약학 석사 논문

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지도교수 신 영 기
이 논문을 약학석사 학위논문으로 제출함
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S. M. Riajul Wahab의 석사학위논문을 인준함

2012 년 8 월

위 원 장 _____ **(인)**
부위원장 _____ **(인)**
위 원 _____ **(인)**

GNAI2 interacts with SMAD4 and negatively regulates the expression of SMAD4 target gene in cervical Cancer (HeLa) Cells

by

S.M. RAJUL WAHAB

A thesis submitted in partial fulfillment of the requirements for the degree of

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at the

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Seoul National University

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Dedicated

To

"My Parents, who sacrificed their happiness for the betterment of me till to their last breath"

Abstract:

GNAI2 (Guanine nucleotide binding protein (G-protein), alpha inhibiting activity polypeptide 2) belongs to a family of Gi alpha proteins that includes three polypeptides: Gi alpha 1 (GNAI1), Gi alpha 2 (GNAI2), and Gi alpha 3 (GNAI3). GNAI2 is a well known proto-oncogene that is involved in onset and propagation of many different types of cancers. Smad4 (Mother Against Decapentaplegic Homologue 4, DPC4) is a well-known tumor suppressor and key elements in TGF-β (Transforming Growth factor- β) signaling pathway. In order to demonstrate the role of GNAI2 in carcinogenesis and to find out its potential interacting partners, we had initially identified Smad4 as one of its interacting partners through a baculovirus protoarray system. In this study we firstly demonstrated the novel interaction between GNAI2 and Smad4 using bimolecular fluorescence complementation (BiFC) assay in cervical cancer (HeLa) cell. We also confirmed the interaction between GNAI2 and Smad4 by endogenous and exogenous immunoprecipitaion (IP) assay. Upon ectopic transfection of GNAI2, the western blot analysis resulted in decrements of the level of Smad4, p15INK4b, p21 protein and an increase the protein expression of C-Myc. In contrast, transient knockdown of GNAI2 resulted in substantial increase in Smad4, p15INK4b, p21 protein and a decrease in the protein expression of C-Myc. However, our qRT-PCR data showed no change at mRNA level of Smad4 but a substantial decrease and increase at mRNA level of p15INK4, p21 after transient overexpression and knockdown of GNAI2 in HeLa cell respectively. Moreover, 26S proteosomal degradation inhibitor MG132 treatment in GNAI2 overexpressed HeLa cell resulted in reduced degradation of Smad4 protein.

Through luciferase reporter assay we have found that the overexpression of GNAI2 resulted in reduced p15INK4b promoter activity. In addition, the si-RNA-based knockdown of GNAI2 significantly reduced the proliferation of cervical cancer (HeLa) cells. Taken together our study demonstrated that GNAI2 interacts with Smad4 and negatively regulates the expression of Smad4 and its targeted gene in cervical cancer (HeLa) cells.

Key Words: GNAI2, Smad4, BiFC assay, p15INK4b, Cervical Cancer.

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LIST OF ABBREVIATIONS

GNAI2	Guanine nucleotide binding protein alpha inhibiting activity polypeptide-2
BiFC	Bimolecular fluorescence complementation assay
GPCR	G-Protein coupled receptor
DPC4	Homozygously deleted in pancreatic carcinoma
EDTA	Ethylene diamine tetraacetic acid
HPV	Human papilloma virus
IP	Immunoprecipitation
LOH	Loss of heterozygosity
NLS	Nuclear localization signal
MAD	Mothers against decapentaplegic
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethylsufonyl fluoride
PVDF	Polyvinyl difluoride
qRT-PCR	Quantitative real-time polymerase chain reaction
SDS	Sodium dodecyl sulfate
TBS	Tris-buffered saline
TGFβ	Transforming growth factor-β
TSG	Tumor suppressor gene
WST	Water soluble tetrazolium salt

Introduction

Heterotrimeric guanosine triphosphate (GTP)-binding proteins, or G proteins, are members of a superfamily of proteins that bind guanine nucleotides with high affinity and have intrinsic guanosine triphosphatase (GTPase) activity. G proteins couple cell-surface receptors to specific intracellular effectors, such as adenylyl cyclase, phospholipase C β (PLCβ), or ion channels, and G proteins are now recognized as signal transducers for many hormones, neurotransmitters, growth factors, and chemotactic substances. G proteins are composed of three subunits (α, β, γ) and the α sub unit can be classified in major four groups such as αq, α12, αs and αi. The α sub unit which is an inhibitory sub unit can further be subdivided into three subunit those are αi1, αi2 and αi3. The αi2 is known as GNAI2 (Guanine Nucleotide Binding Protein Alpha Inhibiting Activity Polypeptide-2). Until recently, α subunits, which bind GTP and possess intrinsic GTPase activity, were thought to be exclusively responsible for the specific functions of G proteins [1-5]. A number of G proteins are suspected of being involved in the regulation of cell growth. Activation of Gq and G12 promotes cell transformation in NIH 3T3 cells and tumor formation in nude mice [6, 7]. Depending on their state of activation, GNAI2 (Guanine Nucleotide Binding Protein Alpha Inhibiting Activity Polypeptide-2) proteins promote or inhibit proliferation of murine fibroblasts [8, 9]. GNAI2, an ubiquitous protein, is a substrate of pertussis toxin. Pertussis toxin uncouples GNAI2 proteins from surface receptors by adenosine diphosphate (ADP)-ribosylating their α subunits, and inhibits the proliferation of various cell types, including fibroblast, epithelial, and myeloid cells [10-12]. This suggests that GNAI2 plays an important role in cell-

growth regulation, which is probably not restricted to fibroblasts or to non-malignant cells, and that modulation of its function might be useful to control cell proliferation. In fact, Moxham and colleagues [13] reported that selective inhibition of GNAI2 expression in adipocytes in syngeneic mice resulted in animals with a considerably decreased weight and size, and a reduced fat mass. Squamous cell carcinoma of the uterine cervix is one of the most frequent cancers affecting women worldwide. The carcinomas arise from cervical intraepithelial neoplasia (CIN) lesions. If left untreated, CIN lesions of all grades (I.III) may progress to invasive and metastatic cancer. Several groups have included analysis of the chromosomal arm 18q in their LOH studies and have reported LOH frequencies of 10.37% for this chromosomal arm [21, 22, 23, 24, 25, and 26]. More importantly, three of these studies have suggested an association of 18q loss with poor prognosis [24, 25, and 26]. One of the genes located on chromosome 18q21 is the tumor suppressor gene Smad4. Functional inactivation of Smad4 is of major importance in gastrointestinal malignancies. Inactivation of Smad4 occurs in one-half of pancreatic adenocarcinomas [27], in one-third of metastatic colorectal cancers [28], in every fourth carcinoma of the small intestine [29] and in smaller subsets of other tumor types (30]. Smad4 is a central transmitter of TGF-β responses and loss of Smad4 has been hypothesized to underlie TGF-b resistance of tumor cells. Smad4 (The mothers against decapentaplegic homolg-4) is a tumor suppressor protein that acts as a signaling molecule in the transforming growth factor-β (TGFβ)-induced anti-proliferative cell signaling pathway. It is already reported that the expression of Smad4 is reduced or completely lost in various cancers. Various gene such as p15,

p21, plasminogen activator inhibitor (PAI)-1 are transcriptionally activated by Smad4 (14). Several mechanisms of Smad4 inactivation in cancer have been reported. These include homozygous deletion, gene mutation, promoter hypermethylation and post-translation modification of Smad4 (15, 16). Till to date, the identification of several Smad4 binding proteins, which determines the cellular status and function of Smad4, simplified the understanding of Smad4 function as a tumor suppressor as well as the mechanisms of Smad4 turnover in cancer. In our study we have identified the novel interacting partner of Smad4 protein. We performed protein microarray and found GNAI2 as a new Smad4-binding protein (Y.K. Shin, unpublished observation). Thus, attempt was made in the present study to elucidate the role GNAI2 in the regulation of Smad4 function and its consequences in cervical carcinogenesis.

MATERIALS AND METHODS

Chemicals and reagents

Water soluble tetrazolium salts (WST, as a component of EZ-Cytox kit) was procured from Daeil Lab Service, Seoul, Korea. Purified TGF-β was purchased from Proteintech, USA. Dulbecco's Modified Eagle Medium (DMEM), RPMI-1640 and fetal bovine serum (FBS) were purchased from Thermoscientific, MA, USA. Primary antibodies for Smad4 (B8 clone, sc-7966) was procured from Santa Cruz Biotechnology (CA, USA). The primary antibody for p15 was obtained from Abcam (Cambridge, UK). The GNAI2 antibody was from Proteintech (USA). Horseradish peroxidase (HRP)-conjugated anti mouse and anti-rabbit antibodies were purchased from Invitrogen (NY, USA) and Thermo Scientific (IL, USA). Flag-HRP conjugated was obtained from Sigma Chemical Co. (MO, USA).

Plasmid constructions

Human GNAI2 cDNA encoding the open reading frame region was PCR-amplified from the cDNA of peripheral blood mononuclear cells (PBMC) using the forward primer TTATCGATAGGCTGCACCGTGAGCGC and reverse primer TTTCTAGAGAAGAGGCCGCAGTCCTTCAGG. A 1065-bp PCR product was cloned into the ClaI and XbaI restriction sites of the mammalian expression vector pFlag-VN173 (Invitrogen, CA, USA) and the sequences of the constructs was verified by sequencing.

For BiFC experiments, a sequence encoding full length GNAI2 was fused to pFlag-VN173 vector. A sequence encoding full length Smad4 was fused to pHA-VC155 vector. Both pFlag-VN173 and pHA-VC155 vector were kind gifts from Dr. Chang-

Deng Hu (Purdue University, West Lafayette, IN, USA). The $p15^{INK4b}$ promoter reporter construct (pGL2-$p15^{INK4b}$ promoter -751/+70) was a kind gift from Dr. Joan Massague (Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, NY, USA).

Cell culture and transfection

The HPV18-positive human cervical cancer cell line HeLa was obtained from the Korean Cell Line Bank (KCLB), Seoul, Korea. The HPV16-positive human cervical cancer cells (SiHa and CaSki), non-HPV infected cervical cancer cells C33A, mouse epidermal JB6 cells and mouse embryonic fibroblasts NIH3T3 were purchased from American Type Culture Collection (Manassas, VA). Human immortalized ovarian surface epithelial cells IOSE80-NIH was a gift from Dr. Michael Birrer (National Cancer Institute, Bethesda, MD, USA). Cells were grown at 37 ℃ in a humidified incubator under 5% CO_2 in DMEM and RPMI containing 10% FBS and combined antibiotics (Thermoscientific, LA, USA). Cells were routinely tested for mycoplasma contamination. SiHa and HeLa cells were transfected with Flag-VN173-GNAI2 plasmid (3 and 5 μg) in each 100mm plate using 2.5 μl lipofectamine LTX (Invitrogen). For BiFC analysis, HeLa cells were co-transfected with the expression vectors indicated in each experiment (1 μg each) using Polyexpress (Excellgen, Gaithersburg, MD).

Western Blot analysis

Cells were harvested and lysed with RIPA buffer [150 mM NaCl, 10 mM Tris (pH 7.2), 0.1% sodium dodecyl sulphate (SDS), 1%Triton X-100, 1% deoxycholate and 5 mM ethylene diaminetetraacetic acid (EDTA)] enriched with a complete protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany), and then incubated on ice for 30 min with regular vortexing before centrifuging at 14,000 rpm at 4° C for 15 min. Protein concentration was determined by using bichinconinic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The protein samples were boiled in 1X SDS sample buffer for 5 min for complete denaturation and were resolved on 10 to 15% SDS-polyacrylamide gel according to the protocol described earlier (10) After electrophoresis, proteins were transferred onto polyvinyl difluoride (PVDF) membrane, which was blocked with 5% nonfat dry milk in 1X TBST (Tris-buffered saline with 0.1% Tween-20) and incubated with primary antibody at the appropriate final concentration followed by hybridization with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:5000). For each step, the membrane was washed with 1X TBST three times for 10 min. Finally, western blot images were developed on photographic film using enhanced chemiluminescence (ECL) reagents.

Quantitative real time reverse transcription–polymerase chain reaction

Total RNA was extracted from cells transfected using Trizol (Invitrogen, NY, USA) and reversed transcribed to cDNA using the Superscript^{TM} II First-Strand Synthesis

System (Invitrogen, NY, USA), according to the manufacturer's protocol. Following cDNA synthesis, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was carried out as described previously (11) in a dual system LightCycler (Roche Applied Science, Mahh heim, Germany) using the primers. Universal Probe library (UPL, Roche Applied Science) probe sequences listed in **Table-1** with the *HPRT)* Taqman probe (TIB MOLBIOL, Berlin, Germany) used as a "reference gene" to normalize gene expression.

Bimolecular fluorescence complementation (BiFC) analysis

The BiFC assay is based on an observation that the association of fluorescent protein fragments can be facilitated by an interaction between proteins fused to the fragments. It enables visualization of interacted proteins at high spatial resolution without the need for other exogenous agents (Tom.K. Kerpolla). HeLa cells were co-transfected with the expression vectors indicated in each experiment (1 µg each) using Polyexpress (Excellgen, Gaithersburg, MD) in each well of 6-well plate. The fluorescence emissions were observed 24 to 30 h after transfection using an Olympus IX71 fluorescence microscope with a TH4-200 camera.

Immunoprecipitation

Cells were harvested in IP lysis buffer containing 20 mM Tris (pH7.5), 15% glycerol, 1% TritonX-100, 1 mM EDTA, 8 mM MgSO4, 150 mM NaCl, 10 mM sodium fluoride, 10 mM glycerophosphate, 0.5 mM sodium orthovanadate, and 0.1 mM phenylmethylsulfonyl fluoride and a mixture of protease inhibitors. Cell lysates (2000 μg) were clarified by centrifugation before incubation overnight at 4 °C in presence of Smad4 monoclonal antibody (2 μg) in same lysis buffer as mentioned above. rProtein G agarose (Invitrogen, 50% slurry) was washed and 50 μg bead slurry was then added and the incubation continued for 2 h 30 min at 4 °C. Immunoprecipitates were recovered by centrifugation, washed three times with IP wash buffer [20 mM Tris (pH7.5), 15%glycerol, 1% TritonX-100, 1 mM EDTA, 8 mM MgSO4, 150 mM NaCl] and resolved by western blotting using specific antibody. In the case of immunoblotting with GNAI2, goat anti rabbit was used as a secondary antibody.

Cell proliferation assay

The effect of GNAI2 siRNA on cell proliferation was measured by the water soluble tetrazolium salts (WST) method (EZ-Cytox kit; Daeil Lab Service, Seoul, Korea). HeLa cells was (1×10^5) transfected with either control si-RNA or GNAI2 si-RNA (25 or 50 nM) were grown in triplicate in 6-well plates for 24, 48 and 72 h at 37 °C. EZ-Cytox solution (200 μL) was added to each well and incubated for 80 min. The number of viable cells was measured in a 96-well plate at an optical density of 492

nm on a Sunrise reader (Tecan Trading AG, Switzerland). Cell viability was described as the percentage of control si-RNA-transfected cells.

siRNA transfection

Five siRNA against GNAI2 were purchased from Genolution Pharmaceuticals, Inc. (Seoul, Korea) as si-GNAI2-01, si-*GNAI2*-02, si-GNAI2-03, si-*GNAI2*-04 and si-GNAI2-5 . The sequences of si-*GNAI2*-01, si-*GNAI22*-02, si-GNAI2-03, si-*GNAI2*-04 and si-GNAI2-05 were CCAUCAUGGCCAUUGUCAAUU, GCUACAUCCAGAGUAAGUUUU, CUGAAUAAGCGCAAAGACUU and GAUGUCAUCAUCAAGAACAUU, GAUAGCAUCUGCAACAACAUU, respectively. HeLa cells were transfected with all 5 siRNA against GNAI2 and further experiments were performed using GNAI2-01-siRNA. The control-siRNA (non-specific control siRNA VIII, cat. No-D-001206-08-20) was purchased from Dharmacon (IL, USA). For transfection with siRNA, cells (5×10^4) were seeded into 6-well plates. After 24 h, cells were transfected with siRNA using oligofectamine (Invtrogen, Carlsbad, CA, USA) following manufacturer's protocol with minor modification. The final concentration of siRNA in the cell culture medium was 25 or 50 nM. Western blot analysis with GNAI2 specific antibody and qRT-PCR showed that GNAI2-01 siRNA produced the best inhibition.

Cell migration assay

Cells (5×10^4) transfected with *GNAI2 si*-RNA (25 and 50 nM) or control si-RNA, were subjected to Millipore's (MA, USA) 24-well CHEMICON QCM™ cell migration assay. After incubation at 37 °C for 24 h, cells were starved in only RPMI for an additional 24 h. Cell number was detected with a GENios Pro microplate reader (Tecan Trading AG, Switzerland) using 485/535 nm filter set (12). All migration and invasion assays were performed in triplicate in at least three independent experiments. Values are expressed as percentages of control.

Luciferase reporter gene assay

Cells were seeded into 12-well plates at a density of 1×10^5 cells per well prior to transfection. Cells were transfected with p15 promoter construct (a kind gift from Dr. Joan Massague, Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, NY, USA) together with an empty vector or pFlag-Vn173-GNAI2 using genefectin transfection reagent. pRL-TK (Promega, Madison, WI, USA) was used as a normalization control. After a further 24-h culture cells were starved for 1 h and the luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega).

RESULT

Expression level of GNAI2 and Smad4 in different cervical cancer cells

To find out the level of Smad4 and GNAI2 the whole cell lysate was made from HeLa, SiHa,Caski and C33A cervical cancer cells with RIPA buffer and the whole cell lysate was separated by SDS-PAGE gel and Immunobloted with Smad4 and GNAI2. To find out the exact band of GNAI2 the whole cell lysate from U937 cell was used. Smad4 protein was higly expressed in SiHa cell whare as there was no Smad4 in Caski. In the HeLa and C33A cell Smad4 was moderately expressed (**Fig-1.A**). The band of GNAI2 was further clarified by si-RNA mediated knockdown of GNAI2 in HeLa cells (**Fig-1.B**).

Interaction between GNAI2 and Smad4 by Bimolecular Fluorescence Complementation (BiFC) analysis

HeLa cells were co-transfected with plasmids harboring full-length GNAI2 (Flag-VN173-GNAI2) in presence of full length Smad4 (Ha-VC155-Smad4) and protein-protein interaction was visualized by BiFC assay as detailed in the Materials and Methods. Co-transfection with full length Smad4 and Smad2-MH1 was used as negative control (upper left panel), whereas transfection of cell with a combination of constructs containing full length Smad4 and Smad2-MH2 domain was used as a positive control (upper right panel). The interaction between full-length GNAI2 and full-length Smad4 are shown (**Fig-2**).

Endogenous and exogenous interaction of GNAI2 with Smad4 in HeLa cells.

To find out the endogenous interaction of GNAI2 and Smad4 in HeLa cell Immunoprecipitation(IP) assay was performed .Hela cell was grown to 80% confluence and whole cell lysate was made using IP lysis buffer. Inacse of exogenous Immunoprecipitation(IP) HeLa cell was co-Transfected with Ha-Smad4 and Flag-VN173-GNAI2 and the whole cell lysate was preapared with IP lysis buffer. Protein extract (2 mg) prepared from HeLa cells was immunoprecipitated with anti-Smad4 monoclonal antibody (S4, 2 μg) or mouse control IgG. Whole lysate (WCL), supernatant (Sup) and immunoprecipitate (IP sample) were separated by SDS-PAGE and immunoblotted with either GNAI2 or Smad4 antibody. The western blot data showed that GNAI2 and Smad4 interact with each other endogenously and exogenously in HeLa cells (**Fig-3 and Fig-4**).

GNAI2 negatively regulates Smad4 expression

After finding out the interaction between GNAI2 and Smad4 we tried to find out if the interaction had any effect on the expression of Smad4. Overexpression of GNAI2 caused decreased Smad4 expression in HeLa cells (**Fig-5.A**). The inhibition of Smad4 expression by GNAI2 was further confirmed by siRNA-based knockdown of GNAI2 which showed increased expression of Smad4 in and HeLa (**Fig-5.B**) cells.

Role of GNAI2 in the expression and activity of Smad4-target genes p15 and p21

Since GNAI2 is involved in the regulation of Smad4 expression, we investigated the role of GNAI2 in the expression and promoter activity of Smad4-target genes. The expression of p15 and p21, which are transcriptionally regulated by Smad4 and inhibit cell proliferation, was markedly decreased in HeLa cells overexpressing GNAI2 as compared to cells empty vector (**Fig-5.B**). Transient overexpression of GNAI2 in these cells also diminished the mRNA expression of p15 and p21 (**Fig. 8.A**). Knockdown of GNAI2 by siRNA, on the other hand, increased the expression of p15 and p21 in HeLa cells (**Fig-8.B**). We also found that overexpression of GNAI2 dose-dependently inhibited p15 promoter activity in HeLa cells (**Fig-10**).

Downregulation of Smad4 expression by overexpression of GNAI2 is mediated through proteasomal degradation

To address whether the reduced expression of Smad4 upon overexpression of GNAI2 is transcriptionally regulated, we performed qRT-PCR analysis of Smad4 mRNA expression from cells transfected with either Flag-VN173-GNAI2 or control vector. Transient transfection with GNAI2 did not cause change in Smad4 mRNA level in Si HeLa cells (**Fig-7.A and 7.B**) as compared to respective vector-transfected cells. Incubation of HeLa cells with proteasomal inhibitor MG132 after transfection with Flag-VN173-GNAI2 or control vector regain GNAI2-mediated downregulation of Smad4 expression (**Fig-6**), suggesting that GNAI2 promotes proteasomal degradation of Smad4.

Overexpression of GNAI2 decrease the Smad4 in cytosolic and nuclear fraction

As we know that in the TGF-β signaling pathway Smad4 form complex with p-Smad2/3 and translocate in the nucleus and regulate the Smad4 target gene. So, the proportion in the cytosol and nuclear fraction plays an important role in the function of Smad4. We found that TGF-β treatment in the GNAI2 overexpressed HeLa cell has decreased Smad4 in cytosolic fraction as well as nuclear fraction as compared to the empty vector transfected HeLa cell (**Fig-9**).

Knockdown of GNAI2 decrease the cell viability

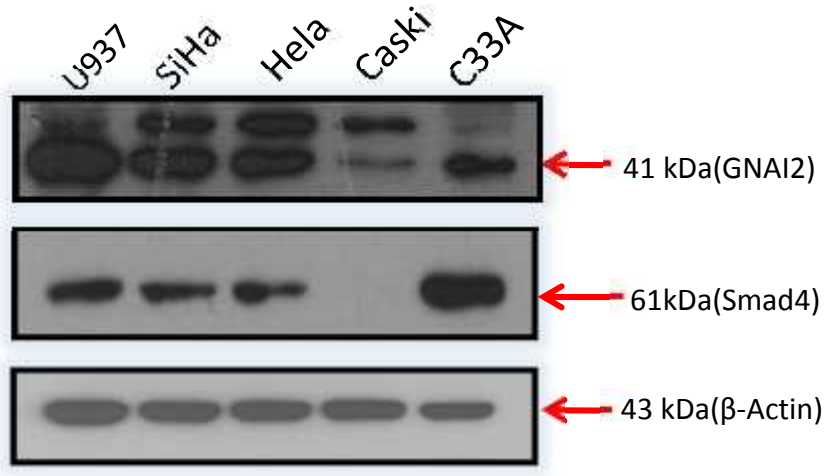
We then attempted to investigate the effect of GNAI2 silencing on the proliferation of cervical cancer cells. Transient transfection of HeLa (**Fig-11.B**) cells with either control siRNA or GNAI2 siRNA (25 or 50 nM) for 48 h reduced the expression of GNAI2 in a concentration dependent manner. In addition, transfection of HeLa with 25 and 50 nM GNAI2 siRNA for indicated periods reduced the expression of GNAI2 in a time-dependent manner. The cell viability assay revealed that transfection with GNAI2 siRNA caused significant decrease in cell viability in HeLa (**Fig-11.A**) cells as compared to cells transfected with control siRNA in time dependent and dose dependent manner.

Knockdown of GNAI2 reduced the migration and invasion of HeLa cells

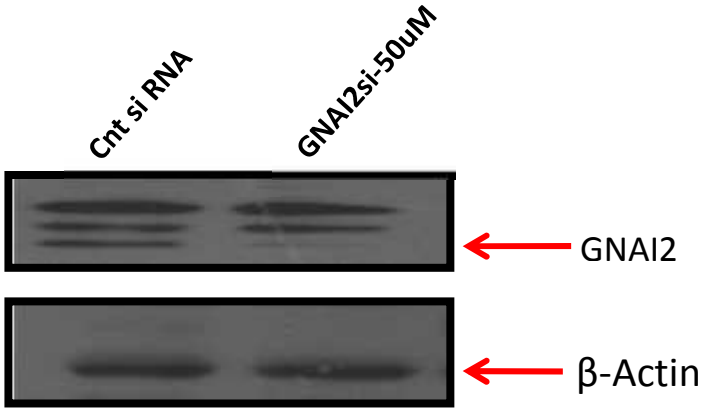
HeLa cells were transiently transfected with either GNAI2 si-RNA (25 and 50 nM) or control si-RNA (50 nM) for 48 h, and cell migration and invasion assay was performed in triplicate as described in the Materials and Methods section.(Cnt: Control).The result shows that knock down of GNAI2 in HeLa cell reduced both th migration and invasion of HeLa cells **(Fig:12.A and 12.B).**

Figure-1: Expression level of GNAI2 and Smad4 in different cervical cancer cells

A.



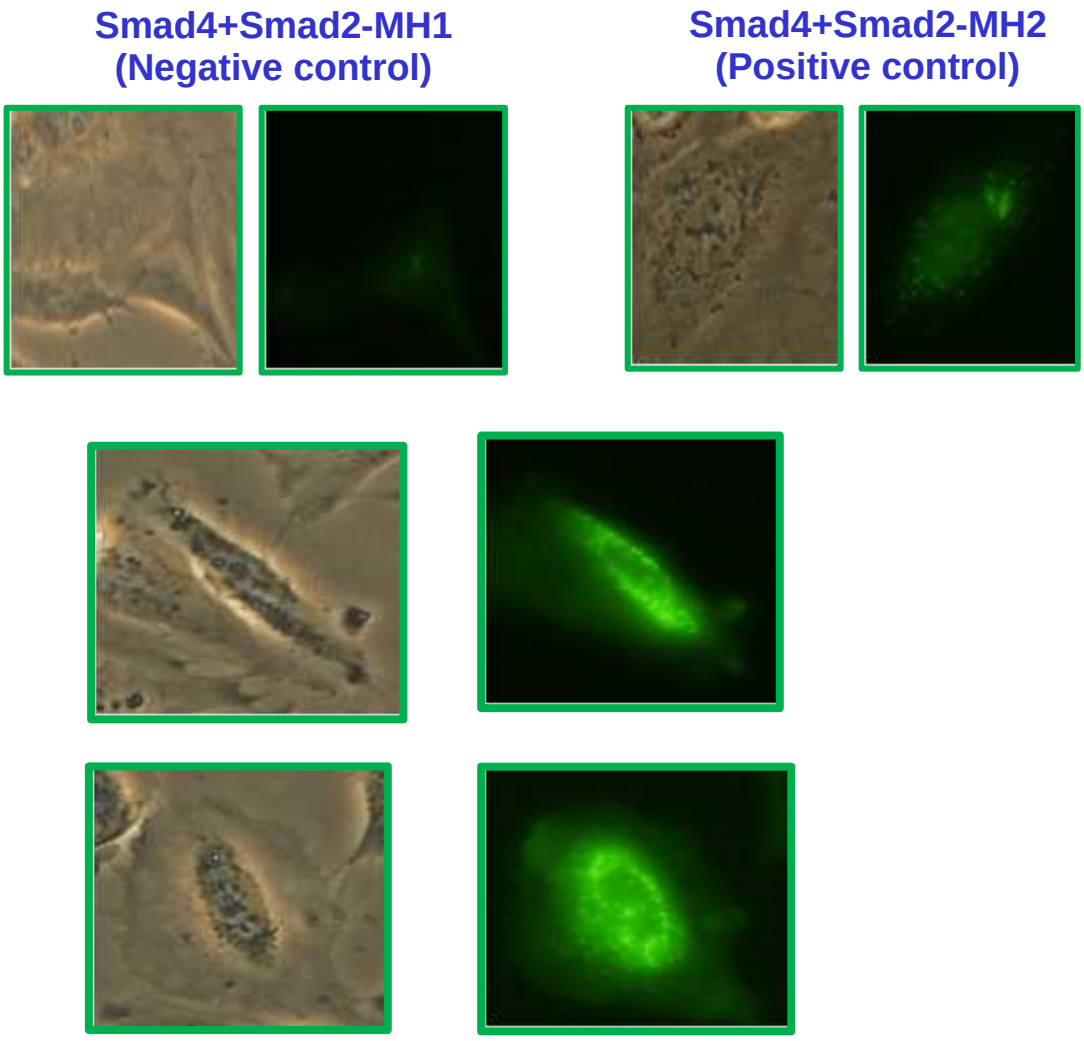
B.



A) Whole cell lysate was made from SiHa, HeLa, Caski and C33A described in Materials and Methods. The expression of GNAI2 and Smad4 was determined by by western blot analysis and actin was was used as loading control. (Cnt: Control).

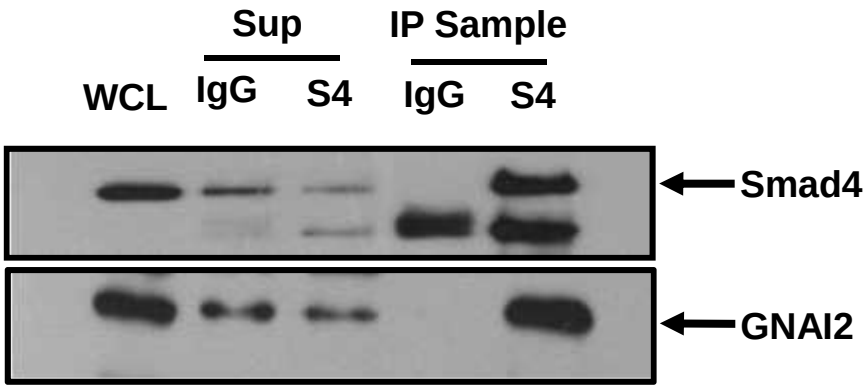
B) Hela cell was transiently transfected with siRNA against GNAI2. After 48 hours whole cell lysate was made as described in the materials and method and GNAI2 was determined by western blot analysis where actin was used as loading control.(Cnt: Control).

Figure-2: Interaction between GNAI2 and Smad4 visualized by BiFC assay



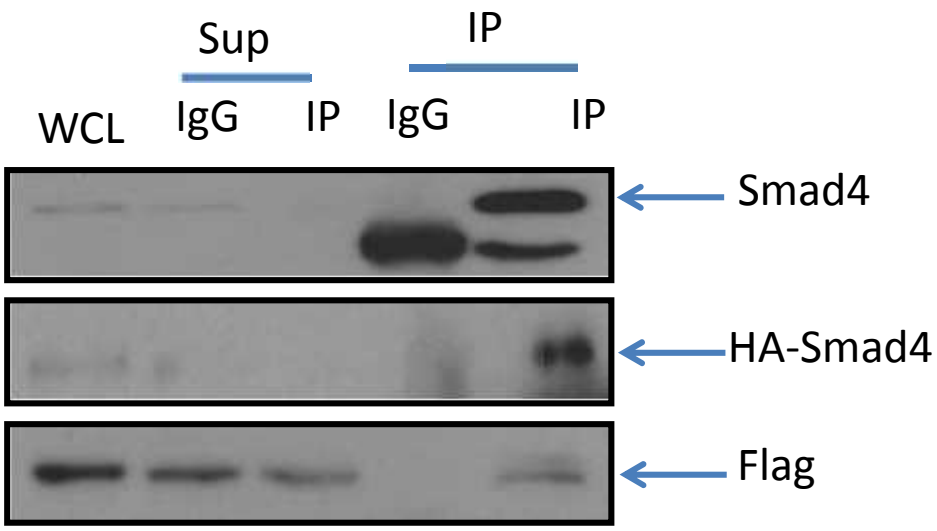
HeLa cells were co-transfected with plasmids harboring full-length GNAI2 (Flag-VN173-GNAI2) in presence of full length Smad4 (Ha-VC155-Smad4) and protein-protein interaction was visualized by BiFC assay as detailed in the Materials and Methods (lower two panels). Co-transfection with full length Smad4 and Smad2-MH1 was used as negative control (upper left panel), whereas transfection of cell with a combination of constructs containing full length Smad4 and Smad2-MH2 domain was used as a positive control (upper right panel).

Figure-3: Interaction between GNAI2 and Smad4 through endogenous Immuniprecipitation(IP)



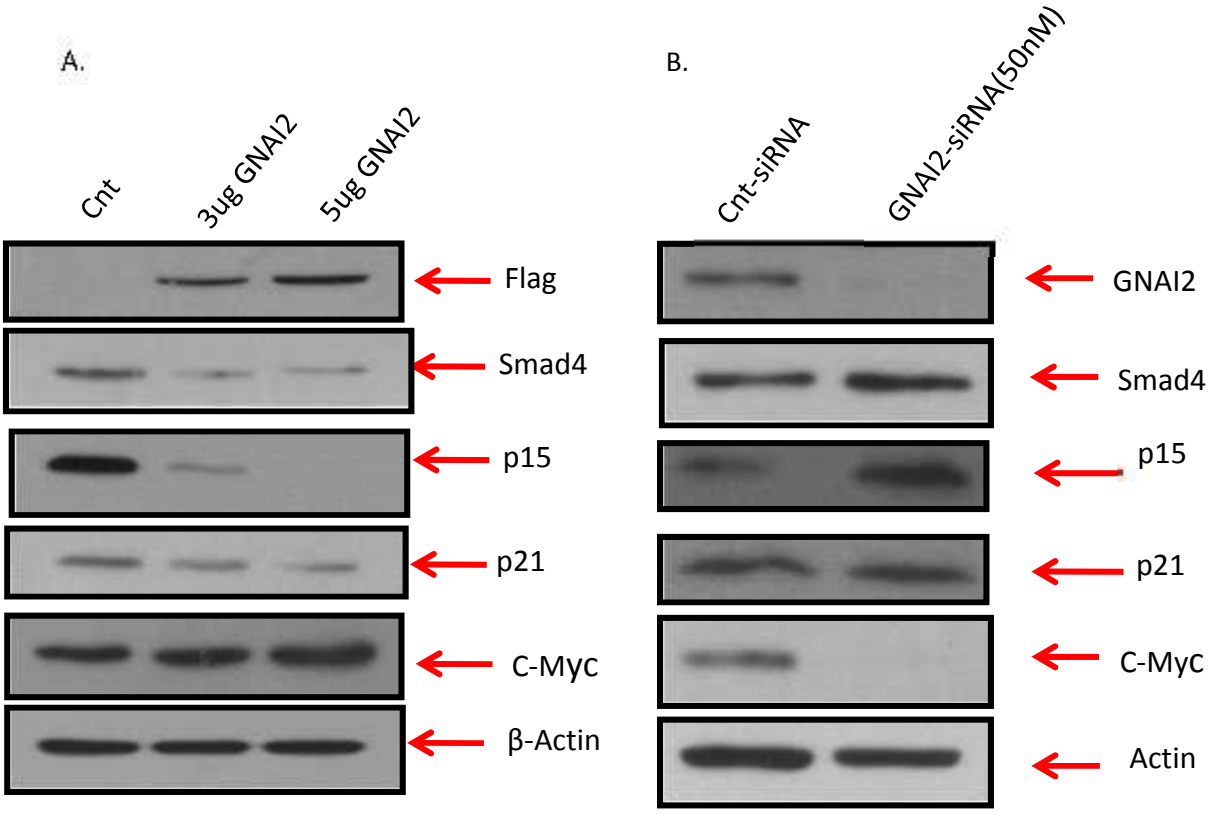
Protein extract (2 mg) prepared from HeLa cells was immunoprecipitated with anti-Smad4 monoclonal antibody (S7, 2 μg) or mouse control IgG. Whole lysate (WCL), supernatant (Sup) and immunoprecipitate (IP sample) were separated by SDS-PAGE and immunoblotted with either GNAI2 or Smad4 antibody.

Figure-4: Interaction between GNAI2 and Smad4 through exogenous Immuniprecipitation(IP)



HeLa cell was co-transfected with plasmids harboring full-length GNAI2 (Flag-VN173-GNAI2) in presence of full length Smad4 (Ha-VC155-Smad4). Protein extract (2 mg) prepared from HeLa cells was immunoprecipitated with anti-Smad4 monoclonal antibody (S7, 2 μg) or mouse control IgG. Whole lysate (WCL), supernatant (Sup) and immunoprecipitate (IP sample) were separated by SDS-PAGE and immunoblotted with either GNAI2 or Smad4 or HA-HRP antibody.

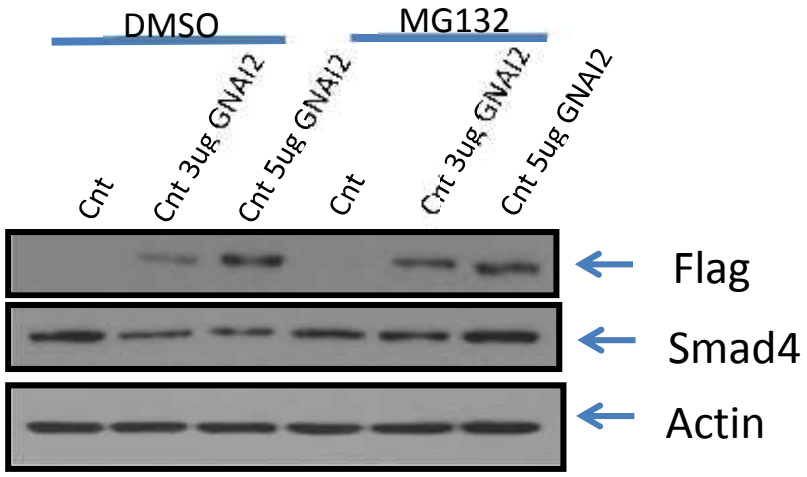
Figure-5: The effect of overexpression and knockdown of GNAI2 on Smad4 and its target gene



(A) HeLa cells were transiently transfected with Flag-VN173-GNAI2 or control vector. Protein lysates were subjected to Western blot analysis to detect the expression level of Smad4, p15, P21 and C-Myc. Flag was measured to confirm the transfection efficiency and actin was used as loading control. (Cnt: Control).

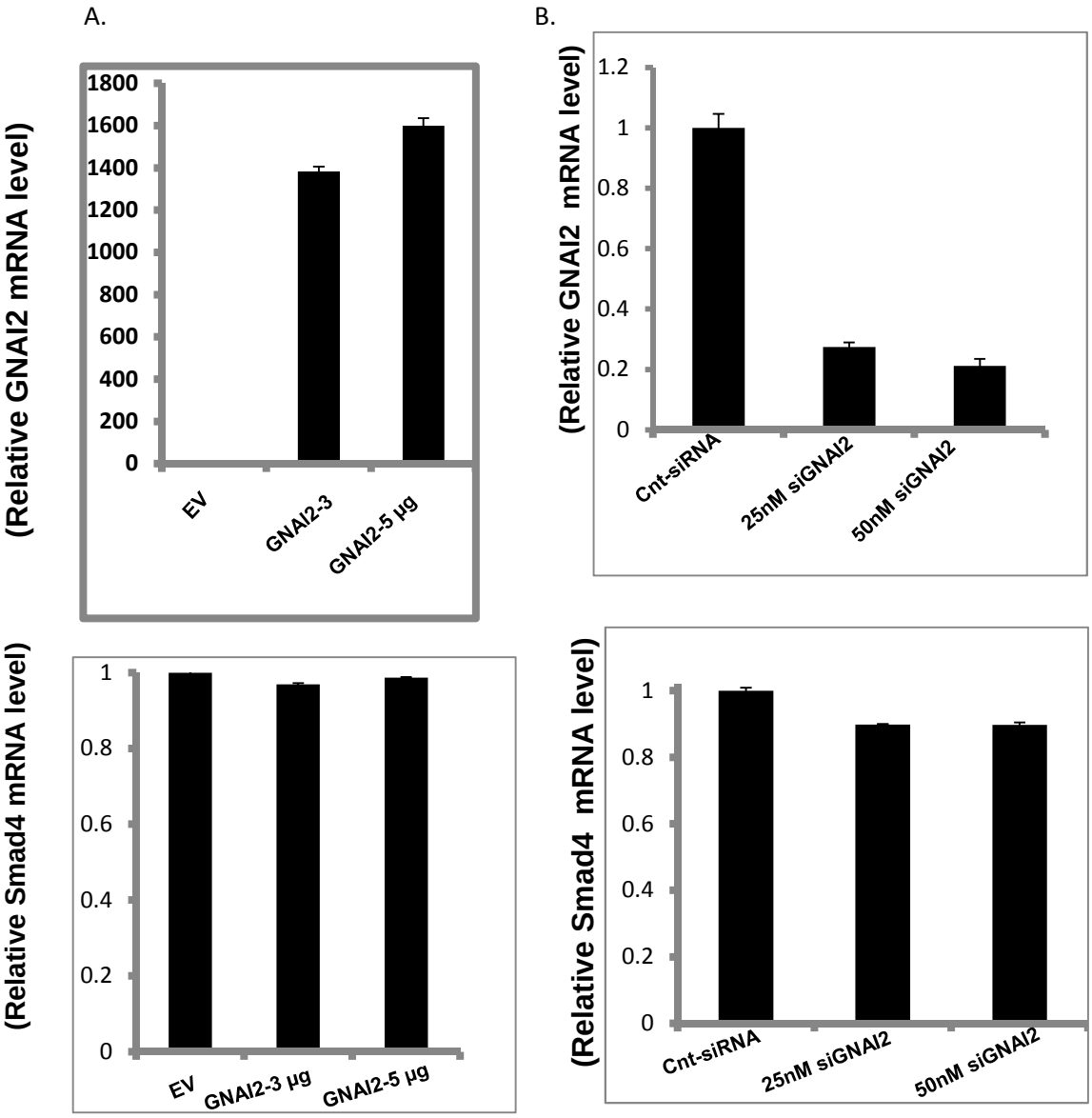
(B) HeLa cells were transiently transfected with control siRNA or GNAI2 siRNA. Protein lysates were subjected to Western blot analysis to detect the expression level of GNAI2, Smad4, p15, P21 and C-Myc. Actin was used as loading control. (Cnt: Control).

Figure-6: Expression level of Smad4 in MG132 treated GNAI2 transient overexpressed HeLa cells



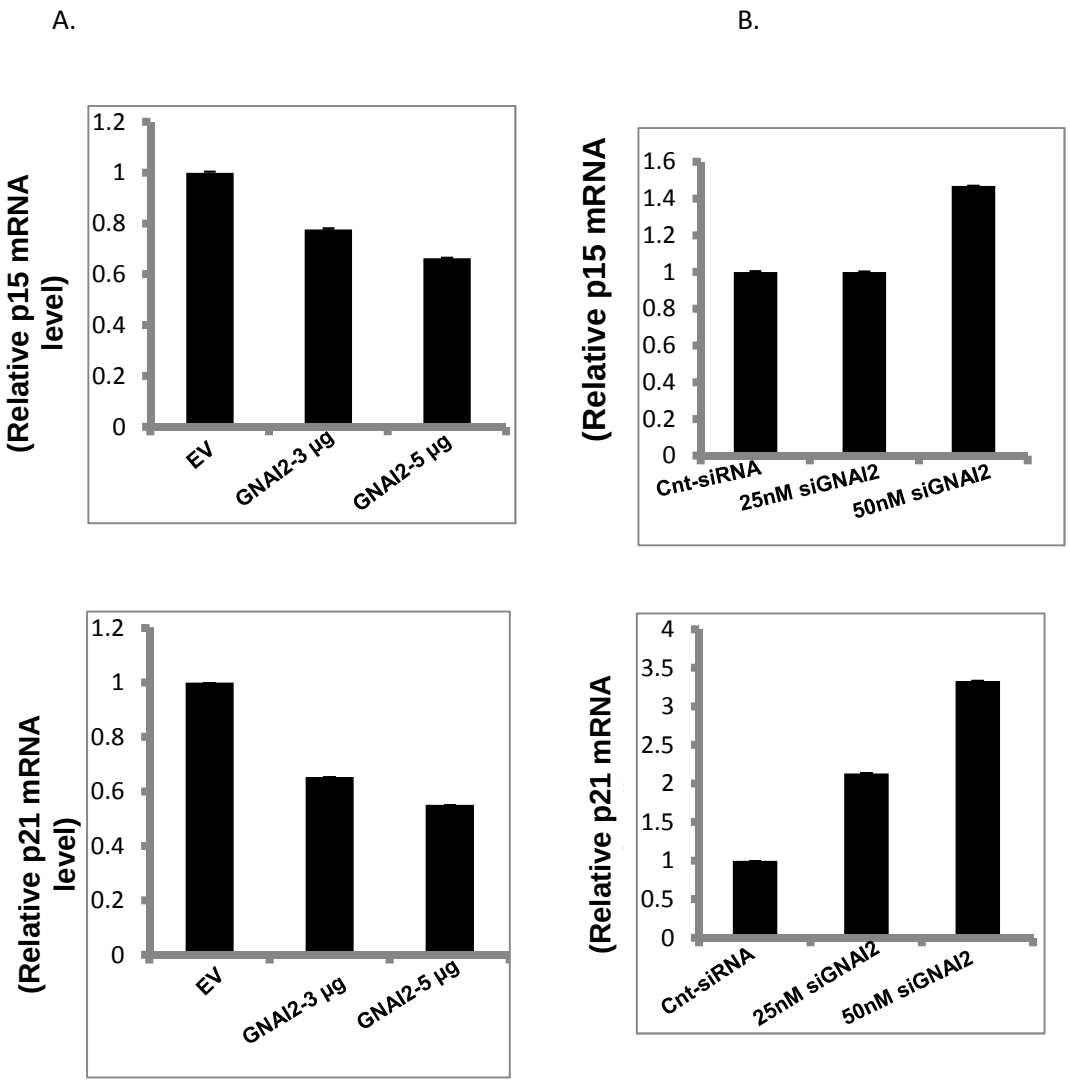
HeLa cells were transiently transfected with Flag-VN173-GNAI2 or control vector. After 24 hours the cell was treated with proteasomal inhibitor MG132 and incubated for 4 hours .The whole cell lysate was prepared as described in the materials and method.The expression of Smad4 was detected by Western blot analysis.(Cnt: Control).

Figure-7: mRNA level of Smad4 after overexpression and knockdown of GNAI2



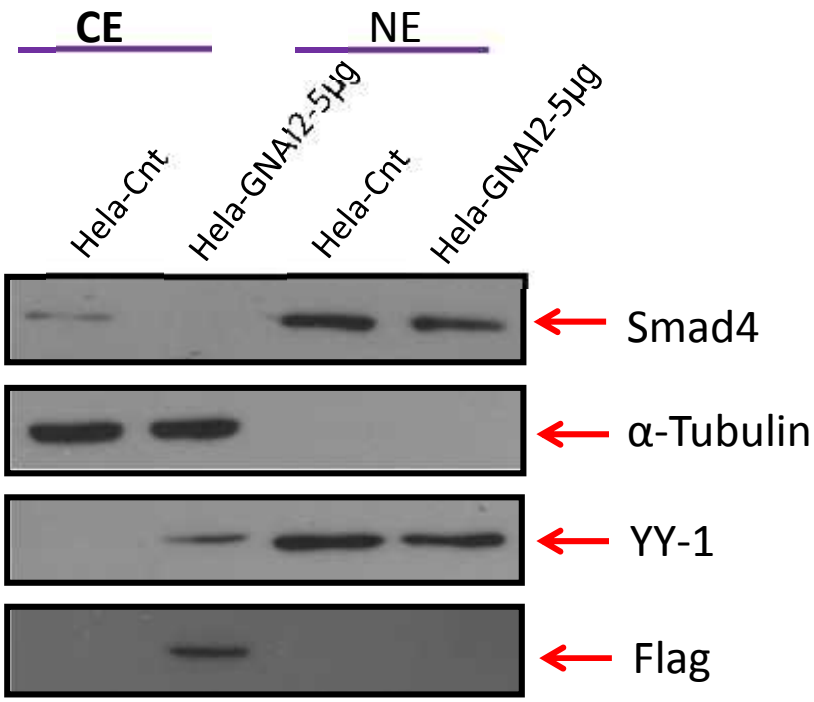
(A-B) (A) HeLa cells were transiently tranfected with Flag-VN173-GNAI2 or control and (B) HeLa cells were transiently transfected with siRNA against GNAI2 or si-Control. Total RNA was isolated and analyzed by qRT-PCR to measure the expression level of Smad4 and GNAI2 mRNA. All experiments were carried out triplicate *, $p < 0.001$ as compared to control siRNA.

Figure-8: mRNA level of p15 and p21 after overexpression and knockdown of GNAI2



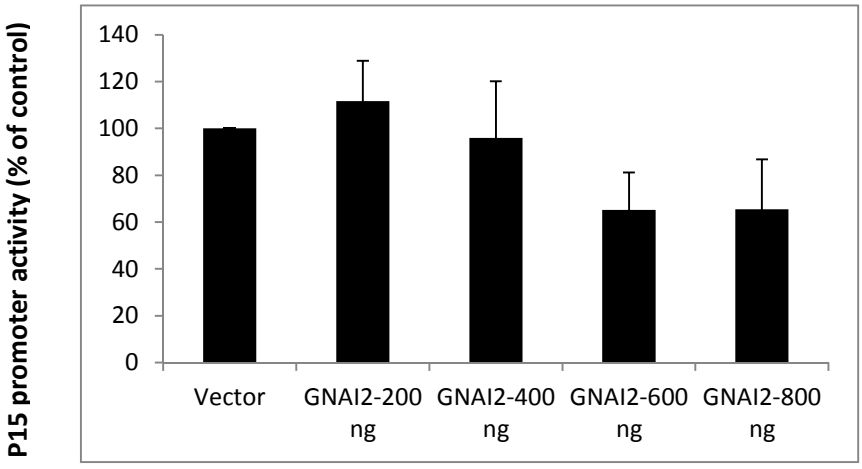
(A-B) (A) HeLa cells were transiently tranfected with Flag-VN173-GNAI2 or control and (B) HeLa cells were transiently transfected with siRNA against GNAI2 or si-Control. Total RNA was isolated and analyzed by qRT-PCR to measure the expression level of p15 and p21 mRNA.(Cnt: Control). All experiments were carried out triplicate *, $p < 0.001$ as compared to control siRNA.

Figure-9: Expression of Smad4 protein in cytosolic and nuclear fraction after overexpression and knockdown of GNAI2



HeLa cells were transiently transfected with Flag-VN173-GNAI2 or control vector. After 24 hours the cell was incubated in serum free media for 1 hour and then treated with TGF-β and incubated for 30minutes. The cytosolic and nuclear fraction was prepared described in the materials and method. The expression of Smad4, YY-1, α-Tubulin and Flag for GNAI2 was detected by Western blot analysis.(CE: Cytosolic fraction, NE: Nuclear fraction, Cnt: Control).

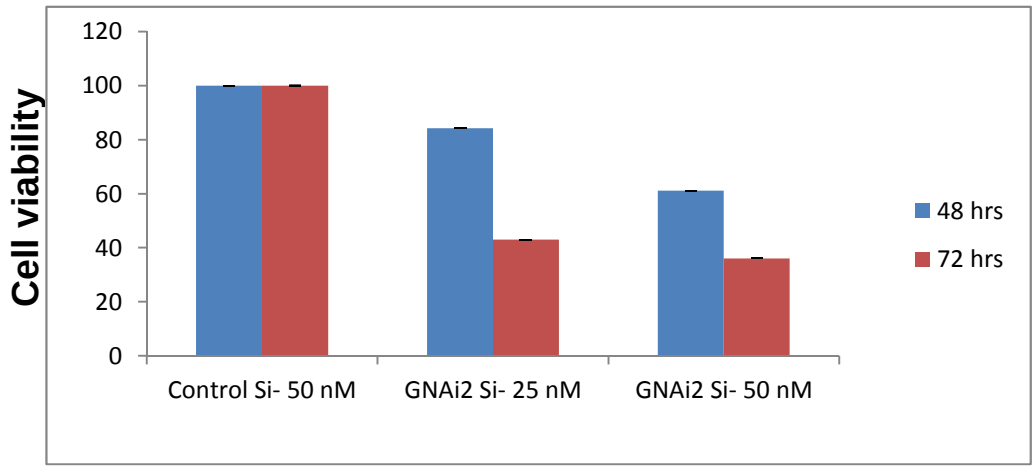
Figure-10: P15 promoter activity after transient overexpression of GNAI2 in HeLa cellS



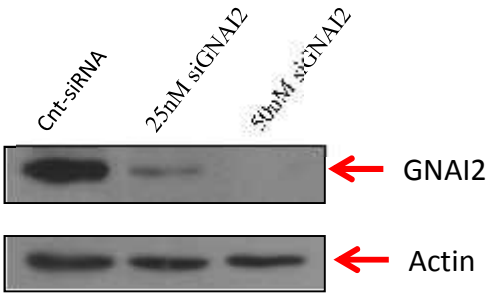
(A) HeLa cells were transiently transfected with Flag-VN173-GNAI2or control vector and incubated for 48hours. The p15 promoter activity from transfected cells was measured as described in Materials and Methods section. All experiments were carried out triplicate *, $p < 0.024$ as compared to control siRNA.

Figure-11: Transfection of GNAI2 (25nM and 50nM) siRNA decreased Cell viability

A.



B.

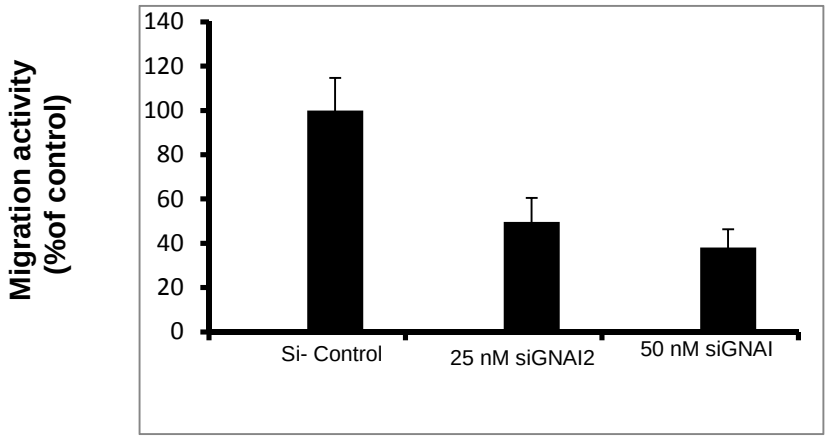


(A) HeLa cells were transiently transfected with siRNA against GNAI2 or si-Control (25or 50nM) for 48 hours and cell viability was evaluated using MTT assay as described in the Materials and Methods section. Cell viability was evaluated using MTT assay as described in the Materials and Methods section. All experiments were carried out triplicate *, $p < 0.001$ as compared to control siRNA.

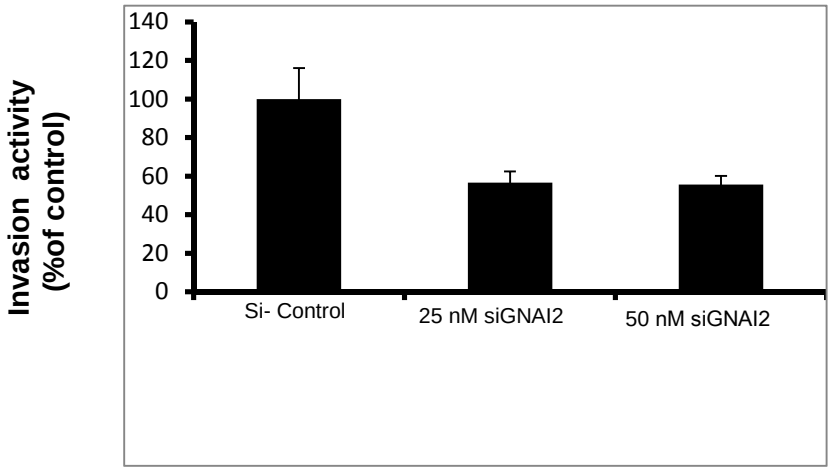
(B) Western blot analysis confirmed the down regulation of GNAI2. Data are representative of three independent experiments showing a similar pattern. (Cnt: Control).

Figure-12: Knockdown of GNAI2 in HeLa cell results in reduced migration and invasion

A.



B.



(A-B) HeLa cells were transiently transfected with either GNAI2 si-RNA (25 and 50 nM) or control si-RNA (50 nM) for 48 h, and cell migration and invasion assay was performed in triplicate as described in the Materials and Methods section.(Cnt: Control). Migration and invasion assays were performed using 24-well transwell after 24 h serum starvation. All experiments were carried out triplicate *, $p < 0.001$ as compared to control siRNA.

Table-1: Primer sequences for quantitative RT PCR

Gene	Orientation	Sequences	Tm (°C)
Smad4	Forward	5'-TGG CCC AGG ATC AGT AGG T--3'	57.1
	Reverse	5'-CAT CAA CAC CAA TTC CAG CA -3'	53.4
CDKN2B	Forward	5'-CAACGGAGTCAACCGTTTC-3'	54.9
	Reverse	5'-GGTGAGAGTGGCAGGGTCT-3'	54.2
GNAI2	Forward	5'- CTCAACGAACTCAGCTGCCTA -3'	57.5
	Reverse	5'- ATGTAGTCTGCTGTGTGGGG -3'	57.5
P21	Forward	5'-CGAAGTCAGTTCCTTGTGGAG-3'	57.8
	Reverse	5'-CATGGGTTCTGACGGACAT-3'	54.9
HPRT	Forward	5'-CTCAACTTTAACTGGAAAGAATGTC-3'	54.1
	Reverse	5'-TCCTTTTCACCAGCAAGCT-3'	55.5

Discussion:

Squamous cell carcinoma of the uterine cervix is one of the most frequent cancers affecting women worldwide. Carcinomas arise from cervical intraepithelial lesions, in which infection with high-risk human papillomavirus types has led to deregulated growth control through the actions of the viral E6 and E7 oncoproteins. The molecular mechanisms underlying progression to invasive tumor growth are poorly understood. One important feature, however, is the escape from growth inhibition by transforming growth factor-β (TGF-β). Loss of chromosomal arm 18q is among the most frequent cytogenetic alterations in cervical cancers and has been associated with poor prognosis.

Tumor suppressor function is lost in many cancers including cervical cancer(17). There are many mechanisms for the loss of Smad4 function such as gene mutation, promoter hypermethylation, homozygous deletion and post translation modification (18, 19). Interaction with various proteins may lead to the loss of Smad4. Research findings show that interaction with Jab1, a co-activator of c-Jun oncoprotein interacts directly with Smad4, and induces ubiquitination and proteosomoal degradation of Smad4 (20). In a protein microarray designed to identify novel Smad4 interacting proteins, GNAI2 appeared as a new Smad4 binding protein (Y.K. Shin, unpublished observation). In this study, the novel interaction between GNAI2 and Smad4 was confirmed by endogenous and exogenous co-immunoprecipitation assay and BiFC analysis.

Our findings show that transient overexpression of GNAI2 decrease the expression of Smad4, p15, and p21 and siRNA-mediated silencing of GNAI2 increase the

expression level of Smad4, p15 and p21.The findings also show that overexpression GNAI2 decrease the mRNA level expression of p15 and p21 and knockdown of GNAI2 with siRNA shows the increased expression of p15 and p21. But in case of overexpression and knockdown of GNAI the mRNA level of SMAd4 remained unchanged. So, we can assume that the degradation of Smad4 protein upon overexpression of GNAI2 may happen through other process such as ubiquitination and proteosomal degradation. The interaction between GNAI2 and Smad4 may result in down-regulation of Smad4-mediated transactivation of genes encoding cell growth inhibitory proteins. The reduced expression of Smad4 protein upon GNAI2 overexpression may also result in decreased expression of p15 and p21. Based on these findings, it may be conferred that GNAI2 enhances cell proliferation and transformation, at least in part, by blocking Smad4-mediated anti-tumor signaling.

The inhibition of GNAI2-mediated down-regulation of Smad4 upon incubation of cervical cancer HeLa cells with proteasome inhibitor MG132 suggest that GNAI2 may promote proteasomal degradation of Smad4. Since Smad4 is a mediator in the transforming growth factor-beta (TGFβ)-induced signal transduction pathway, and upon formation of complex with Smad2/3, Smad 4 translocate in the nucleus and regulate the gene expression. So, the level of Smad4 protein in cytoplasm and nucleoplasm may play an important role in TGF-β tumor suppression. Our findings shows that TGF-β treatment in GNAI2 overexpressed HeLa cell has reduced level of Smad4 protein in cytosolic fraction as well as nuclear fraction.

The dose-dependent inhibition of TGF-β-induced p15 promoter activity upon transient overexpression of GNAI2 suggests that GNAI2 may affect p15 promoter activity via interacting with Smad4 which is an important mediator in TGF-β signaling pathway. Moreover, the decreased level of cell viability, cell migration and cell invasion after dose dependent and time dependent si-mediated down-regulation GNAI in HeLa cell shows the functional relevance of the interaction between GNAI2 and Smad4.

In summary, the present study convincingly demonstrate that GNAI2 plays an oncogenic role in cervical carcinogenesis by interacting with Smad4 and inhibiting Smad4 expression which results in the reduced expression of Smad4 target genes p15 and p21 and increase the C-Myc level. Thus, additional studies are necessary to find out the role of GNAI2 in the modulation of TGF-β signaling pathway through the interaction with Smad4 and its consequences in cervical carcinogenesis.

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초록

GNAI2 (Guanine nucleotide binding protein (G-protein), alpha inhibiting activity polypeptide 2)는 polypeptides 인 Gi alpha 1 (GNAI1), Gi alpha 2 (GNAI2), 그리고 Gi alpha 3 (GNAI3)를 포함하는 Gi alpha 단백질에 속한다. GNAI2 는 잘 알려진 protooncogene 으로 다양한 종류의 암에서 암 발생과 증식에 관여된다. Smad4 (Mother Against Decapentaplegic Homologue 4, DPC4)는 잘 알려진 tumor suppressor 이며 TGF-β (Transforming Growth factor- β) signaling pathway 에서 주요 요소이다. 발암 과정에서 GNAI2 의 역할을 밝히고 잠재적 결합 대상을 찾기 위해, 첫째로 baculovirus protoarray system 을 통하여 Smad4 를 그 결합 대상으로 확인하였다. 이 연구에서, bimolecular fluorescence complementation (BiFC) assay 를 이용하여 자궁경부암세포(HeLa cell)에서 GNAI2 와 Smad4 간의 새로운 결합함을 처음으로 확인하였고, 또한 immunoprecipitaion (IP) assay 에서 내적으로나 외적으로 그 결합을 확인하였다. GNAI2 를 인위적으로 넣어서 western blot 을 한 결과, Smad4, p15INK4b, p21 protein 의 발현은 감소하였고, c-Myc 은 발현이 증가하였다. 반대로, GNAI2 의 발현감소는 Smad4, p15INK4b, p21 protein 의 발현을 유의적으로 증가시켰고 c-Myc 의 발현을 감소시켰다. 그러나, qRT-PCR 실험에서는 Smad4 mRNA 양에는 변화가 없었으며, GNAI2 의 과발현과 발현 감소 시에는 각각 p15INK4, p21 mRNA 양에서 상당한 감소와 증가가 보였다. 또한, GNAI2 가 과발현되는 HeLa cell 에서 26S 프로테오좀을 억제하는 MG132 를 처리하였을 때, Smad4 단백질의 분해가 감소하였다. Luciferase reporter assay 를 통해서 GNAI2 의 과발현이 p15INK4b promoter 의 활성을 감소시킴을 확인하였다. 또한 siRNA 에 의한 GNAI2 의 발현감소에 의해 HeLa cells 의 증식이 저하되었다. 이 연구를 통해 자궁경부암세포에서 GNAI2 가 Smad4 와 결합하여 Smad4 와 그 타겟 유전자의 발현을 음성적으로 조절함을 확인하였다.

주요어 : GNAI2, Smad4, BiFC assay, p15INK4b, Cervical Cancer.

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