6000 patients, hormone treatment after surgery + radiation + chemotherapy gave a 20% total death rate, 11% of death rate came from recurrence ³. There are no efficient treatments for HER2 negative/hormone receptor negative breast cancer. In sum, breast cancer has a high death rate according to current treatment. New treatment methods need to be developed for breast cancer.

1.2 G-protein coupled receptor-associated sorting protein 1 (GASP-1)

G-protein coupled receptor-associated sorting protein 1 (GASP-1) was identified in breast cancer for the first time in 2011⁴. A new protein separation technology, 2-D High Performance Liquid Electrophoresis (2-D HPLE), was developed in Dr. Chang's laboratory (US Pat 7,326,326). Using 2-D HPLE, serum albumin complexes were separated in the sera of breast cancer patients and normal control according to the isoelectrical points. After mass spectrometric analysis, a peptide representing 850-865 amino acids of GASP-1 was identified to be absent in normal controls, but present in breast cancer patients. Antiserum against this peptide was prepared by injecting the peptide into the rabbit and antibodies against this epitope were raised. By using this antiserum, clinical tissue lysates from breast cancer patients were analyzed by western blot. The result showed that breast cancer tissue lysates had a higher expression level of GASP-1 than lysates from adjacent normal tissue⁴. These results support the idea that GASP-1 peptide epitope has a higher expression level in the tumor and sera of breast cancer patients as compared to normal controls. Therefore, GASP-1 protein or peptide (850-865 a.a.) levels have a positive correlation with breast cancer, both in serum and tissue specimens.

In a study in 2004, Frederic Simonin tested the level of GASP-1 mRNA in tissue lysates of different human organs and tissues by northern blot. The results showed that GASP-1 mainly expressed in CNS such as brain, hippocampus, amygdala, etc. Other organs such as heart, lung, and liver do not have mRNA transcription. These results were verified by *in situ* hybridization on coronal mouse brain sections⁵. In 2005, Selena E. Bartlett detected GASP-1 expression in neuron dendrites and cell body by immunofluorescence⁶.

In 2007, Lene Martini detected GASP-1 protein expression in rat striatum, hippocampus and dentate gyrus/hilusregion ⁷ by immunofluorescence. In summary, GASP-1 was expressed mainly in CNS in healthy, non-cancerous animals and human.

The first paper that identifies GASP-1 was published in Science in 2002 by Dr Jenifer Whistler ⁸. She identified the proteins that were involved in trafficking membranous delta-opioid receptor (DOR) into lysosome for degradation. She used yeast 2-hybrid system with C-terminal tail of the DOR as the bait to scan HEK293 cDNA library. Many clones were identified to be bound by DOR. Sequencing of these clones revealed that these sequences correspond to the same C-terminal of a predicted protein. Upon validation by affinity chromatography, she named this protein as “G-protein coupled receptor-associated sorting protein one” (GASP-1). Further functional studies showed that inhibition of GASP-1 by dominant negative mutants blocked the trafficking of DOR into lysosome and degradation. Her study concluded that GASP-1 was a cytoplasmic protein that binds to DOR, facilitating its lysosome translocation and degradation.

In the recent decade, more and more GASP-1 binding receptors were discovered by affinity chromatography and immunoprecipitation. Almost all of these receptors were G-protein coupled receptors, including kappa-opioid receptor (KOR), Mu-opioid receptor (MOR), beta-1-adrenergic receptor (beta-1-AR), dopamine receptor 2 (D2R), cannabinoid receptor 1 (CB1R), GPR55, etc ^{5, 6, 7, 8, 9, 10}. However, only DOR, D2R, CB1R and GPR55 were proved to be regulated by GASP-1 for lysosome translocation and degradation ^{6, 7, 8, 10}. In animal studies, GASP-1 was shown to down-regulate D2R upon ligand treatment. For example, in an electrophysiological study in 2005, dopaminergic neurons of the ventral tegmental area (VTA) in rat brain were tested for

hypopolarization. Treatment of D2R ligand for the second time on these neurons does not induce hypopolarization. However, after anti-GASP antibodies were delivered into the cell via patch pipette, 2nd hypopolarization was recovered, indicating GASP-1 promoted translocation and degradation of D2R, which de-sensitized the neurons in response to D2R ligands ⁶. In another study in 2010, GASP-1 knock-out mice were generated. Treatment of cocaine decreased the D2R protein level in striatal tissue in wildtype mice, but knock-out GASP-1 mice recovered the protein level of D2R, indicating GASP-1 promoted the degradation of D2R *in vivo* ¹¹. As a consequence, both *in vitro* and *in vivo* studies showed that GASP-1 promoted the lysosome translocation and degradation of G-protein coupled receptors such as D2R, CB1R, DOR, and GPR55.

GASP-1 occurred very early in sera and tissue specimens of breast cancer patients. Can I use it as a cancer marker? If so, I can detect breast cancer at a very early stage by just analyzing its serum level for diagnosis of breast cancer. Early detection will greatly increase the survival rate of the patients. On the other hand, what is the biological function of GASP-1 in breast cancer? Since it is significantly over-expressed in breast cancer cells at a very early stage, it may contribute to breast cancer tumorigenesis, and thus, might be developed into a new therapeutic target. In this study, I report that GASP-1 contributed to tumorigenesis in breast cancer cell lines and has a great potential to be used as a tumor marker in breast cancer diagnosis.

1.3 Epithelial to mesenchymal transition (EMT)

Epithelial to mesenchymal transition (EMT) process is an important process in both tissue/organ development and cancer progression, during which the epithelial cells loses

polarity and cell-cell adhesion, while acquiring migratory and invasive characteristics ¹². In clinical studies, EMT has been characterized at the invasive front of various types of cancer by either morphological or molecular evidence, and some EMT markers such as Snail and Slug correlated with disease relapse and poor clinical outcomes ¹², indicating EMT may play an important role in cancer metastasis and even tumorigenesis. In tissue culture studies, EMT had been proven to promote self-renewal ability; by generating cancer stem cells in breast cancer cell line ¹³, indicating EMT process might influence cancer cell proliferation, migration, and colonization in a new microenvironment.

EMT process could be activated by some transcription factors, including snail, slug and ZEB2 ¹⁴, and therefore, the mRNA and protein levels of these transcription factors can be used to determine an active EMT process ^{15, 16, 17, 18, 19}. Vimentin is type III intermediate filaments. During EMT, vimentin expression increased. During the reverse process called MET(mesenchymal to epithelial transition), vimentin expression is decreased. Therefore, vimentin, which mainly expressed in mesenchymal cells, has been used as a marker for mesenchymal cell state and EMT process ²⁰. N-Cadherin is normally expressed in mesenchymal cells and is over-expressed in many invasive cancer types ¹⁴, and thus is also applied as a mesenchymal marker in some cancer studies ²¹. These transcriptional factors were usually used as EMT markers to show EMT activation, and were often correlated with cell migration, proliferation, and colony formation in soft agar assays.

There are many cancer related signaling pathways regulating EMT markers. For example, inhibition of c-Src by siRNA decreased vimentin, slug and ZEB2 protein level in MDA-MB-231 and MCF-7 breast cancer cell line, indicating c-Src pathway contributed to

EMT activation²². ZEB2 was a downstream target of NFκB in breast cancer cells¹⁶. Wnt and TGFβ were required to activate and maintain EMT markers in human immortalized epithelial cells (HMLE) and human breast cancer cell line MDA-MB-231^{23, 24}. IGF-1 was proven to contribute to EMT in human benign mammary epithelial cells (HME)²⁵. ERK signaling pathway regulated cell migration through slug expression level in a breast cancer cell line²⁶. p53 inhibited EMT and cell invasiveness through inhibition of Slug in MCF-7 breast cancer cells, but mutant p53 lost this inhibition²⁷. These research works proved that many tumorigenic signaling pathways regulated EMT markers, and thus suggest that EMT markers may play a role in tumorigenesis.

EMT markers have been proven to regulate cell proliferation, migration, invasion and soft agar colony formation in many cancer types. For example: SIP1 (ZEB2) induced vimentin expression (both mRNA and protein) in breast cancer cells²⁸, while vimentin was necessary for wound healing migration²⁹. Snail mediated E-cadherin methylation was necessary for EMT³⁰. Snail induced EMT, increased cell invasion, but decreased cell migration and soft agar colony formation in SCC-9 cells³¹. Snail and slug increased cell invasion in a TGFβ dependent manner in breast cancer cells³². SLUG induced EMT through its SNAG domain³³. Slug increased the cell migration ability in pancreatic cancer cells through metalloproteinase activity³⁴. Slug positively regulated cell proliferation, migration, and *in vivo* tumorigenesis in esophageal cancer cell line³⁵. Over-expression of Slug increased cell proliferation, invasion, migration, and *in vivo* tumor growth in human glioblastoma cells³⁶. Over-expression of slug increased cell invasion in bladder cancer cell lines 5637³⁷. Over-expression of Slug increased migration and invasion in human prostate cancer PC3 cell line³⁸. On the other hand, down-

regulation of slug decreased cell migration, invasion, and lung metastasis (in vivo) in MB231 cells ²⁶. Knocking down Slug abrogated IK1-mediated increases in migration and invasion in ovarian cancer cells ³⁹. These results suggest that EMT markers regulated tumor growth and metastasis, and thus contribute to tumorigenesis.

In summary, EMT markers are involved in the tumorigenesis process, and might be a downstream effector of many tumorigenic signaling pathways such as p53 mutation, ERK, TGF β , Wnt, etc. The question is: Are EMT markers downstream targets of GASP-1?

1.4 NF κ B signaling pathway

NF κ B had been shown to be activated in breast cancer patients. In a study involving 7 breast cancer patient samples, all samples showed higher NF κ B activity, as detected by EMSA, than normal adjacent tissue ⁴⁰. Another study analyzing 6 breast tumor patients showed that ER negative patients are more likely to have higher NF κ B activity, indicating a positive correlation between NF κ B and tumor malignancy ⁴¹.

NF κ B pathway had been proven to be a tumor promoter in animal experiments. In rats, DMBA-induced mice mammary tumor shows an elevated level of NF κ B activity, as detected by EMSA ⁴². The most definitive evidence showing NF κ B tumorigenicity in breast cancer is Debajit's work ⁴³. He over-expressed IKK β dominant negative to inhibit IKK, which then stabilize I κ B and abolish PMA induced NF κ B activation. After injecting this stable transfected cell model, the tumor volumes were determined. The results were: inhibiting NF κ B reduced tumor volume *in vivo*, and this reduction depends on the level of NF κ B inhibition.

NFκB is activated in breast cancer cell lines. For example, breast cancer cell lines showed higher NFκB total activity than non-transformed MCF-10F breast epithelial cell line as determined by IKK activity assay ⁴⁴. The amount of NFκB does not change as determined by western blot. But the level of IκB, an NFκB inhibiting protein, is low in ER- breast cancer cells and a higher activity of NFκB is detected, indicating a positive correlation between malignancy and NFκB activity ⁴¹. Breast cancer cell lines have high levels of nuclear NFκB activity as determined by EMSA. The increase of NFκB activity mainly comes from NFκB subtype p65, as determined in SKBR3 cell lines by EMSA, indicating canonical pathway activation is predominant in breast cancer cell lines ⁴⁰.

In this study, I want to test whether NFκB activity is regulated by GASP-1. Western blot on the phosphorylated NFκB p65 and NFκB luciferase assay was performed, in MDA-MB-231 cells under-/over-expressing GASP-1.

1.5 Src signaling

Src signaling is an important tumorigenesis signaling pathway. It was summarized by Ricardo H. Alvarez (2006) ^{45,46}. Viral Src (v-Src) was first identified from Rous sarcoma virus to induce solid tumor in birds. Later, researchers found out an intracellular counterpart, the proto-oncogene c-Src. C-Src influences proliferation, survival, migration, and angiogenesis, but is insufficient to induce tumor in human cell lines. Numerous human malignancies display increased Src expression and activity (colon, breast, lung, ovarian, esophageal, gastric, and pancreatic cancer). Y530 phosphorylation inactivated Src by association of different domains and thus folding of the protein. Dephosphorylation of Y530 and autophosphorylation of Y419 activates Src. Src was involved

in many cancer related signaling. Growth factor RTK activates Src. Src signaling increases HER2/HER3 hetero-dimer. Src is required for PDGFR signaling. SRC maintains cyclin D1 and myc expression through activating beta-catenin. Src positively correlated with VEGF expression and IL-8. MDA-MB-231 cells stably over-expressing SRC increase bone metastasis in mice model. Src has become a good potential target for cancer treatment. In this study, I wanted to test whether the activity of Src was regulated by GASP-1 in breast cancer cells.

1.6 GPR30

Edward J. Filardo, carried out a clinical case study. 321 breast cancer tissue specimens were analyzed in his study. Among the 122 ER negative samples, half of them are GPR30 positive, indicating GPR30 may be an important ER receptor in estrogen negative breast cancer cells ⁴⁷. In tissue culture experiments, GPR30 regulates ERK phosphorylation by two different pathways. Activation of GPR30 activated ERK phosphorylation by releasing HB-EGF and activating EGFR. On the other hand, upon EGF treatment, GPR30 inhibited ERK phosphorylation by activating PKA and cAMP signaling. GPR30 expression is discovered in MCF-7 cells, with 60kDa of glycosylated protein and 30kDa non-glycosylated protein ⁴⁸. However, its expression in MDA-MB-231 cells is still contradictory. GPR30 was first identified in 1997 by Carmeci C ⁴⁹. By using cDNA library, he identified a gene that is overexpressed in ER+ MCF-7 cell line rather than ER- MDA-MB-231 cell line. This expression pattern was further validated by western blot. On the other hand, a group of Japanese research detected GPR30 by western blot in MDA-MB-231 cells, as well as MCF-7 and SKBR-3 cells ⁵⁰. In this study, I tested GPR30 expression in MDA-MB-231 cells by western blot. With the help

of LICOR highly sensitive western blot system, I hope to verify whether GPR30 is expressed in MDA-MB-231 cells and whether its expression level is regulated by GASP-1.

1.7 Competitive ELISA

The method I used to quantify GASP-1 peptide level in patient sera is called “competitive ELISA”. I developed this method specially for detecting GASP-1 peptide epitope. The basic idea was as follows: Sera and antibody were reacted in solution with the antibody present in excess. The resulted solution containing antibody-antigen complex and excess free antibody was incubated with immobilized antigen in a microplate. Free antibody bound to the immobilized antigen, and was detected by a secondary antibody that generated the signal. This method would give an inverse relationship to signal: the higher the antigen concentration was in the sample, the lower the signal will be. The first point in the standard curve will be 0ng/ml, which produce the highest signal. This method has very high sensitivity, because antigen and antibody were reacted in the solution so that researcher does not have to worry about the efficiency of antigen immobilization. However, in order to perform this method efficiently, the optimal antibody concentration and amount of immobilized antigen have to be determined for a specific antigenic protein. In this study, I developed a competitive ELISA detecting GASP-1 levels in sera of cancer patients.

1.8 Objectives and hypothesis

The first goal of this study is to discover and validate the correlation between GASP-1 and breast cancer in clinical samples. The second goal is to study tumorigenicity and signaling pathways mediated by GASP-1 in MDA-MB-231 breast cancer cell line.

The hypothesis is: GASP-1 promotes breast cancer tumorigenesis in the MDA-MB-231 breast cancer cell line, and that epithelial to mesenchymal transition transcription factors may mediate GASP-1 promoted tumorigenesis.

Rationale:

GASP-1 is overexpressed in both sera and tissue specimens of breast cancer patients, indicating GASP-1 may play a role in breast cancer tumorigenesis. EMT transcription factors have been reported to be regulated by these tumorigenic signaling pathways, and contribute to cancer cell proliferation, invasion, migration, and colony formation, suggesting EMT transcription factors may be involved in breast cancer tumorigenesis mediated by GASP-1.

CHAPTER 2: MATERIALS AND METHODS.

Synthesize GASP-1 peptide:

GASP-1 peptide EEASPEAVAGVGFESK (850-865a.a. of GASP-1 amino acid sequence) had been identified from breast cancer patient sera by 2-dimensional high performance liquid electrophoresis (2-D HPLC) analysis (Tuszynski and Chang, 2011). This peptide was synthesized by Covance, Princeton, NJ.

Prepare GASP-1 antisera:

GASP-1 antiserum was prepared by Covance, Princeton, NJ. Briefly, a rabbit was immunized with the GASP-1 peptide (850-865a.a. of GASP-1 amino acid sequence) and the serum containing polyclonal antibody against GASP-1 peptide was isolated.

Purify and biotinylate polyclonal anti-GASP-1 antibody:

Anti-GASP-1 polyclonal antibody was purified from GASP-1 antisera by Protein A/G (Pierce 20421) and biotinylated by EZ-link NHS-PEO4-Biotinylation Kit (Thermo Scientific 21455) following the manufacturer's instruction.

Competitive ELISA:

Sera of glioma patients were kindly provided by the Temple University Center of Neurovirology. Sera of lung cancer patients were kindly provided by Dr. Hossein Borghaei from the Fox Chase Cancer Center, Philadelphia, PA. Sera of liver cancer and cirrhosis patients were kindly provided by Dr. Anand Mehta, Drexel University School of Medicine. Sera of stage 2 and stage 3 breast cancer patients with controls were purchased and obtained from the Biomarker Shared Resource directed by

Dr. Christopher Loffredo, Associate Professor of Oncology & Biostatistics Leader, Cancer Genetics and Epidemiology Program Director, Clinical & Molecular Epidemiology Shared Resource, Georgetown University. Additional sera were obtained from normal healthy volunteers.

Competitive ELISA protocol detecting GASP-1 protein level was developed according to a previous competitive ELISA protocol detecting thrombospondin-1 in this lab (Tuszynski et al., 1992). Briefly, two ELISA strip plates were labeled “plate A” and “plate B”. The plate A was coated with 300ul 1% BSA in PBS solution for at least overnight at 4°C. The plate B was coated with 200ul 0.0625ug/ml GASP-1 peptide in PBS for 48-72h. Tris-buffered saline Tween20 (TBST) containing 0.5M NaCl was used in ELISA. Serum samples were diluted in TBST. The dilution factor was optimized from 1:10 to 1:480. The standard curve was prepared with GASP-1 peptide diluted in TBST. The standard solutions and diluted serum samples were incubated with equal amount of biotinylated antibody (1:25,000 dilution in TBST) at 4°C overnight in plate A (BSA coated). The next day, plate B was blocked with 1% BSA in PBS solution with shaking at room temperature for 30min. After washing plate B with 250ul TBST 5 times, an aliquot of 200ul sample-antibody mixture or peptide-antibody mixture was transferred from plate A into plate B. Plate B was incubated at room temperature for 90min with shaking. Free antibody in the mixture, which did not bind to GASP-1 in samples, will bind to the peptide immobilized on the plate B. Plate B was aspirated and washed with 5 times of 250ul TBST. The antibody bound to the plate B was detected by HRP-Streptavidin (Vector Lab SA5004, 1:54000 dilution in TBST) by shaking at room temperature for 60min. After washing 5 times with 250ul TBST, the plate B was

incubated with 100ul 1-Step Ultra TMB-ELISA (Thermo Scientific 34028) for 20min with shaking. The HRP enzymatic reaction was stopped by adding 50ul 1M HCl, and absorbance (450nm) was measured. The standard curve was generated by fitting the data to a one-site-total binding equation using Graphpad Prism version 5.01 (San Diego, CA). The GASP-1 epitope concentrations were calculated according to the standard curve.

Immunohistochemistry:

Brain cancer tissue array slides BS17016, breast cancer tissue array slides BR1003 and BR1503, and pancreatic tissue array slides PA2081 were purchased from US Biomax, Inc, Rockville, MD. Slides were deparafinized in xylene 3 times for 30min and rehydrated in ethanol gradient 100%, 90%, 70%, and finally in distilled water. The slides were then incubated in citrate buffer pH6.0 at 95°C for exactly 30min to break antigen cross links. After cooling for 20min at 4°C, the slides were washed 3 times with PBS and incubated in freshly made 6% hydrogen peroxide diluted in methanol for exactly 20min at room temperature. The slides were washed in PBS 3 times and then blocked at room temperature for 2h, in PBS containing 5% horse serum (Vectastain PK8800), 0.1% BSA, and 4 drops/ml of streptavidin (Vector SP-2002). Slides were washed in PBS 3 times, and incubated at 4°C overnight with GASP-1 antisera diluted (1:4000) in PBS containing 0.1% BSA and 4 drops/ml biotin (Vector, SP-2002). The next day, slides were washed 3 times with PBS, and incubated with Uni-kit biotinylated secondary antibody (Vectastain PK8800, 1 drop/ml in PBS containing 0.1% BSA) at room temperature for 1h. Slides were washed 3 times in PBS, and incubated with HRP-streptavidin (Vectastain PK8800) at room temperature for 30min. Slides were washed 3 times in PBS and color was developed using the DAB kit (Vector SK-4100), following manufacture's instruction.

After a brief PBS wash, the slides were counterstained with hematoxylin, dehydrated using reverse ethanol gradients: 70%, 90%, 100%, finally in dehydrated 2 times in xylene for 30min and mounted using mounting medium (Vector, H-5000). The slides were scored by a breast cancer pathologist (Dr. Xinmin Zhang, Department of Pathology and Laboratory Medicine at Temple University) using a staining index from 0 (weakest) to 3 (strongest). The scoring was conducted in a blinded manner. Staining scores were plotted and analyzed by a student T-test to calculate the statistics for each group. (Graph Pad Prism version 5.01). Two groups having a p value of less than 0.05 were considered statistically different.

Plasmid Construction:

shRNA-GASP-1 was constructed by Origene (Catalog number: TG312634). Briefly, sequences targeting GASP-1 were inserted into pGFP-V-RS shRNA vector (TR30007). The scrambled control vector is provided by the company (TR30013). The vector structure was shown in Figure 2. pGFP-V-RS plasmid has a short hairpin RNA sequence right after U6 promoter. The left and right sequences are complementary to each other. When this sequence is transcribed into RNA, the RNA will fold at the loop and the target sequences will complement with its RC (reverse complement sequence) to form a short hairpin structure. shRNA will bind to dicer, lose its loop structure, then bind

shGASP-1_29 (cat#: GI350529): TGGCAGATGAAGCCAGCATAGAGTCCAGT

shGASP-1_30 (cat#: GI350530): CCAGTCTACAAGTGGAGGATGAGTCCATA

shGASP-1_32 (cat#: GI350532): CATCCTGTAACTGCATACAATGTGAGCTG

GASP-1 over-expression plasmid was constructed by Origene. cDNA sequence of GASP-1 (transcription variant 1, NCBI accession NO.: NM_014710.4) was inserted into

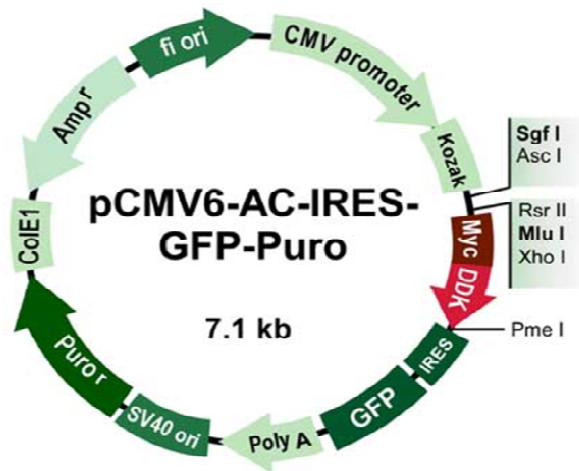


Figure 3. pCMV-AC-IRES-GFP-puro plasmid was applied to over-express GASP-1. The GASP-1 cDNA was inserted at Sgf-I and MLU I site, driven by CMV promoter. Myc-DDK tag was connected to the c-terminal of GASP-1. Internal ribosomal entry site (IRES) was applied to control GFP expression. Ampicillin and puromycin resistant gene is shown.

the pCMV6-AC-IRES-GFP-Puro vector (Origene PS100059) after CMV promoter. The protein was fused with myc-DDK-tag at the C-terminal. Internal ribosomal entry site was applied to control GFP expression. GASP-1 and GFP share the same mRNA, with IRES

in the middle. IRES is the binding site of ribosome in the middle of the mRNA, so that one mRNA contains two genes, under the control of two different promoters. The advantage of this design is that GFP still shows the expression level of GASP-1, but GASP-1 is only linked to a small myc-DDK tag, which had less possibility to influence GASP-1 function. The structure is shown in Figure 3

Cell culture and Stable Transfection

GASP-1 under-expression clones: MDA-MB-231 breast cancer cells were plated in 96 well plates in DMEM supplemented with 10% FCS. shRNA-GASP-1 was transfected with Fugene transfection reagent (Promega E2311). 48h post-transfection, cells were expanded from the 96 well plate into a 6 well plate containing regular DMEM. 24h later, media was changed to DMEM containing 0.6ug/ml puromycin and cells were grown for about 10 days until 95% confluent. Total RNA was isolated from each clone in order to identify GASP-1 down-regulation by RT-PCR.

GASP-1 over-expression clones: MDA-MB-231 cells were plated in 6 well plates. The GASP-1 over-expression plasmid and the control vector were transfected by Fugene HD Transfection Reagent (Promega E2311). 48h post-transfection, media was changed to DMEM containing 0.6ug/ml puromycin. Cells were in 6 well plates for 10 days to select for puromycin resistant cells. The cells were then serially diluted in 96 well plates until only one cell was seeded in each well. These clones were then grown to 95% confluency (with puromycin in the media). Clones were expanded in 6 well plates and then grown in 10cm dishes (with puromycin in media). The cell lysates were evaluated by indirect ELISA to identify those that over-expressed GASP-1.

RT-PCR:

Cells were plated in 6 well plates and grown to 95% confluency. Total RNA was isolated with Trizol reagent (Invitrogen, 15596-018) following the manufacture`s instruction. RNA concentration was determined by measuring O.D. at 260 nm using a spectrophotometer (NanoDrop, ND-1000) and the RNA concentration calculated using its software. RNA quality was controlled by running RNA samples on non-denaturing agarose gel electrophoresis for 18S, 28S rRNA and mRNA smears. Four ug RNA was applied to DNase I digest (Fermentas, EN0521) according to the manufacture`s instruction. 1.5 ug of digested RNA was applied for reverse transcription. The RT system contains: random primers (Promega C1181), M-MLV (Promega M1701), buffer (Promega, M5313), dNTP mix (Promega, U1511) and rRNasin (Promega, N2511). DNA contamination was monitored by running non-enzyme negative reverse transcription control. cDNA was applied for PCR amplification. Proper dilutions of cDNA were optimized. PCR master mix was purchased from either BioRad (cat#: 172-5121). PCR primers were listed in the table with respective T_m and product size ^{19, 22, 51}[Primer 3 website]. The PCR program was optimized as: 95°C 30s (for BioRad master mix) or 10min (for Agilent master mix), 33 cycles of amplification, 95°C 30s, T_m for 1min, and 72°C for 1min (all products are less than 500bp). An additional 72°C for 3min is added at the end of the last cycle. All products were applied to DNA agarose gel electrophoresis with 2% gel. The 50bp gene ruler DNA ladder was purchased from Fermentas (SM0373). Pictures were taken by Gel Logic 112 camera system (Kodak). For qPCR, samples were run in the LightCycler 480 (Roche), the program was run for 45 cycles. The relative quantification was calculated by its software.

Table 2. RT-PCR primers

Name	sense	antisense	Tm	Product size:
Vimentin	ctcttccaaacttttctccc	agtttcgttgataacctgtcc	52	134bp
ZEB2	caagaggcgcaacaagc	ggttggcaataccgcatcc	55	128bp
Slug	cgcctccaaaaagccaaac	cggtagtccacacagtgatg	55	149bp
Snail	cgaaggccttcaactgcaaat	actggacttcttgacatctg	50	262bp
GAPDH	tgatgacatcaagaaggtggtgaag	tccttgaggccatgtgggcat	56	240bp
GASP-1	tggttgctgctgttctacactcta	ccagtactatactcctccccagt	56	213bp
N-Cadherin	aagacaaagagaccaggaaaagt	ccagtctcttctgcctttgtag	56	164bp

Western Blot

Cells were grown in 6 well plates until 95% confluency and lysed with Mammalian Protein Extract Reagent (M-PER, Thermo scientific, 78501) containing Halt Protease and Phosphatase inhibitor Single – Use Cocktail (Thermo Scientific 1861280). Protein concentration was determined by BCA method (Pierce 23224 and 23221) following manufacturer’s instructions. Samples were denatured in Laemmli buffer at 100°C for 5min. Urea was added to insure denaturation of protein larger than 100kDa. Samples containing 20-30ug of total lysate were separated on SDS-PAGE (10-12% gel for proteins less than 100kDa, 7% gel for proteins larger than 100kDa), transferred to PVDF membrane (Thermo Scientific 88518) in Tris-Glycine buffer (pH8.3) at 4°C, 40V, overnight. The membrane was blocked by Odyssey Blocking Buffer (LICOR). The

primary antibody includes: anti-GASP-1 antibody (proteinTECH). Anti-Slug antibody was purchased from Cell signaling (cat#: 9585). Anti-N-cadherin antibody was kindly provided by the Neuroscience Department of Temple University School of Medicine. Anti-Vimentin was purchased from Calbiochem (cat#: IF01). Primary antibody incubation conditions were applied according to manufacturer's instructions, respectively. The primary antibody was detected by IR-dye conjugated goat anti-rabbit or goat anti-mouse secondary antibody (LICOR), and was visualized by the Odyssey Imaging system (LICOR). Beta-actin staining was used to determine loading levels. To perform the screening western blot, the 12 wells of the 15 well western blot comb was taped with regular tape, so that all 12 wells became one big well. The SDS-PAGE gel was prepared to have one well for protein marker, and one big well for the sample. 340ug total protein lysate of MDA-MB-231 cells was loaded in the big well. After electrophoresis, transfer, and blocking, the membrane was fixed in the multi-channel western blot chamber (Immunetics) according to the instruction manual. Pyronin Y was added into the Laemmli buffer ahead of time to track the position of the sample on the membrane. Different antibodies were prepared and added into the channels on the chamber, so that one big sample was analyzed by different antibodies solutions at the same time. Secondary antibody and signal detection was accomplished as described above.

Dot Blot:

1-2ug antigenic peptide, 1-2ul MDA-MB-231 lysates (3ug/ul), or 1-2ug BSA was spotted on methanol treated PVDF membrane. Antibody or antigen blocked antibody was applied and incubated for 2h at room temperature. Either biotinylated antibody or antiserum was used in experiments. Biotinylated antibody was detected by HRP linked

streptavidin (Pierce, 1:20 000 dilution). Antiserum was detected by HRP linked goat anti-rabbit secondary antibody.

Amplifying of Plasmid:

All plasmids were transformed into E.coli and cloned according to Origene`s instruction manual. DNA mini-preparation (Qiagen, 27106) was used to validate cloning efficiency. Maxi-preparation (Qiagen 12163 and 12263) was used to produce enough plasmid for transfection. BamH1 and HindIII restriction digestion was applied to verify the insertion of DNA fragments.

Cell Proliferation Assay:

Cells were plated in 96 well plates, 2000 cells in 100ul DMEM complete media per well for triplicates. 20ul of MTS reagent (Promega) was added to each well. Cells were incubated at 37°C for 1h. Absorbance at 490nm was measured on day 0, 2, 4, 6 and 8, using a spectrophotometer, and was plotted on a graph. Standard deviation was calculated from triplicate readings. Media control without cells were used to blank the absorbance.

Boyden Chamber Migration Assay:

100,000 cells were plated in 24-well Millicell hanging cell culture inserts (Millipore, 8.0um pore size, tissue culture treated, PIEP12R48), in 200ul serum-free DMEM supplied with penicillin/streptomycin. 1000ul serum-free DMEM was added to the bottom chamber. Plates were incubated at 37°C for 4h. Cells in both the upper and lower chambers were fixed with 2.5% Glutaraldehyde-PBS solution for 30min at room

temperature, and stained with 0.5% Crystal Violet in 25% methanol overnight at room temperature. After a water rinse, cells on the upper chamber were wiped off by a cotton swab. The cells on the lower side of the membrane were counted using a normal optical microscope. 5 views were counted and standard deviations were calculated.

Wound Healing Assay:

Aliquots of 300,000 cells were plated in 6 well plates in DMEM complete media. After 4h, cells attached to the bottom of the plate. Scratches were made by 1ml pipet tips, and media was replaced with fresh media to remove detached cells. Plates were incubated for 24h at 37°C in 5% CO₂. Pictures were taken at both 4h and 24h under the same magnification. Pictures were aligned on the same paper, with the same amplification, and printed out on the same paper. Migration fronts were drawn by hand and the distance between the two migration fronts at 4h and 24h were measured with a ruler. The relative migration was calculated as the width at 4h minus the width at 24h. The relative migration was plotted on the graph.

Soft Agar Assay:

DMEM containing 0.5% melted Noble Agar (Sigma A-5431) was loaded in 6 well plates as the bottom agar layer. A cell suspension of 2000 cells was mixed with DMEM containing 0.35% melted agarose (Fisher BP1356-500) at 40°C, and loaded on top of the bottom agar. After the top agarose solidified, 1ml of DMEM media was added to the top of the agarose layer to prevent drying. 15 days later, colony number was counted in 8 fields, and colony size was determined by eye. Photos were taken under PHASE contrast of the fluorescent microscope (Nikon).

Flow Cytometry:

A total of 500,000 cells were trypsinized and washed 3 times with Stain Buffer (Thermo scientific, 554657). The cells were then pelleted at 300g for 5min and vortexed a little bit, and fixed in cytofix/cytoperm (Thermo scientific) buffer for 35min. The cells were washed two times with cytoperm/wash buffer (Thermo scientific), and incubated with anti-CD44 antibody (BD pharmingen) at 0.03ug per 100ul perm/wash buffer, on ice for 30min. After washing the cells 2 times with perm/wash buffer, cells were resuspended in stain buffer and CD44+ cells measured by flow cytometry (Quavaminicyte). The percentage of CD44+ cells were plotted on a graph and compared to that measured for allcell clones and controls.

NFκB Luciferase Assay

NFκB promoter-adenovirus was kindly provide by Dr. Hu (Department of Neuroscience, Temple University). 10,000 cells were plated in 96 well tissue culture plates. The following day, replace media with 10% FCS-DMEM containing NFκB promoter adenovirus. The multiplicity of infection (MOI) equals to 50. Incubate 6-8h, followed by replacing the media with or without 10% FCS-DMEM containing 10ng/ml TNFα. The following day, lyse the cells in 100ul lysis buffer (20mMTris·HCl pH7.4, 1mMEDTA, 150mM NaCl, 1% Triton X-100) for 3min at room temperature. 40ul were aliquoted into 2 separate white plates. 40ul of OneGLO reagent (Promega) and CellTiter reagent (Promega) were added into these two well respectively. The luciferase signals were read by WALLAC EnVision 2104 Multilabel Reader (PerkinElmer). Luciferase signals were normalized to the respective cell titer signal, the normalized again to the scrambled

control or vector control, respectively. Four replicated wells were performed in each experiment.

CHAPTER 3: RESULTS

3.1 GASP-1 epitope has a higher serum level in cancer patients rather than normal control.

GASP-1 was identified in the serum of breast cancer patients by 2-D HPLC, but not in normal controls. This result suggested that GASP-1 is a potential new cancer marker. However, further validation needs to be carried out to provide stronger evidence, showing GASP-1 serum level is positively correlated with cancer. Therefore, I used antisera against GASP-1 peptide identified in the mass spec analysis of new cancer serum albumin complex from breast cancer patients, and developed a competitive ELISA to measure GASP-1 peptide levels in serum samples from various cancer types and controls. I hope to validate that GASP-1 epitope has a higher level in cancer sera rather than normal controls, and this higher level is present in cancer only, not other diseases.

3.1.1 Antibody characterization

The first step in developing a competitive ELISA method is to use an appropriate antibody directed against the GASP-1 epitope identified from the 2D-HPLC analysis. I used a dot blot assay to validate the immune-specificity of the antiserum. I used the antigenic peptide as the “blocking peptide” to block the immunoreactive site of the antiserum (Figure 4A). The antibodies were pre-incubated with different amounts of antigenic peptide at 4°C overnight, and then applied to the antigenic peptide spots adsorbed on the methanol-treated PVDF membrane. The results showed that pre-incubation of antigenic peptide can block the immunoreactivity of antiserum in a dose-dependent manner, indicating that our antiserum is specific to peptide epitope of GASP-

1, identified by 2D-HPLE. Non-immunized serum was applied as a control to exclude any non-specific signals resulting from serum components.

In order to make our competitive ELISA assay more sensitive, IgG was purified from anti-GASP-1 antisera by protein A/G, and was linked to biotin using a biotinylation kit from Pierce. The resulting biotinylated antibody was characterized by doing dot blot experiments (Figure 4B). Three different protein samples were spotted on methanol-treated PVDF membranes: 1mg/ml BSA solution, 1mg/ml GASP-1 antigenic peptide, and cell lysate (3mg/ml) of MDA-MB-231 breast cancer cell line. The horse-radish peroxidase (HRP) linked streptavidin was applied to detect the biotinylated antibody. The results showed that BSA does not have immunoreactivity with biotinylated antibody. Both antigenic peptide and breast cancer cell lysate show strong immunoreactivity, indicating biotinylated anti-GASP-1 antibody specifically targets the peptide epitope of GASP-1. Taken together, these two dot blots validated the specificity of biotinylated antibody against GASP-1 epitope (a.a. 850-865), and thus this antibody was used in the following ELISA experiments.

To further validate the specificity of GASP-1 antiserum for western blot application, MDA-MB-231 cell lysate was analyzed by blocking peptide absorption assay by using multichannel western blot chamber as described in materials and methods. Addition of blocking peptide completely abolished the bands showed in the GASP-1 antiserum lane, indicating all of those bands containing GASP-1 antigenic peptide fragment. Secondary antibody control and control IgG lanes did not show any bands, excluding the possibility of non-specific bands resulted from IgG non-specific binding (Figure 4C).

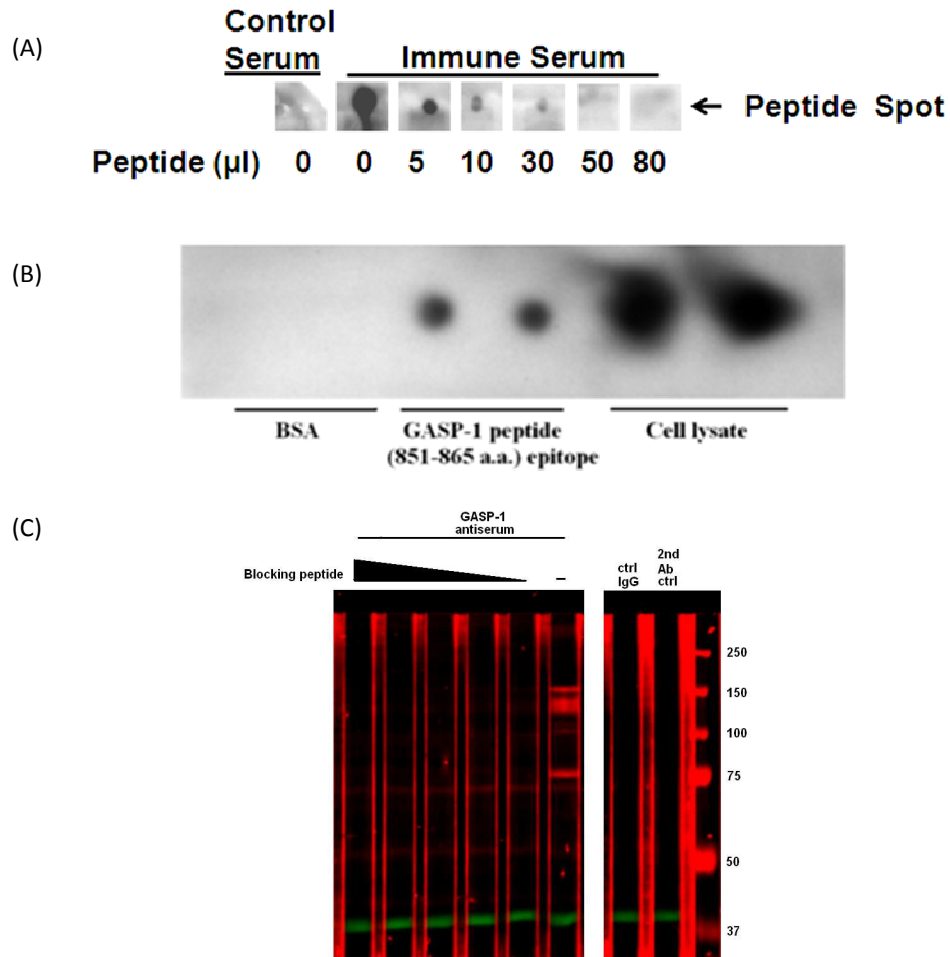


Figure 4. Characterize the biotinylated antibody. (A) Blocking peptide experiment. Antiserum was first incubated with different volume of 1 mg/ml antigenic peptide and applied to antigenic peptide spot on the membrane. Non-immunized serum is the control. (B) Dot blot of BSA, antigenic peptide, and MDA-MB-231 breast cancer cell lysate. Biotinylated antibody was detected by HRP linked streptavidin. (C) MDA-MB-231 cell lysate was analyzed by indicated antibodies. A multi-channel western blot chamber was used. The red background between the two lanes resulted from tight sealing by the chamber. Green color is the beta-actin.

3.1.2 Checker Board Titration and optimal analysis range

I characterized the best combination of antibody dilution and the amount of antigenic peptide immobilized on the ELISA microplate. The general steps of ELISA are in Figure 5A. Figure 5B showed a sample standard curve of competitive ELISA. The 0ng/ml peptide had the highest signal, while the 32ng/ml peptide had the lowest signal. The larger the absorbance range, the more sensitive the method will be. To get the largest absorbance range, the first point of the standard curve (0ng/ml) should be as high as possible. According to the measurement range of the spectrophotometer, I preferred the absorbance (450nm) to be approximately equals to 1.0. There were two factors that controlled the absorbance (450nm) of the first point: the antibody concentration and the amount of the peptide immobilized on the ELISA microplate. Therefore, I modified these two factors to acquire the highest signal for the first point of the standard curve. A checker board titration was performed (Figure 5C). A serial dilution of biotinylated antibody was applied into microplate wells that contained different amounts of immobilized antigenic peptides. After incubation, the microplate-bound biotinylated antibody was detected by HRP linked streptavidin and then TMB system. Absorbance at 450nm was measured. A series of graphs was created by plotting absorbance 450nm vs. antibody dilution factors. Each amount of immobilized antigenic peptide had a separate curve (Figure6). The higher the antibody dilution factor is, the lower the absorbance is at 450nm. The less the immobilized peptide, the smaller the absorbance 450nm values were and the sharper the curve was. I picked up all points on the curve that give a 450nm absorbance of 1.000, and extrapolated their respective combinations of antibody dilution factors and amount of immobilized antigenic peptides.

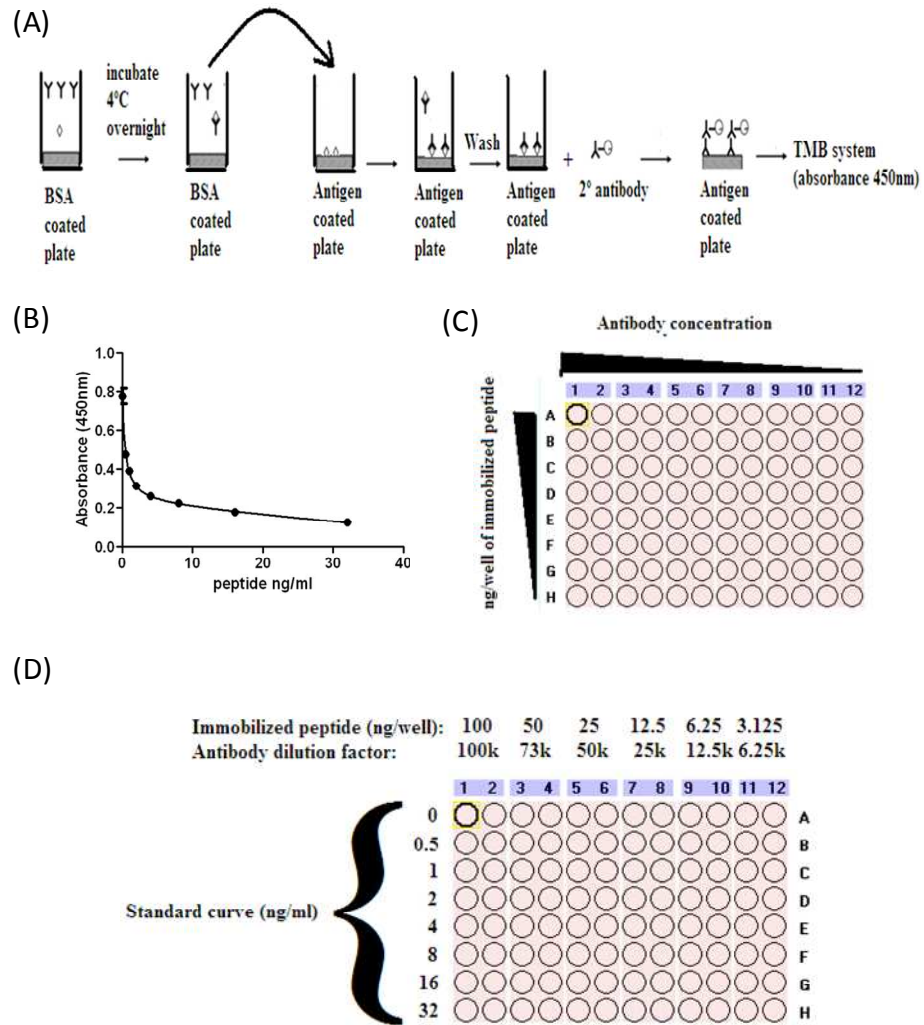


Figure 5. The strategy of developing competitive ELISA. (A) flow chart of competitive ELISA. (B) A sample of standard curve of competitive ELISA. (C) checker board titration. The black triangles represent decreasing antibody concentration and decreasing immobilized peptide amount respectively. (D) Identifying the best analysis range. The numbers on the left are the antigenic peptide (ng/ml) in the standard curve. The numbers on the top represent the combinations of immobilized peptide amounts and antibody dilution factors.

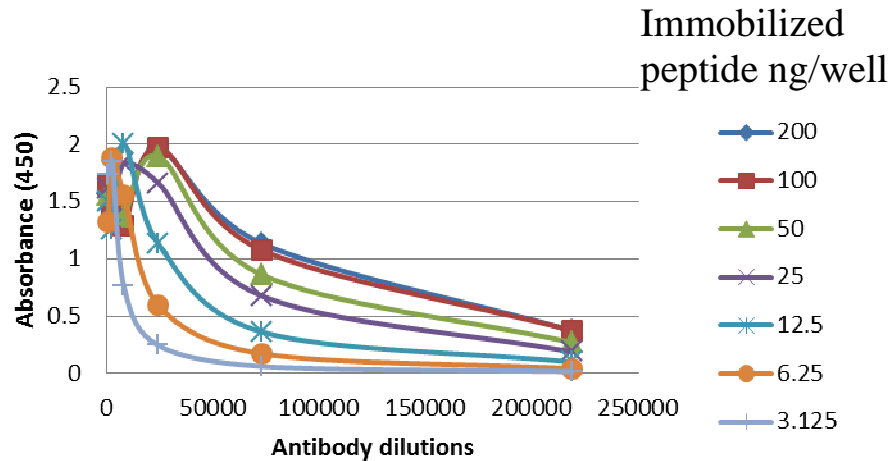


Figure 6. Checker board titration is applied to find out the best antibody concentration and amount of immobilized antigenic peptide. Antibody was incubated with antigen-coated microplate for 1.5h, and developed by HRP-streptavidin and TMB system. Absorbance vs. different antibody dilution factors were plotted for each peptide amount. Any combination of antibody and antigen that gave an absorbance of 1.0 was selected.

The best analysis range for the standard curves was then identified. As shown in Figure 5B, the first point in the standard curve was close to 1.0. In order to make the absorbance range as wide as possible, I also had to try all of the antibody-peptide combinations acquired from checker board titration on the same standard curve (Figure 5D). I wanted to find out the combination that gave out the largest absorbance range (450nm). As is shown in the Figure 7A, 12.5ng/well immobilized antigenic peptide on the microplate, with 1:25,000 dilution factor of biotinylated antibody, gave the highest absorbance range (from 0.15 to 0.8). Therefore, this combination of antibody dilution factor and amount of immobilized antigenic peptide was applied to future ELISA assays. The final standard

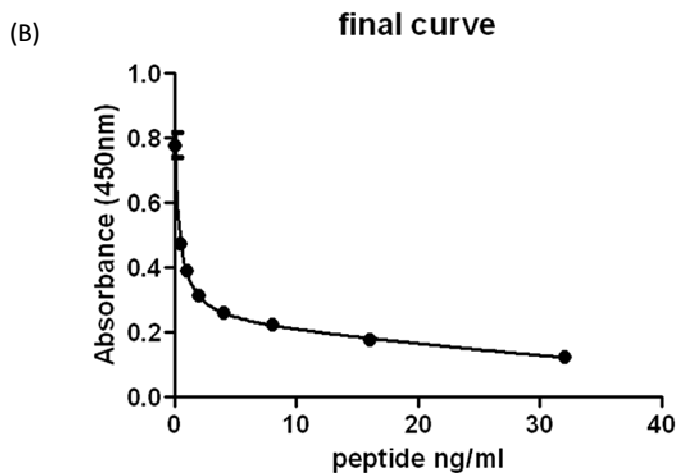
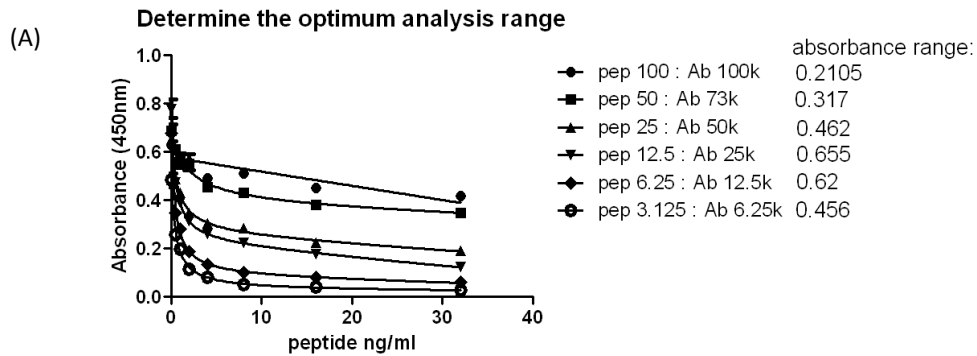


Figure 7. Determine the optimum analysis range. (A) Six combinations that gave an absorbance 450nm of 1.000 were used to generate standard curves. The resulting curves were plotted on a graph. The one that gave the largest Absorbance range is the best curve for future experiments. (B) A representative standard curve. All points are in triplicates.

curve was shown in Figure 7B. This standard curve was performed for ELISA to calculate all the samples tested within the same experiment, so that the influence of environmental factors such as temperature and humidity were eliminated.

3.1.3 Detect GASP-1 epitope level in sera from cancer patients and controls.

The final step is to quantify the GASP-1 epitope level in the sera of cancer patients and normal controls. The ELISA method was performed to quantify the GASP-1 epitope level as follows: 12.5 ng antigenic peptide was immobilized in each well of the ELISA microplate, and 1:25,000 dilution factor of biotinylated antibody was applied. A standard curve was performed for each experiment. Serum samples from various types of cancer were analyzed, including brain cancer, breast cancer, lung cancer and liver cancer. GASP-1 epitope levels were calculated according to the standard curve. The resulting values were plotted in the graph (Figure 8). Median values and P values were calculated by the computer software Graphpad. The results showed that normal control serum had a low level of GASP-1 epitope. In comparison, all cancer types had significantly higher levels of GASP-1 epitope, and their values were similar to each other. This result suggested that GASP-1 is over-expressed in the sera of cancer patients, but not normal controls. GASP-1 is not a breast cancer specific marker, but might be a potential general cancer marker for multiple cancer types. To validate GASP-1 as a potential cancer marker, not just a marker for any kind of disease, serum samples from cirrhosis patients were also tested. Interestingly, GASP-1 epitope level in the sera of cirrhosis patients was similar to normal controls, and was significantly lower than the level in liver cancer patients. This result indicated that GASP-1 was a cancer marker, not just a general

disease marker. Taken together, I validated the positive correlation between GASP-1 and cancer.

GASP-1 is over-expressed in cancer patients as compared to normal control and other non-cancerous disease types, and has the potential to become a general cancer marker in clinical applications.

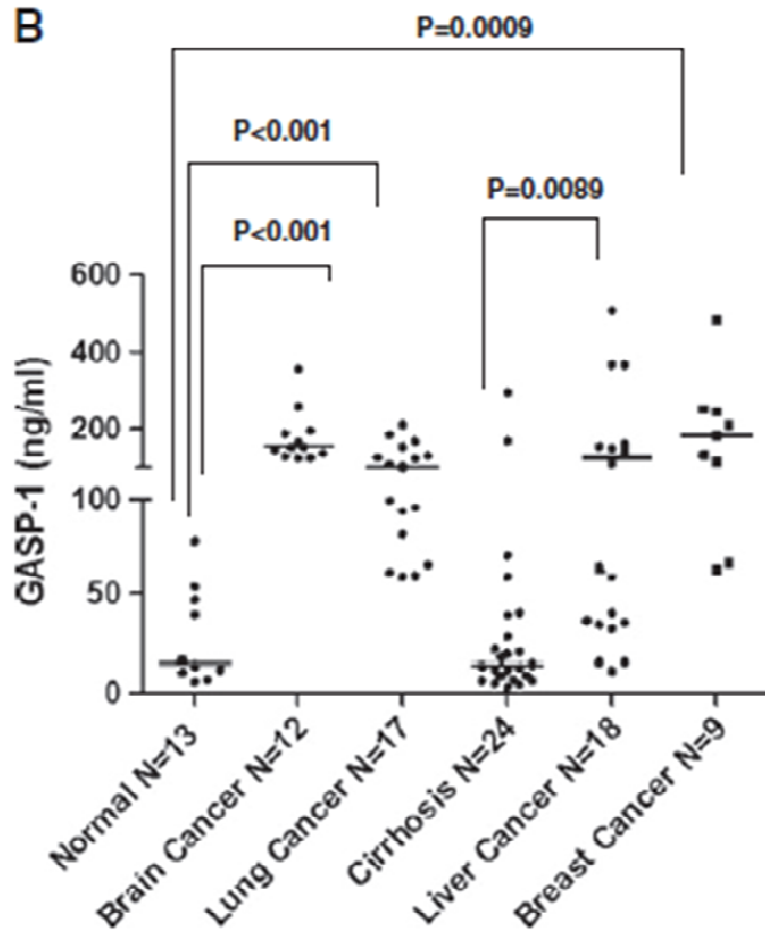


Figure 8. GASP-1 is over-expressed in the sera of cancer patients.

Competitive ELISA was performed to test serum samples of cancer patients, non-cancerous patients, and normal controls. The resulting values were plotted. Median and P values were calculated by computer software Graphpad.

3.2 GASP-1 peptides containing the epitope discovered by 2D-HPLE is more highly expressed in cancer cells than in normal cells.

GASP-1 epitope has a higher level in cancer patient sera, but I don't know its expression level inside the cell. If I hypothesize GASP-1 promotes tumorigenesis, then it will be natural to predict GASP-1 has a higher expression level intracellularly in cancer cells rather than normal controls. Therefore, I tested GASP-1 expression levels by immunohistochemistry in tumor tissue specimens from cancer patients and normal tissues from controls.

3.2.1 GASP-1 is over-expressed in tissue specimens of breast cancer patient

GASP-1 was first detected in breast cancer serum by 2D-HPLE, therefore I tested tissue specimens of breast cancer patients. Immunohistochemistry was applied by using GASP-1 antisera to determine GASP-1 intracellular levels. Tissue specimens were bought from US Biomax. Many tiny biopsies from cancer and normal tissue were immobilized on the same glass slide, and were arranged in an array. The advantage of tissue specimen arrays was that all samples was incubated, stained, and tested at the same time, so that the inter-experimental variation was eliminated. A disadvantage of tissue arrays is that only a very small section of tissue is sampled. The stained slides were analyzed by a Temple University pathologist, Dr. Xinmin Zhang, and a score representing stain intensity was given to each samples. The scale of the score was from 0 to 3. The results showed that normal tissues had very little staining or no staining. Benign breast cancer had moderate staining. Cancer samples as a whole had the highest staining intensity. The P value showed that the differences among them were statistically significant (Figure9). The

result suggested that GASP-1 epitope had a higher expression level in cancer cells as compared to normal and benign tumor. Benign tumor is considered as a non-metastasis tumor, which occurs very early in the breast cancer progression. The detection of GASP-1 in benign tumor indicated GASP-1 might become a very early cancer marker in the clinical diagnostic process in the future. According to the sample information provided from the biological company, samples were grouped into ADH/DCIS (ductal carcinoma in situ), stage 1, stage 2 and stage 3 (Figure9). However, no significant differences were found among them, indicating GASP-1 levels could not distinguish different cancer stages, but that fact GASP-1 was highly expressed in ADH/DCIS, an early stage tumor, suggests that it may be an important early stage cancer marker.

Representative pictures of immunohistochemistry staining GASP-1 are shown in Figure10. Normal ducts showed little or no staining at all. In cancer specimens, GASP-1 staining is confined in cancer cells only. Ductal carcinoma *in situ* showed clustered cancer cells (Figure 10A). Breast cancer does not invade at this stage. Invasive ductal carcinoma is later stages of breast cancer. Cancer cells started to invade into the surrounding tissue, and thus showed scattered staining of invaded cancer cells (Figure10 B and C). Taken together, GASP-1 intracellular expression level positively correlates with breast cancer, and might have the potential to become a new cancer marker, which can diagnose breast cancer in very early stages such as DCIS. However, its expression level may not reflect the later stages of breast cancer progression.

Hyperplasia is another early stage of breast cancer. It is characterized by increased proliferation of epithelial cells, and has been considered as a pre-cancerous stage. In order to compare the GASP-1 expression level between hyperplasia and other stages, I

grouped samples in benign, hyperplasia, and carcinoma. The result showed that hyperplasia has a GASP-1 level as high as carcinoma, and is higher than benign, indicating GASP-1 may influence cell proliferation (Figure 11A).

Triple negative breast cancer is hard to cure because it doesn't have hormone receptor and HER2 receptor, making hormone receptor treatment and monoclonal antibody treatment ineffective. Does GASP-1 correlated with triple negative breast cancer cells? To answer this question, I grouped samples in benign, DCIS and triple negative breast cancer. The result shows that GASP-1 expression correlates with triple negative breast cancer (figure 11B).

In summary, GASP-1 is over-expressed in the breast cancer cells by immunostaining the tissue specimens of breast cancer patients. Its expression is as early as DCIS and hyperplasia, indicating GASP-1 might become an early marker of breast cancer. Its intracellular over-expression also suggested that GASP-1 may play a role in breast cancer cell proliferation and metastasis, and thus might become a novel target for the development of therapeutic strategies.

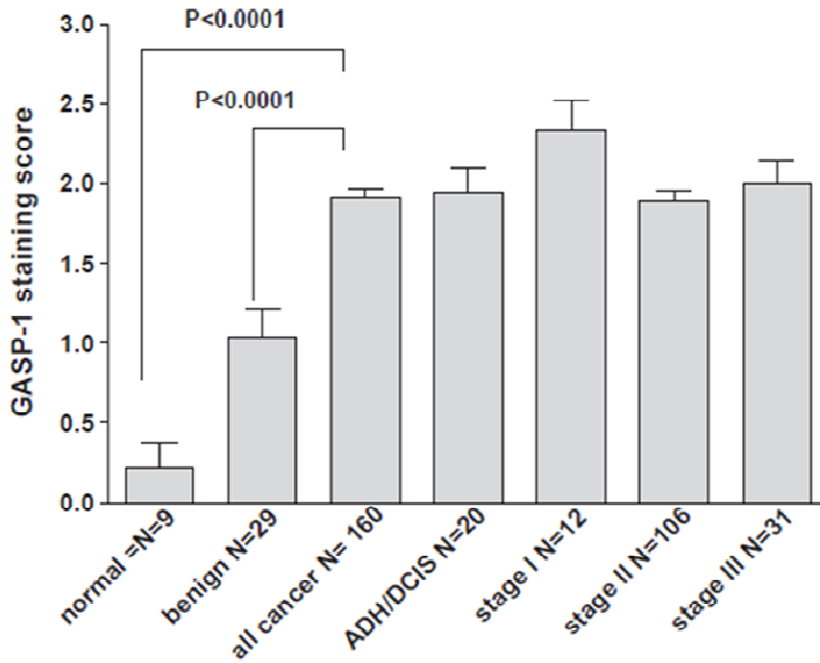


Figure 9. GASP-1 is over-expressed in breast cancer cells of tissue specimens. Immunohistochemistry was performed to analyze the GASP-1 expression in breast cancer tissue samples and normal controls. N is the number of samples. Standard deviation and p values were calculated by computer software Graphpad.

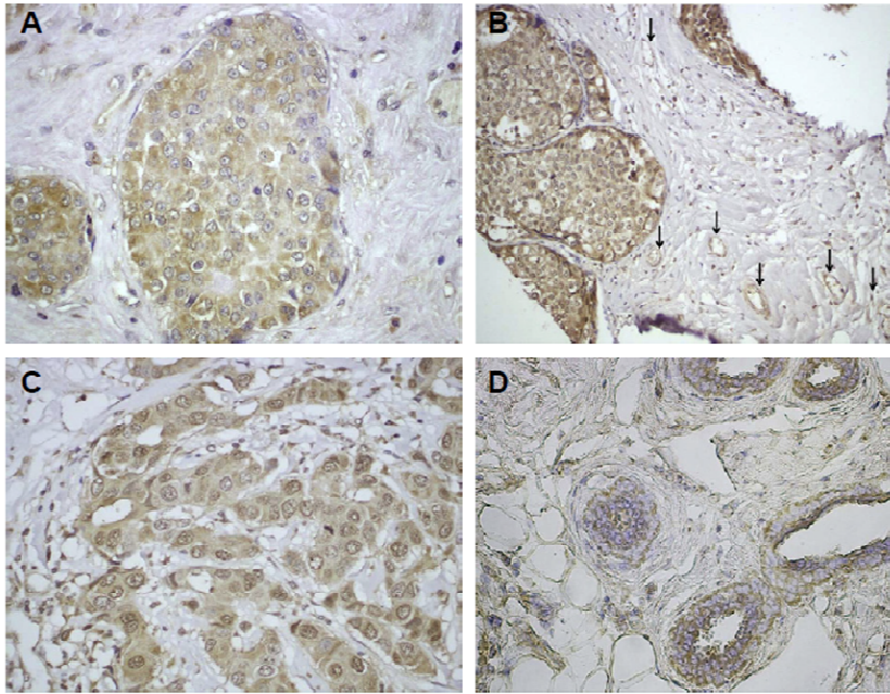
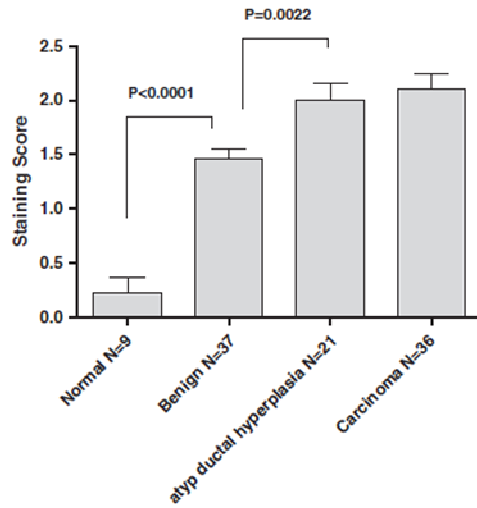


Figure 10. A typical immunohistochemistry picture was shown. (A) DCIS (B and C) invasive ductal carcinoma. (D) normal duct.

(A)



(B)

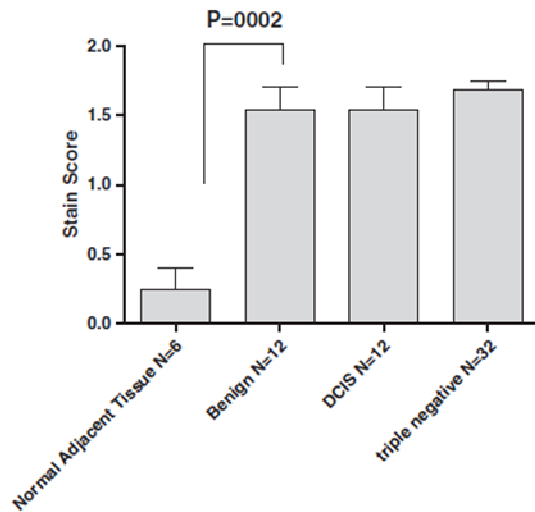


Figure 11. GASP-1 did not correlate with hyperplasia and triple negative breast cancer. Immunostained slides were grouped to compare hyperplasia (A) and triple negative breast cancer (B) with normal and other stages. N is the number of the specimens analyzed.

3.2.2 GASP-1 is over-expressed in pancreatic cancer and brain cancer

Since GASP-1 was shown to be over-expressed in breast cancer cells, I wanted to know whether GASP-1 was over-expressed in cells of other cancer types. Tissue arrays of pancreatic cancer and brain cancer were bought from biological companies. Immunohistochemistry was performed to detect GASP-1 levels in these cancer cells. The results showed that GASP-1 has a higher staining intensity in pancreatic cancer cells. Interestingly, hyperplasia/inflammation has a higher GASP-1 level than later stage cancer, indicating GASP-1 could become an early cancer marker for pancreatic cancer (Figure 12A). GASP-1 was over-expressed in all stages of brain cancer. Glioma stage 2 has a smaller staining intensity than later stages, indicating GASP-1 level increases during cancer progression (Figure 12B). Several typical pictures of immunohistochemical staining are shown in Figure 13. In the pictures, both pancreatic cancer and brain cancer has a higher staining intensity than normal controls, and normal controls hardly had any staining. These results suggests that GASP-1 has a higher expression level in pancreatic cancer and brain cancer cells at very early stages, and thus could become an early cancer marker for multiple cancer types.

In this chapter, I quantified the GASP-1 epitope serum levels and intracellular expression levels in clinical samples. In conclusion, I validated that GASP-1 is over-expressed in tumors from cancer patients compared to normal controls. GASP-1 could become a new cancer marker for several cancer types including brain, lung, liver, breast and pancreatic cancer.

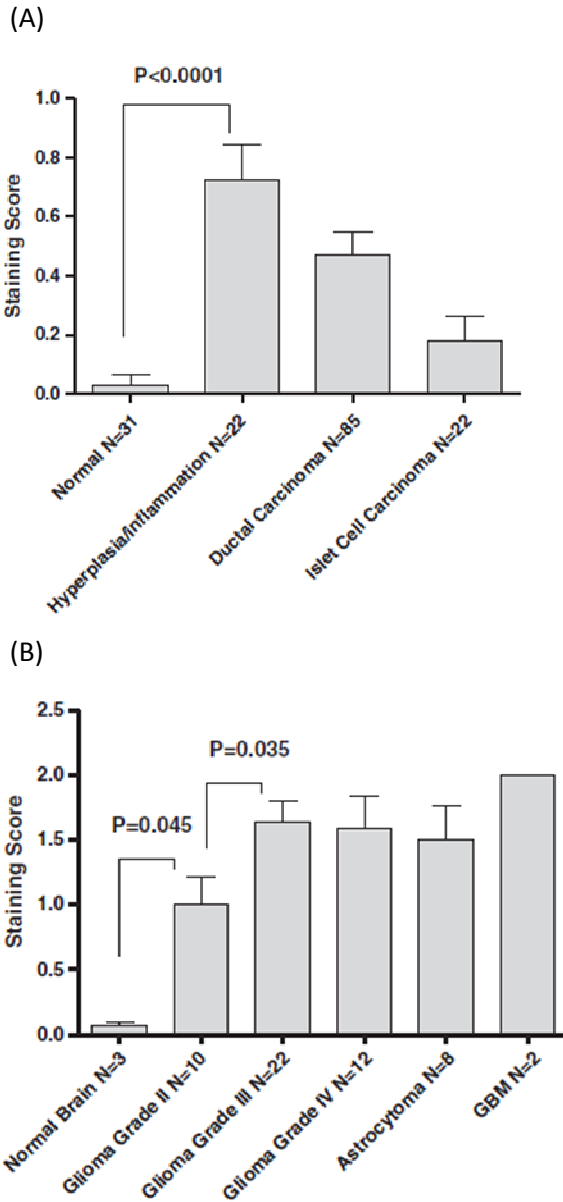


Figure12. GASP-1 is overexpressed in pancreatic cancer and brain cancer. Immunohistochemistry analysis was performed among pancreatic cancer (A) and brain cancer (B) arrays. The staining intensity was scored and plotted on the graph. P values were calculated by computer software Graphpad.

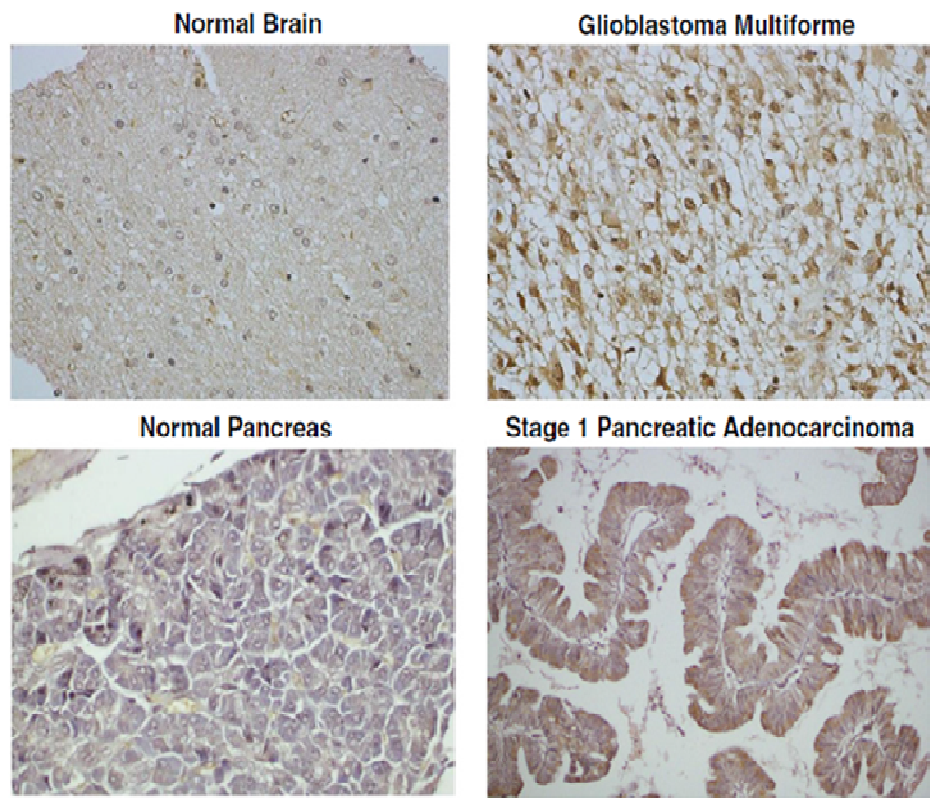


Figure 13. A typical immunohistochemistry picture is shown. (A) normal brain. (B) glioblastoma. (C) normal pancreas. (D) pancreatic cancer.

3.3 GASP-1 Promoted Tumorigenesis in MDA-MB-231 breast cancer cells.

3.3.1 Establish MDA-MB-231 cell clones that stably over-/under-express GASP-1.

Clinical sera and tissue specimen samples shows that GASP-1 expression level had a positive correlation with breast cancer. This correlation began at very early stages of the cancer progression, indicating GASP-1 may play a role in breast cancer tumorigenesis. Therefore, I propose that GASP-1 promotes tumorigenesis in breast cancer cells. To prove this hypothesis, MDA-MB-231 breast cancer cells were transfected with either shRNA against GASP-1 (shGASP-1), or plasmid encoding full-length GASP-1. The controls were scrambled shRNA and empty plasmid, respectively. Cells were selected by 0.6ug/ml puromycin for 2-3 weeks. Two clones were selected from GASP-1 over-expressed cells by limiting serial dilutions in 96 well tissue culture plates. GASP-1 under-expressing clones could not be maintained longer than several weeks. They either died out or restored GASP-1 expression as measured by RT-PCR assays. This time period was not long enough to select single cell clones. Therefore, single cell clones were not selected from shGASP-1 clones. Instead, heterogeneous cells after selection in puromycin were evaluated in the following experiments: mRNA level of GASP-1 was tested by RT-PCR and RT-qPCR. 3 polyclones containing different shRNA against GASP-1 were compared. Two GASP-1 over-expressed single cell clones were also tested. The results are shown in the Figure 14A. From the agarose gel, shRNA number 30 (shGASP-1_30) did not down-regulate GASP-1. shRNA number 29 (shGASP-1_29) and 32 (shGASP-1_32) showed a down-regulation of GASP-1. GASP-1 clone number 3 (pGASP-1_3) and 9 (pGASP-1_9) have shows obvious over-expression of GASP-1 as compared to their vector control.

In the qPCR result, shGASP-1_30 did not down-regulate GASP-1 (Figure 14B). shGASP-1_29 and 32 showed down-regulation, but shGASP-1_32 showed more down-regulation than shGASP-1_29. pGASP-1_3 and 9 both showed over-expression of GASP-1.

Since mRNA does not necessarily translate into protein, I performed western blot analysis to detect GASP-1 protein levels from the cell lysates of these clones (Figure 15). Band intensity was calculated by densitometry software and listed below the picture. The results showed that shGASP-1_32 has the best down-regulation efficiency. Both pGASP-1_3 and pGASP-1_9 had a higher GASP-1 expression level than their empty vector control.

Taken together, shGASP-1_32 (shorted as shGASP-1 in the following results), pGASP-1_3 and 9 were evaluated in the following experiments with their respective controls.

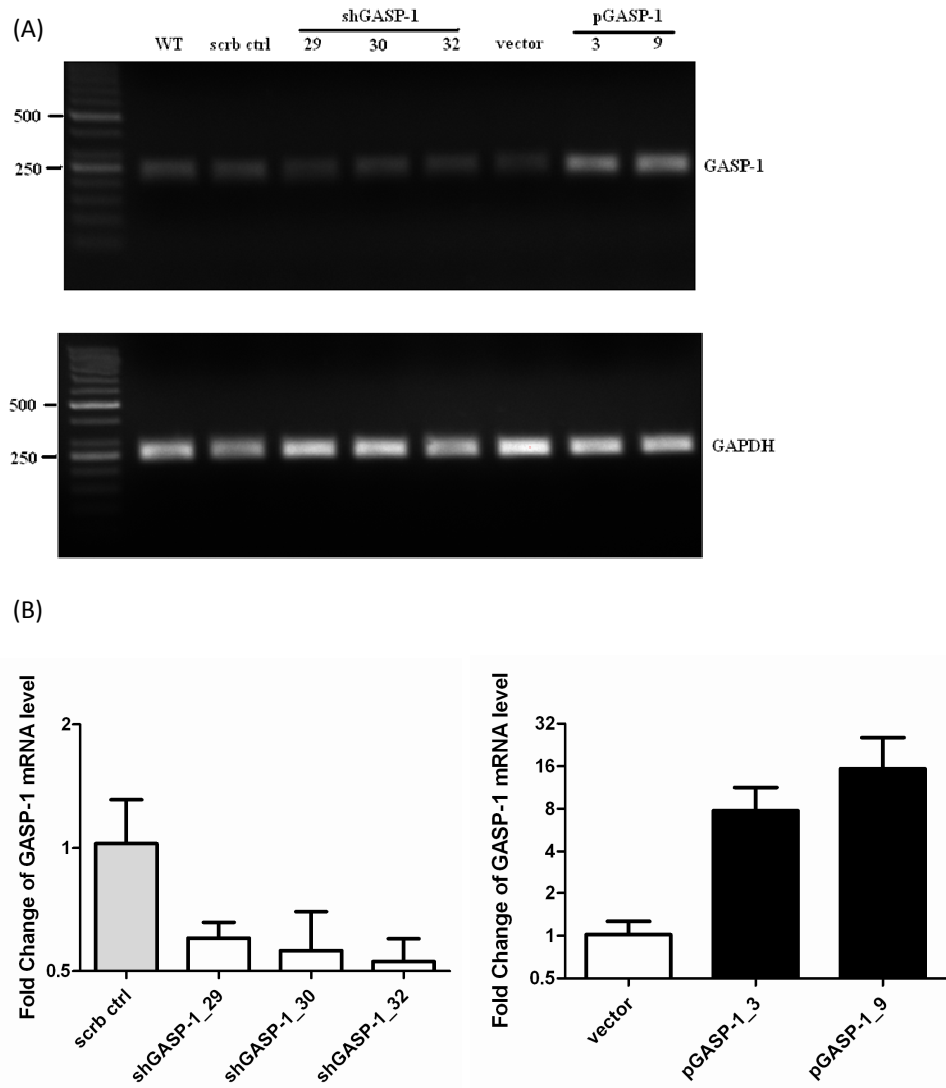


Figure 14. GASP-1 was down-regulated in shGASP-1 clones and is over-expressed in pGASP-1_3 and 9 clones. MDA-MB-231 breast cancer cells were stably transfected with shRNA against GASP-1 and plasmid over-expressing GASP-1. After antibiotic selection, monoclonal pGASP-1_3 and 9 were isolated. GASP-1 mRNA transcriptional level was measured by RT-PCR (A) and RT-qPCR (B). The standard deviation represents triplicates in RT-qPCR.

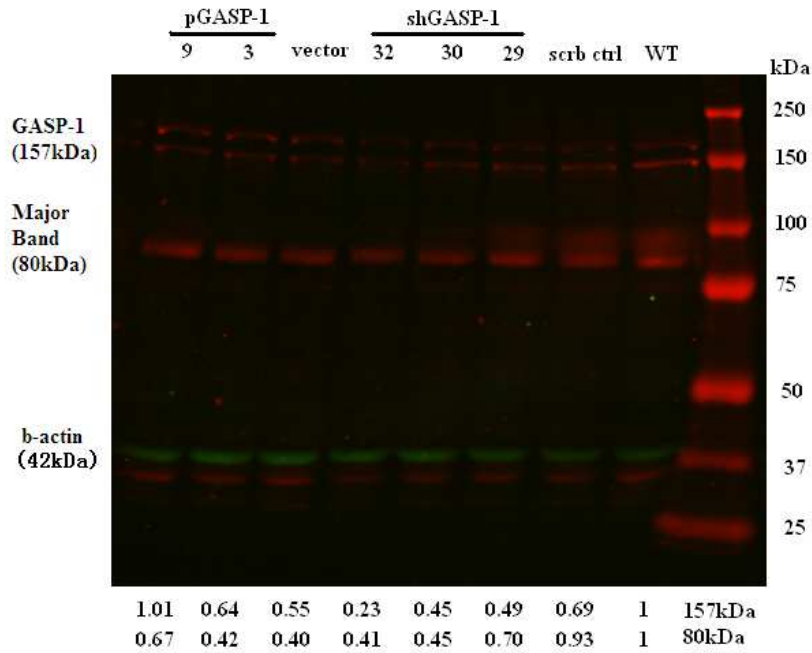


Figure 15. GASP-1 modification was confirmed by western blot. 20ug cell lysates were loaded on the gel. Transfer was performed at 40V, 4°C overnight. GASP-1 was detected by anti-GASP-1 antibody and then secondary antibody. Picture was scanned and analyzed by LICOR computer software. Normalized band density was listed below the picture.

3.3.2 Down-regulation of GASP-1 decreased the cell proliferation rate in MDA-MB-231 Cells

Unlimited cell proliferation is a characteristic of cancer cells. Does GASP-1 regulate cell proliferation in breast cancer cells? To answer this question, MTS cell proliferation assay was applied on the established stable cell clones. Absorbance at 490nm was taken at day 0, 2, 4, 6, and 8. The result showed that down-regulation of GASP-1 decreased the cell proliferation (Figure 16). shGASP-1 clone stop growing after day 4. Under microscope, they showed a lot of cell death. Over-expression of GASP-1 did not influence the cell proliferation rate. Their doubling time is the same as the vector control. These results suggested that GASP-1 may maintain the cell proliferation in MDA-MB-231 cells, while over-expression of GASP-1 might not increase the cell proliferation rate any further.

3.3.3 Down-regulation of GASP-1 decreases the migration rate in MB231 cells

Another characteristic of cancer cells is the capacity of the cells to invade surrounding tissues from the primary tumor, finally migrating to another part of the body and colonizing distant organs. Since our hypothesis was that GASP-1 is a tumorigenic promoter, I predict that GASP-1 would positively regulate breast cancer cell migration ability. Therefore, I tested the migration of our established stable cell clones. Boyden chamber chemotaxis assay and wound healing migration assay were performed.

In Boyden chamber chemotaxis assay, 10% FCS was used as the chemoattractant and 3h incubation period was applied. The results showed that shGASP-1 clone had significantly decreased cell migration ability as compared to scrambled control in response to 10% FCS. pGASP-1_3 and 9 had significantly increased cell migration

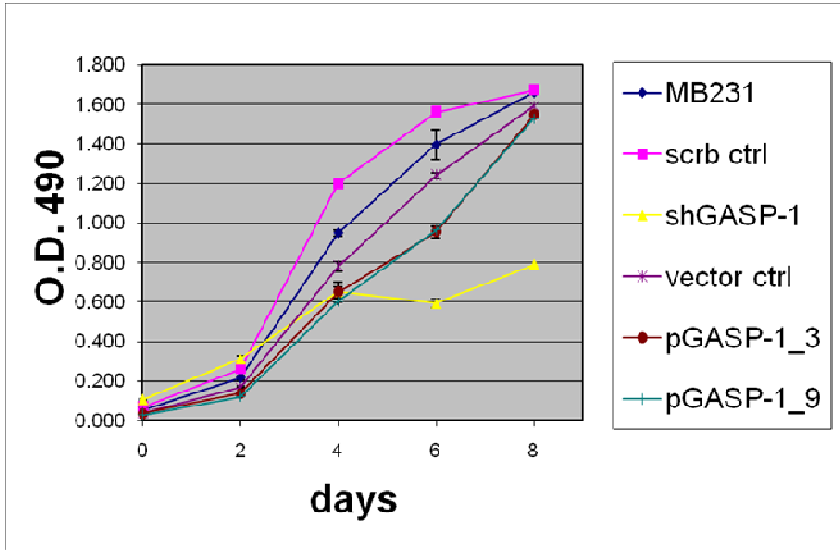


Figure 16. Down-regulation of GASP-1 decreased the cell proliferation rate in MDA-MB-231 cells. 2000 cells were plated in 96 well plates with 100ul DMEM complete media. 20ul MTS reagent was added and absorbance 450nm was measured by spectrophotometer. Continue measurements on day 0, 2, 4, 6 and 8. The resulted readings were plotted against days. Standard deviation represents triplicate experiments.

ability as compared to empty vector control in response to 10% FCS (Figure 17). This result suggested that GASP-1 positively regulated the migration ability of MDA-MB-231 breast cancer cells under the chemotaxis conditions of the assay.

In wound healing assay, shGASP-1 clone showed a decrease in migration distance as compared to scrambled control. pGASP-1_3 and 9 had a larger migration distance than empty vector control (Figure 18). This result illustrated that GASP-1 positively regulates migration ability of MDA-MB-231 breast cancer cells under the non-chemotaxis conditions of this assay.

Taken together, these different experiments suggest that GASP-1 positively regulates cell migration ability and invasiveness, and may play a role in breast cancer invasion and metastasis.

3.3.4 Down-regulation of GASP-1 in MDA-MB-231 Cells Decreased the Number and the Size of Colonies in Soft Agar Assay

The MTS assay was used to measure the cell proliferation rate when cells were grown on a flat surface. However, this is not the physiological condition where cancer cells grow into a 3-dimensional tumor and are contacted with surrounding connective tissues. Therefore, soft agar assay was performed to measure cancer cell growth. In soft agar assay, cancer cells will be disseminated into single cells, and suspended in a 3-dimensional agarose matrix. Cells will then grow in a 3-dimensional manner, into sphere like colonies. There are two advantages of soft agar. First, it better mimics the physiological condition than the MTS assay. Second, soft agar assay not only tests tumor growth in single colonies (the size of the colonies), but also gives out the information of

GASP-1 positively regulated cell migration in MB231 cells (Boyden Chamber Assay)
(A) (B)

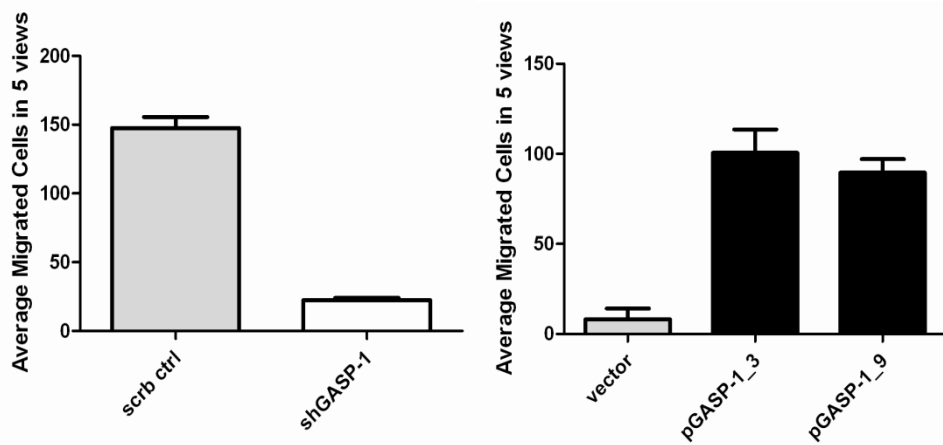


Figure 17. GASP-1 positively regulates the migration ability of MDA-MB-231 breast cancer cells in the Boyden chamber migration assay. 100,000 cells were plated in the upper well of the chamber. The upper well was tissue culture compatible, and no protein coating was performed. 10% FCS was applied in the bottom well as a chemoattractant. Cells were incubated at 37°C for 3h. Cells were fixed with Glutaraldehyde and stained with crystal violet. Non-migrated cells were removed by swabbing the upper surface of the insert. Migrated cells were counted in 5 fields at 200 x magnification. Standard deviation was calculated from the number of cells in the 5 fields.

GASP-1 positively regulated cell migration in MB231 cells
(cell scratch assay)

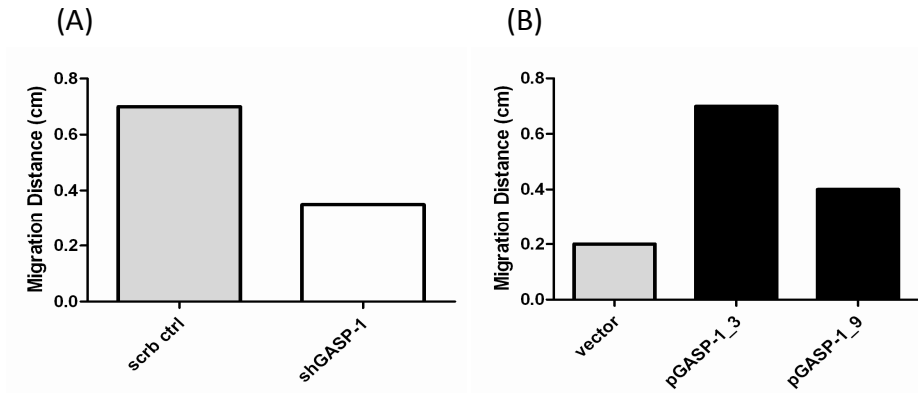


Figure 18. GASP-1 positively regulates the migration ability of MDA-MB-231 breast cancer cells in a wound healing assay. 300,000 cells were plated in wells of 6 well plates. Cells were allowed to attach for 4h, and the monolayer was scratched by a 1 ml pipet tip. Media was replaced with fresh media to remove detached cells. Cultures were then incubated for 24 h. Pictures were taken at 4 h and 24 h. The width of the scratch was measured by a ruler and the relative migration rate was calculated as initial width minus final width.

how many colonies can be formed (the number of the colonies), indicating the number of cells that have the ability to grow into a colony. 2000 cells were plated into one well of a 6-well plate, so that the cells were scattered enough to prevent the merging of neighboring colonies. Triplicates were performed for each cell clone. Colony growth was allowed to develop after 15 days of incubation.

For the colony number, the results showed that shGASP-1 clone had a decreased colony number as compared to scrambled control. pGASP-1_3 and 9 had a slightly increased colony number compared to vector control and wild type (Figure 19A). These results suggested that down-regulation of GASP-1 decreased the number of cells that could form a colony, but over-expression of GASP-1 did not increase the colony number dramatically, indicating GASP-1 is required for the colony formation of MDA-MB-231 breast cancer cells.

For the colony size, the pictures were taken to evaluate the size of the colonies. The results showed that shGASP-1 clones had very little colonies, and these colonies had a very small size as compared to all other cells (Figure 19B). pGASP-1_3 and 9 had regular colony size just as the vector control. These results suggested that GASP-1 is required for the colony growth.

In this chapter, I showed that GASP-1 positively regulated cell migration and invasion. For cell proliferation and soft agar colony formation, GASP-1 seems only to maintain the ability of cells to grow and form colonies. These results suggested that GASP-1 contribute to breast cancer tumorigenesis, which may be related to breast cancer pathogenesis and disease progression.

Down-regulation of GASP-1 in MDA-MB-231 cells decreased the number of colonies in soft agar assay

(A)

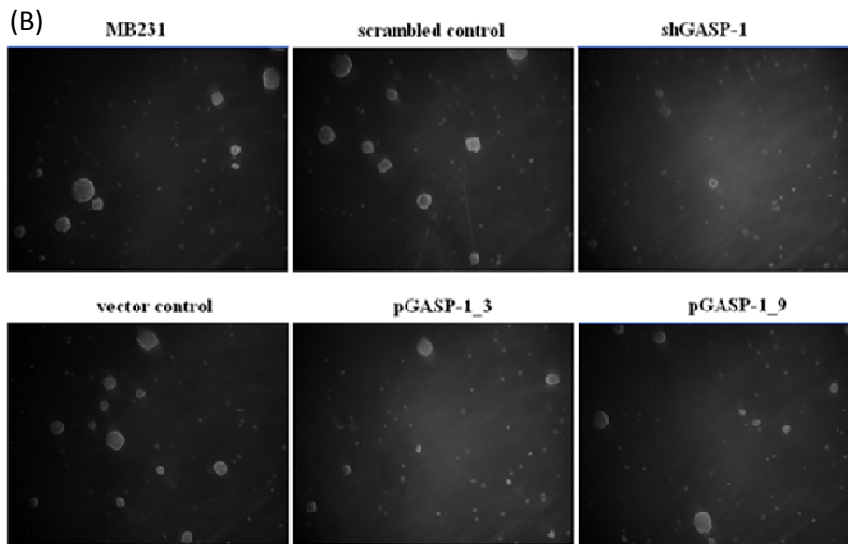
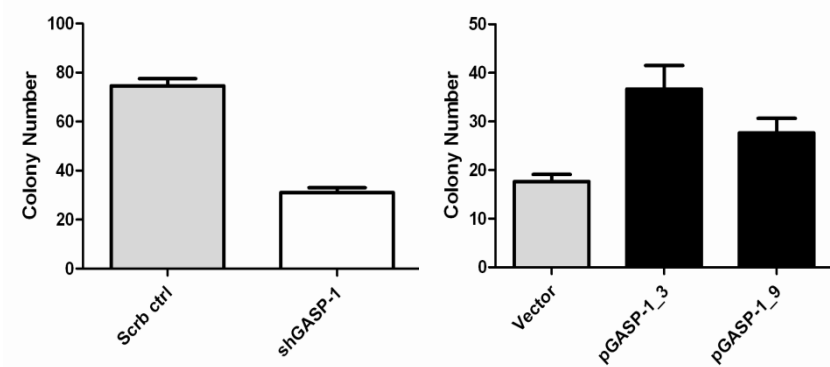


Figure 19. Inhibition of GASP-1 decreased the number of the colony formed in the soft agar assay. Stable cell clones were plated in 0.35% soft agarose, on top of the 0.5% soft agar, in 6 well plates. 13 days later, the number of the colonies was counted (A) and the size of the colonies was compared in the picture (B). Error bars represent triplicate parallel experiments.

3.4 IDENTIFY THE SIGNALING PATHWAYS MEDIATED BY GASP-1 IN BREAST CANCER CELLS *IN VITRO*.

3.4.1 Epithelial to Mesenchymal Transition (EMT)

There are 3 reasons that made us decide to test EMT markers in MDA-MB-231 breast cancer cells that stably over-/under-express GASP-1. First of all, EMT markers are involved in tumorigenesis and are regulated by many cancer signaling pathways. Second, MDA-MB-231 cells that stably under-express GASP-1 shows decreased cell proliferation, migration, and soft agar colony formation ability, indicating EMT markers may be inhibited. Colony formation ability in soft agar has been shown to be related to cancer stem cell formation. For example, ESA+/PROCR+ cells, characterized as cancer stem cells, generated more colonies in soft agar assay⁵². CD133+ cells, Another cancer stem cell marker, positively correlated with colony formation in soft agar⁵³. Whether or not EMT and cancer stem cells influence colony formation in soft agar and proliferation in MDA-MB-231 breast cancer cells havenot been reported. Finally, MDA-MB-231 cells that stably under-express GASP-1 cannot be maintained in tissue culture conditions longer than several weeks. The cells died out unless the GASP-1 expression reversed back to normal level, indicating cell self-renewal ability is impaired and EMT markers are inhibited. Therefore, mRNA level of EMT transcriptional factors, including snail, slug and ZEB2, were evaluated by RT-qPCR and further validated by western blot. These experiments will tell us whether EMT transcription factors are regulated by GASP-1. Vimentin and N-cadherin are markers of the mesenchymal cell state. RT-qPCR analysis for these markers will tell us whether they are regulated by EMT. Western blot analysis will tell us whether they are regulated on a post-translational level. These

experiments will provide evidence whether GASP-1 can promote mesenchymal characteristics in MDA-MB-231 breast cancer cells. Moreover, since EMT has the ability to generate cancer stem cells¹³, I wanted to evaluate the proportion of cancer stem cell in MDA-MB-231 cells that stably over-/under-express GASP-1. CD44, a cell surface molecule, is used as a cancer stem cell marker. It has been reported that CD44+ breast cancer cells can colonize and grow into a tumor after injecting into mice, while CD44- cells cannot⁵⁴, suggesting CD44+ cells have the self-renewal ability and thus comprise the cancer stem cell subpopulation in breast cancer. Therefore, the proportion of CD44+ cells in MDA-MB-231 cells that stably over-/under-express GASP-1 were evaluated by flow cytometry and compared to the control. These experiments will tell us whether the EMT markers influenced by GASP-1 will influence cancer stem cell formation.

We first used RT-PCR to test the mRNA level of these EMT markers. The results showed that slug mRNA level decreased in shGASP-1 cells, but not in pGASP-1 cells (Figure 20). N-Cadherin mRNA decreased in GASP-1 over-expression clones. Other EMT markers were not influenced at the mRNA level (Figure 20). These results suggested that GASP-1 maintained slug mRNA level, but decrease N-Cadherin mRNA level.

Since mRNA levels do not always correlate with protein levels, I also used western blot to verify the expression level of slug (snail levels were too low to be detected by western blot) (Figure 21A). The results showed that shGASP-1 had no bands of slug, indicating a decrease of slug protein level. pGASP-1_3 and 9 clones showed no differences between vector control, suggesting slug is not further up-regulated by GASP-1 over-expression. Therefore, GASP-1 maintains slug expression levels. This result is consistent with cell

proliferation assay and soft agar assay, suggesting that slug may play a role in cell growth in breast cancer cells.

Vimentin and N-cadherin are mesenchymal markers. Therefore, I tested their protein levels in case any post-translational modifications occurred, which made the cell more mesenchymal. The results showed that vimentin and N-cadherin showed no difference among the clones according to western blot results (Figure 21B and C), indicating no post-translational modifications occurred. The cells epithelial characteristics were not changed.

Since EMT markers have the ability to increase cancer stem cell formation, I evaluated the proportion of cancer stem cell (CD44+ cells) in all stable cell clones by flow cytometry. The results showed that shGASP-1 pGASP-1_3 and 9 had the same percentage of CD44+ cells as their respective controls and wild type MDA-MB-231 cells (Figure 21D).

3.4.2 NF κ B signaling pathway

NF κ B positively correlated with breast cancer incidence and malignancy. NF κ B activation had been proven to be a required factor for breast cancer in animal experiments and *in vitro* tissue culture experiments (See the introduction). In order to test NF κ B activation, I performed luciferase assay and western blot assay. Luciferase signal increased in GASP-1 over-expression cells, while only with a slight reduce in GASP-1 under-expression cells. TNF α treatment does not enlarge the differences (Figure 22A). phospho-NF κ B p65 (Ser 536) increased in GASP-1 over-expression cells, but not in GASP-1 under-expression cells. NF κ B total expression level had no differences among

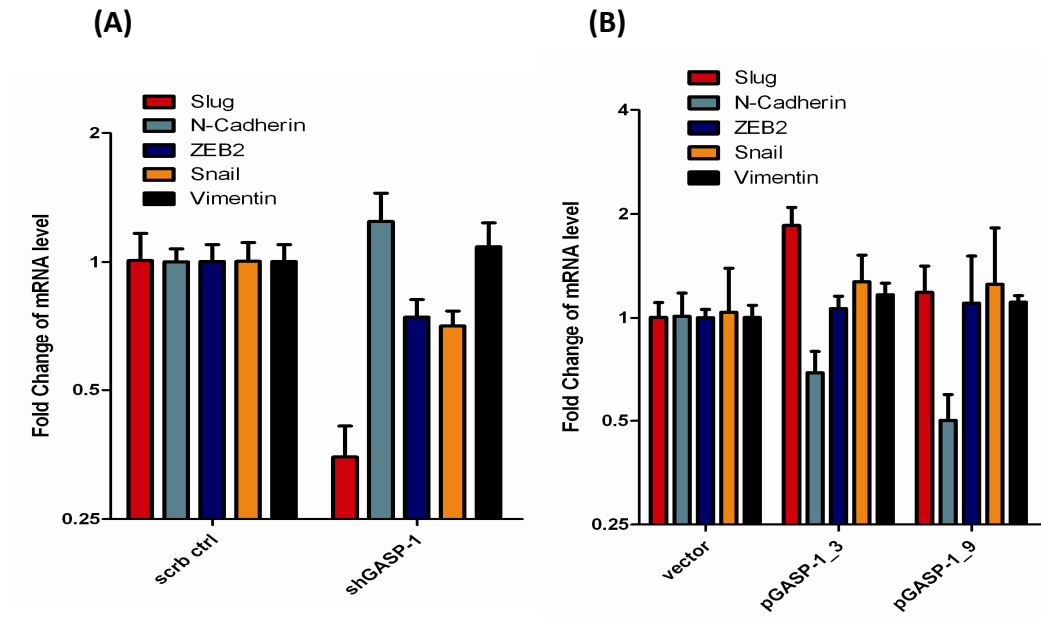


Figure 20. GASP-1 maintains slug mRNA, and decreased N-Cadherin mRNA level in MDA-MB-231 breast cancer cells. RT-qPCR analysis was performed to test EMT markers at the mRNA level. 1.5ug RNA was reverse transcribed into cDNA in a 25ul system. 1ul cDNA samples was added to a 20ul PCR system. Tm is listed in table 2. 45 cycles was applied. Single detection of syber green I was designed at the extension step. The result was calculated by computer software of the ROCHE Lightcycler 480, using basic relative quantification method. STDEV was calculated from triplicate experiments.

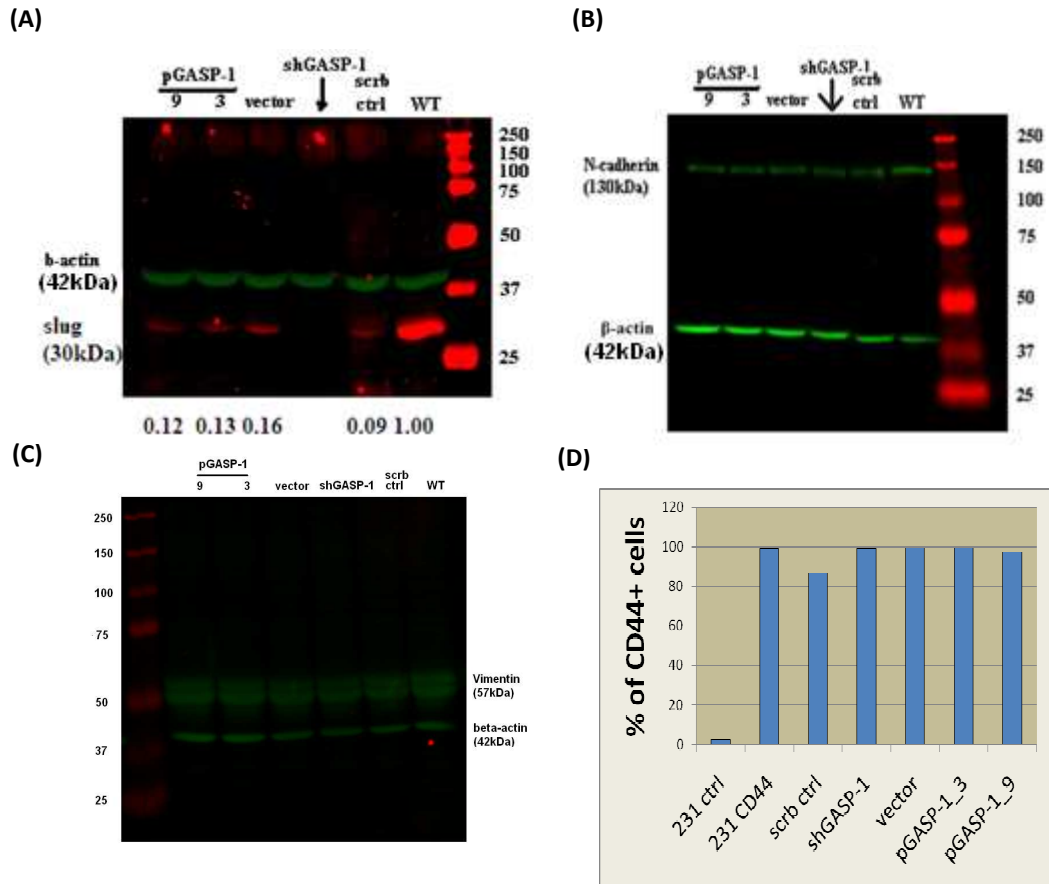


Figure 21. GASP-1 maintained slug protein expression, but N-cadherin and vimentin protein levels did not change. Western blot analysis was performed on (A) slug, (B) N-cadherin and (C) vimentin. 20ug/well total cell lysate was loaded. Beta-actin was applied as loading control. (D) CD44+ cell population was not changed by modification of GASP-1 in MDA-MB-231 breast cancer cells. 500,000 cells were fixed and stained by anti-CD44 antibody. CD44+ cells were detected by flow cytometry. The same concentration of isotype control was applied as a negative control.

cells (Figure 22B). These results suggested that GASP-1 increased NF κ B activation. However, down-regulation of GASP-1 did not change NF κ B activation level, possibly due to other complementary signaling in breast cancer cells.

3.4.3 Src signaling pathway

Src promote proliferation, survival, migration, and angiogenesis in cancer. Its over-expression and hyper-activation had been reported in many cancer types. Src also increased bone metastasis in animal models (See introduction). Phosphorylation at Tyr 530 inhibited src activity, while phosphorylation at Tyr 419 increased its activity. To test whether src activity is regulated by GASP-1, I performed western blot on phosphor-src at both Tyr 419 and Tyr 530. GASP-1 inversely regulated Tyr 530, but did not change Tyr 419 level (Figure 23), indicating GASP-1 may contribute src activation through decreasing inhibitory src phosphorylation.

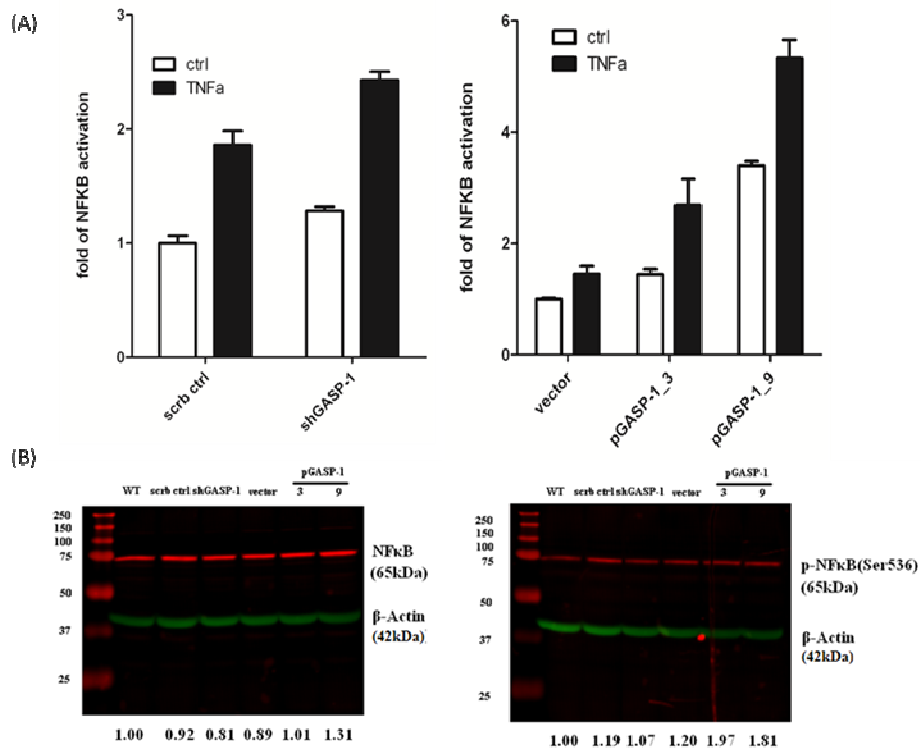


Figure 22. NFκB activity increased in GASP-1 over-expression cells. (A) Luciferase Assay was performed to test NFκB activity. Adenovirus was used as the vector. Luciferase was driven by a promoter for NFκB. After 6-8h of infection (MOI=50), cells were treated with/without TNF-alpha (10ng/ml) overnight. Luciferase signal was measure the following day and was normalized to cell titer signal. 4 replicates were performed in each experiment. (B) western blot was performed to test total NFκB level and phospho-NFκB (Ser536) level. Beta-actin was used as loading control.

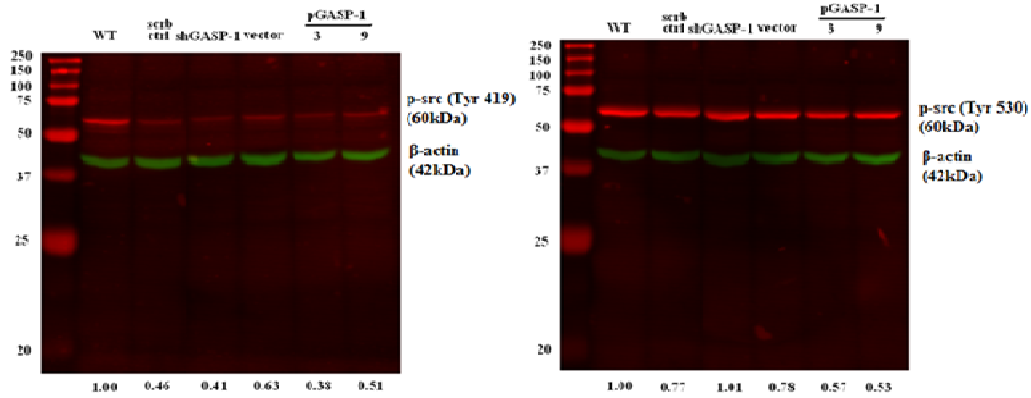


Figure 23. GASP-1 inversely regulate phospho-src Tyr 530, but did not change Tyr 419 level. Western blot was performed on p-src Tyr 419 (left) and Tyr 530 (right). 20ug was loaded on SDS-PAGE. Beta-actin is the loading control.

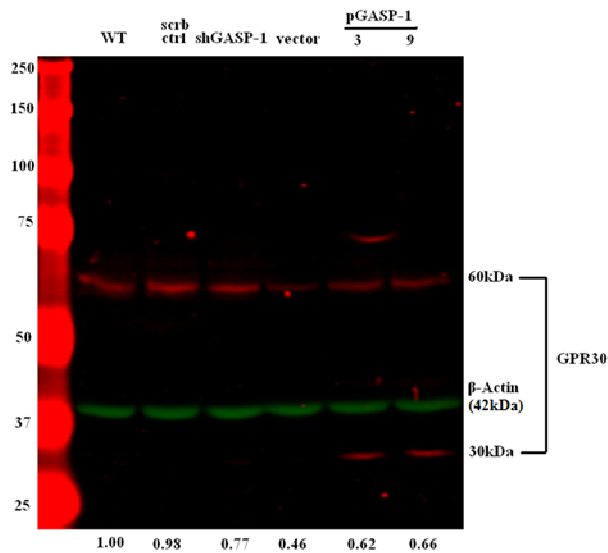


Figure 24. Over-expression of GASP-1 increased GPR30 protein level. Western blot was performed on GPR30 protein level. 20ug was loaded on SDS-PAGE. Beta-actin was used as loading control.

3.4.4 G-protein coupled receptor 30 (GPR30)

GPR30 is an estrogen receptor. It had been hypothesized to be the complementary estrogen receptor in estrogen receptor (ER) negative breast cancer, and had been proven to regulate ERK phosphorylation through EGFR dependent mechanism (See introduction). To test whether GPR30 is regulated by GASP-1, I perform western blot on GPR30 protein level among stable cells. GPR30 protein level increased in GASP-1 over-expression cells, but did not change in GASP-1 under-expression cells. Interestingly, both 30kDa and 60kDa bands showed up in pGASP-1 cells, indicating GASP-1 may increase the de novo translation of GPR30, so that non-glycosylated GPR30 accumulated in the cells (Figure 24).

In summary, I tested the expression level of EMT markers, NFκB activity phosphor-src, and GPR30. RT-qPCR and western blot analysis concludes that GASP-1 maintained slug expression, increase NFκB activity and GPR30 expression level, while decrease the inhibitory phosphor-src (Tyr 530), which possibly contributes to tumorigenesis in breast cancer cells.

CHAPTER 4: GENERAL DISCUSSION AND POTENTIAL FUTURE DIRECTIONS

GASP-1 was first identified and characterized as a regulator protein targeting G-protein coupled receptors. However, Dr Chang's discovered its expression in sera of breast cancer patients. Because of this positive correlation, I bring up the hypothesis that GASP-1 may have a contribution to breast cancer tumorigenesis. Both ELISA and immunohistochemistry studies in this thesis demonstrated that GASP-1 positively correlate with breast cancer and other cancer types. This strong positive correlation indicated that GASP-1 had a potential to become a new cancer marker in clinical diagnoses. The *in vitro* tissue culture studies demonstrated that GASP-1 is a tumorigenic promoter, contributing to breast cancer cell proliferation, migration, invasion and colony formation. The signaling pathway analysis revealed that EMT transcriptional factor slug may be involved in promoting tumorigenesis. For the first time, our work discovered GASP-1 over-expression in breast cancer patients, describing its potential clinical application, its role in breast cancer tumorigenesis, and a possible signaling mediator.

We want to validate the positive correlation between GASP-1 expression level and cancer. Therefore, I tested serum samples by ELISA and tissue specimens by immunohistochemistry. The results suggested that both GASP-1 serum level and intracellular expression is high in breast cancer patients and other cancer patients. This result suggested a new diagnostic methods, which might be more efficient and economical advantageous than current methods. X-ray mammogram and clinical breast exam are the current method to detect new breast cancer. However, these methods cannot detect tumor until the tumor size is large enough to be seen or touched. Only 20% of the diagnosed breast cancer could be discovered before cancer cells began to invade.

On the contrary, GASP-1 epitope could be detected as early as benign tumor and ductal carcinoma *in situ*, both of those are stages when cancer cells did not begin to invade. These results indicated its potential to become an early cancer marker in clinical diagnostic processes. It is also a convenient diagnostic method. People may just take bloodtestannually, followed by ELISA analysis, and wait for the result of GASP-1 level. It is much better than having an X-ray mammogram every year. Taking blood and do ELISA will be an economical way for patients, since ELISA need less expensive equipments as mammogram. In the future, more than one cancer marker can be used together with GASP-1 to make diagnoses more accuracy. Different cancer markers may even give the information to distinguish cancer types. Furthermore, IHC staining showed a high specificity to cancer cells. If the specimens have to be taken for further testing, GASP-1 staining could be another accurate indicator showing the presence of breast cancer cells.

For serum samples, I don't have detailed information of stages. Therefore, I do not know the correlation between breast cancer stages and GASP-1 serum level. IHC shows no change of GASP-1 level. However, intracellular level does not necessarily mean serum level. Factors such as half life, enzyme digestion, secretion, and carrier protein would contribute to the serum level. Does GASP-1 serum level correlate with stages? Further study is needed by using much more detailed characterized serum samples, especially with stage information.

From the IHC staining and serum ELISA, GASP-1 is positively correlate with a number of cancer types. Therefore, I hypothesized that GASP-1 is a tumorigenic promoter in breast cancer. Tissue culture studies showed that GASP-1 promoted cell migration and

invasion, and contributed to cell proliferation and colony formation, indicating GASP-1 is a tumorigenic promoter. These GASP-1 mediated functions might facilitate breast cancer tumorigenesis and progression. GASP-1 is over-expressed at early stages of breast cancer (for example, stage 0, also called ductal carcinoma *in situ*). In this stage, breast cancer cells formed a primary tumor at the ductal epithelium, but cancer cells are restricted within the primary tumor. They do not invade to the surrounding tissue. If the primary tumor is cut away by surgery and no cancer cells are left, the cancer is cured. At this stage, GASP-1 maintains cancer cell proliferation to sustain tumor growth. At later stages, GASP-1 also increased the migration and invasion of breast cancer cells so that the cancer cells can disseminate from primary tumor and invade to the surrounding tissue, getting into the lymph nodes and blood, migrate to other parts of the body.

Down-regulation of GASP-1 makes breast cancer cells hard to be maintained in tissue culture condition for long period of time, indicating the self-renew ability is impaired. Because of this phenomenon, I decided to study EMT transcriptional markers. EMT markers have the ability to regulate cell proliferation, migration, invasion, and colony formation in breast cancer and other cancer types. According to my results, these effects are also mediated by GASP-1, indicating there must be some link between GASP-1 and EMT markers. Furthermore, many cancer-related signaling pathways target EMT markers, such as c-Src, p53, ERK, etc. As a consequence, I tested the EMT markers expression by RT-PCR and western blot. GASP-1 positively regulated snail, and maintains slug expression, but did not change the CD44⁺ cell population. Therefore, GASP-1 mediated tumorigenesis might involve snail, slug, and signaling pathways other than breast cancer stem cell formation, and do not need to increase all EMT markers. Similar discoveries in

the literature supported this conclusion. For example, hypoxia, resulted from low oxygen tissue culture conditions only increased snail and cell migration, but do not increased vimentin and N-cadherin protein level ⁵⁵. Furthermore, slug itself positively regulate breast cancer cell tumorigenesis. For example, down-regulation of slug decreases cell migration, invasion, and lung metastasis (in vivo) in MB231 cells ²⁶. In MCF-7 breast cancer cells, p53 abolished EMT and cell invasiveness through down-regulation of Slug ²⁷. On the other hand, GASP-1 also regulate NFκB activity, src phosphorylation, and GPR30, indicating GASP-1 influenced more than one tumorigenic signaling, making GASP-1 a good potential therapeutic target in the future.

The down-regulation of GASP-1 was accomplished by transfecting plasmid. One limitation on the plasmid based down-regulation may influence the percentage of down-regulating cells. Integration site on the plasmid is not certain. Plasmid can break at any site to integrate into the host genome. If the break point happens in the shRNA sequence, the integrated host cells will not show RNA interfering, but still have GFP and antibiotic resistance. If the down-regulation decreased the cell viability, for example GASP-1, then the GFP+/antibiotic resistant cells without down-regulation will overgrow and finally, the whole population will lose the down-regulation characteristics. Using lentivirus may avoid this problem. Lentivirus will integrate through predictable site on its genome, excluding the possibility of losing shRNA.

In conclusion, GASP-1 is a tumorigenic promoter. It contributed to breast cancer cell proliferation in early stages, and promote breast cancer cell invasion into the surrounding tissue, and finally metastasized to distant organs. EMT transcription factor snail and slug may be involved, and may through signaling pathways other than cancer stem cell

formation. A possible link between GASP-1 and EMT markers is G-protein receptors, since GASP-1 has been reported to regulate GPCR expression level in a post-translational manner⁸. On the other hand, I cannot exclude the possibility that GASP-1 might directly bind and influence the signaling pathway components such as Akt, NFκB, p53, MAPK, etc.

There are several future directions. First, I did not do mice work because MDA-MB-231 cells cannot be maintained in the tissue culture condition for long period of time after GASP-1 is down-regulated. Therefore, another strategy of mice study will be applying Tet on/off promoter to drive the expression of shRNA. In this way, I can inject mice with the stable cell clones and then feed mice with or without tetracycline to down-regulate GASP-1. Lentivirus will be a good vector system. The second direction is to apply competitive ELISA on clinical diagnoses. One issue in this process will be: are proteins in the serum may possibly bind to GASP-1 peptide? This binding may interfere with the detection. To solve this issue, an immunoprecipitation could be performed on the peptide, followed by mass spectrometric analysis to identify the potential binding protein. Further identification of the binding protein will be accomplished by western blot. Among these proteins, the one that interferes with the ELISA detection will be identified, so that these interfering proteins will be used to prepare standard curve in each experiment, or be used to prepare positive controls representing different stages of cancer. The third direction is to discover other breast cancer markers by 2D-HPLC. Multiple markers will increase the accuracy of diagnose, and may even distinguish cancer types with ease. ELISA is one method, other methods may also be considered such as antibody chips. The final

direction is to identify downstream targets of GASP-1 to find out potential therapeutic targets for breast cancer.

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