



Oncogenic Fibulin-5 Promotes Nasopharyngeal Carcinoma Cell Metastasis through the FLJ10540/AKT Pathway and Correlates with Poor Prognosis

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Abstract

Background: Nasopharyngeal carcinoma (NPC) is known for its high metastatic potential and locoregional recurrence, although the molecular alterations that are driving NPC metastasis remain unclear at this time. This study aimed to examine the expression of fibulin-5 in NPC, correlate the results with clinicopathological variables and survival, and to investigate the role of fibulin-5 in human NPC cell lines.

Material and Methods: Standard semi-quantitative-RT-PCR, quantitative-RT-PCR, immunoblotting, and immunohistochemistry were used to investigate the mRNA and protein expression profiles of fibulin-5 in normal and NPC tissues. Immunohistochemistry of fibulin-5 was correlated with clinicopathological characteristics by univariate analyses. NPC cells overexpressing fibulin-5 or fibulin-5-siRNA cells were generated by stable transfection to characterize the molecular mechanisms of fibulin-5-elicited cell growth and metastasis.

Results: Our results demonstrated that fibulin-5 overexpression in NPC specimens and significantly correlated with advanced tumor metastasis indicating a poor 5-year overall survival. Fibulin-5 was mainly expressed in the nucleus in human NPC specimens and cell lines. Functionally, fibulin-5 overexpression yielded fast growth in NPC cells. In addition, fibulin-5 promotes cell metastasis in NPC cells through increased FLJ10540 and phosphor-AKT activity. In contrast, siRNA depletion of fibulin-5 suppressed FLJ10540 expression and phosphor-AKT activity. Suppression of either fibulin-5 or FLJ10540 can cause significant inhibition with regards to cell motility in NPC cells. Finally, immunohistochemical analysis of human aggressive NPC specimens showed a significant and positive correlation between fibulin-5 and FLJ10540 expression.

Conclusion: Higher fibulin-5 expression is not only an important indicator of poor survival, but also contributes to the development of new therapeutic strategies in the FLJ10540/AKT pathway for NPC treatment.

Citation: Hwang C-F, Shiu L-Y, Su L-J, Yu-Fang Yin, Wang W-S, et al. (2013) Oncogenic Fibulin-5 Promotes Nasopharyngeal Carcinoma Cell Metastasis through the FLJ10540/AKT Pathway and Correlates with Poor Prognosis. PLoS ONE 8(12): e84218. doi:10.1371/journal.pone.0084218

Editor: Chih-Pin Chuu, National Health Research Institutes, Taiwan

Received: July 9, 2013; **Accepted:** November 13, 2013; **Published:** December 27, 2013

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Funding: This study was funded by grants obtained by Dr. Chung-Feng Hwang (CMRPG890461-2, CMRPG8A0871, NSC 98-2314-B-182A-046-MY3 (NMRPG886051-3), NSC 101-2314-B-182A-138 (NMRPG8B0214), and NSC 102-2314-B-182A-115 (NMRPG8C0271)), and by Dr. Chang-Han Chen (CMRPG8A0391-2, CMRPG890921, CMRPG8B1251, NSC 100-2320-B-182A-001 (NMRPG8A0011), and NSC 102-2320-B-182A-010 (NMRPG8C0141)). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

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Introduction

Nasopharyngeal carcinoma (NPC) arises from the epithelial cells that cover the surface and line the nasopharynx [1]. NPC is one of the most common malignancies in Southern China and Southeast Asia with an incidence rate of 20-30 per 100,000. Globally, NPC accounts for 80,000 new cases and 50,000 deaths annually [2]. There is a large body of evidence suggests that the etiology of NPC is associated with multiple factors such as smoking, alcohol consumption, intake of salted food, EBV infection, and genetic factors [3]. NPC is characterized by distant recurrence, which are the major causes of therapeutic failure and the reported 5-year survival rate of 19% for all disease stages (25% for stage III and IVB subgroups) [4]. However, NPC patients with the same clinical stage often undergo different clinical courses, suggesting the TNM staging and tumor size are insufficient to accurately predict prognosis [5]. The molecular mechanisms of the progression and metastasis of NPC remain unclear at this time. Thus, it is quite important to identify valuable molecular biomarkers to facilitate an early diagnosis, support prognosis prediction, and to develop novel therapeutic strategies.

The fibulins, an ancient family of proteins, are conserved in species as evolutionarily distant as worms and humans. Structurally, fibulins are comprised of a globular C-terminal fibulin-type module, and calcium-binding epidermal growth factor-like modules. The fibulin family includes 7 mammalian members, termed fibulins 1-7. Mutations in fibulin genes are associated with several human diseases [6]. The fibulins are associated with basement membranes and interact with a variety of extracellular matrix components, such as laminin, elastin, aggrecan, endostatin, and fibronectin to stabilize the structures of the extracellular matrix [7,8]. Fibulins also regulate tissue organogenesis, vasculogenesis, fibrogenesis, and tumorigenesis [8,9]; however, the molecular mechanisms underlying the various biological activities of fibulins in human disease or cancer remain to be elucidated.

Fibulin-5, also known as DANCE, or EVEC, a TGF- β -induced glycoprotein, is a multifunctional extracellular matrix protein. Fibulin-5 contains an RGD sequence that binds integrins and modulates endothelial cell adhesion [10,11]. Initial reports suggest fibulin-5 is expressed in the embryonic vasculature and neural crest, but is downregulated in most adult vascular beds; however, its expression is reactivated in injured vessels during neointima formation and in atherosclerotic plaques in the mouse model of hypercholesterolemia, suggesting that fibulin-5 regulates vasculogenesis [12,13]. Several studies have indicated fibulin-5 modulates cancer cell proliferation, migration, and invasion in a cell- and context-specific manner [8,14]. For example, overexpressed fibulin-5 stimulates DNA synthesis and motility in fibroblasts, fibrosarcoma, and breast cancer cells. However, in stark contrast, fibulin-5 expression is suppressed dramatically in human tumor tissues such as kidney, urothelial carcinoma, prostate, lung, and colon [11,13,15,16]. To date, the expression patterns and significance of fibulin-5 for tumor histological grade, stage, and patient outcome in NPC patients have not yet been

determined, and the precise roles of fibulin-5 in NPC tumor growth and metastasis are largely unknown.

The aim of this study was to investigate the expression patterns and clinicopathological implications of fibulin-5 in NPC progression, and to identify potential underlying molecular mechanisms that may lead to an increased understanding of NPC.

Materials and Methods

Patients and tumor samples

Eighty-four patients undergoing biopsies for NPC were enrolled and fresh biopsy tissues were then analyzed. Controls included fresh normal nasopharyngeal mucosal tissues from patient biopsies for other non-neoplastic diseases. The collection of NPC specimens and clinical and pathological information were reviewed and approved by the human research committees of the Chang Gung Memorial Hospital and the written informed consents were obtained from each patient involved prior to this study commencing. Clinicopathological information for each subject, including gender, age, tumor-(T) stage, nodal-(N) status, distant metastasis (M), TNM stage, and overall survival, was obtained retrospectively from clinical records and pathology reports. NPC patients received local head and neck examinations before treatment, along with staging examinations, including whole body bone scans, abdominal ultrasonography, computed tomography, and/or magnetic resonance imaging. Using the 2010 American Joint Committee on Cancer system, 26 patients were classified as T1, 30 as T2, 4 as T3, and 24 as T4. Thirty patients were classified as N0, 20 as N1, 27 as N2, and 7 as N3. Seventy-five patients were classified as M0 and 9 as M1. Twelve patients were determined to be in stage I, with 22 in stage II, 18 in stage III, and 32 in stage IV. The method of radiotherapy was, in general, uniform within this period of time. All patients were regularly monitored after radiotherapy and/or chemotherapy until death or their last appointment, according to the intervals and protocols of follow-up detailed in a previous study [17]. Survival data was obtained from the cancer registry of our hospital or collected from the patients' attending physicians. Locoregional failure was determined by pathological diagnosis or progressive deterioration, as demonstrated by consecutive imaging studies. To identify distant metastases, patients underwent chest radiography yearly and bone scan or abdominal ultrasonography when indicated.

RNA Extraction and Quantitative RT-PCR (Q-RT-PCR)

Tissue samples were frozen in liquid nitrogen and stored at -80°C prior to RNA extraction. RNA extraction and quantitative RT-PCR assays were performed as described previously [3,18]. *Fibulin-5* and *FLJ10540* Taq-Man probes (ABI) were used for Q-RT-PCR. Data are presented as means \pm SD. To analyze the distribution of normal and tumor areas, we used the Wilcoxon signed-rank test between 2 groups for statistical analysis. A *P*-value of <0.05 was considered to be statistically significant. *GAPDH* was used as an internal control for

comparison and normalization. Assays were performed in triplicate on an Applied Biosystems 7500 Fast instrument.

Immunoblot analysis

For protein extraction, frozen samples were homogenized in RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, and 0.1% SDS). Western blotting was performed as described previously [3]. Antibodies included against fibulin-5 (Epitomics), FLJ10540 (generated by us) [19,20], DDK (Origene), phosphor-AKT and AKT (Cell Signaling Technology, Beverly, MA, USA), lamin A/C, and β -actin (monoclonal; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were applied. The proteins were investigated using X-ray films.

Immunohistochemistry and indirect immunofluorescence

Normal and tumor NPC tissue samples were selected by a pathologist based on diagnosis and microscopic morphology. Immunohistochemical staining was performed as previously described [3]. After antigen retrieval, the sections were incubated with diluted anti-FLJ10540 antibody (polyclonal; generated by us; 1:500; polyclonal; Abnova, Taiwan 1:100) and anti-fibulin-5 (polyclonal; Abnova, Taiwan 1:100; monoclonal; Protein Tech Group, Inc, Chicago, USA; 1:50) at room temperature for 1 hour, followed by washing with PBS. Horseradish peroxidase/Fab polymer conjugate (PicTure™-Plus kit; Zymed, South San Francisco, CA, USA) was then applied to the sections for 30 min followed by washing with PBS. Finally, the sections were incubated with diaminobenzidine for 5 min to develop the signals. A negative control was run simultaneously by omitting the primary antibody. Reactivity of the immunostained tissues was evaluated independently by 2 pathologists who were blinded to the subjects' clinical information. Between 15 and 20 high-power fields were viewed. Criteria were developed for quantitating the immunoreactivities of fibulin-5 staining in the normal and tumor sections using a score range of 0 to +3, where 0 indicated no positive cell staining, +1 less than 5% positive cell staining, +2 5-50% positive cell staining, and +3 more than 50% positive cell staining. Similarly, the stain intensity was graded as +0, +1, +2, or +3 as previously described [21]. High expression of fibulin-5 was defined as +2 or higher for both scoring methods. Indirect immuno-fluorescence staining on the NPC cell lines were performed with monoclonal antibody directed against fibulin-5 and DDK at RT for 1 hour. The sections were then washed three times with PBS and incubated with goat-anti-rabbit-FITC (1:200; Jackson ImmunoResearch) and DAPI (1:1000; Jackson ImmunoResearch) at RT for 1 hour. After washing with PBST, the sections were mounted with GEL/Mount (biomeda corp) and the fluorescence images on the slips were analyzed using a Zeiss microscope.

Cell culture, establishment of stable clones, and gene silencing using siRNA

NPC-TW01, and Hone1 cell lines derived from primary nasopharyngeal tumors of untreated NPC patients were used for functional assays [22,23]. All cell culture-related reagents

were purchased from Gibco-BRL (Grand Island, NY, USA). TW01 cells were grown in DMEM; Hone1 cells were grown in RPMI containing 10% FBS and 100 U/mL penicillin and streptomycin (Gibco-BRL). DDK-vector and DDK-fibulin-5 were transiently transfected into cancer cells using Lipofectamine (Invitrogen) based on the manufacturer's instructions. Mixed TW01 and Hone1 cells stably expressing fibulin-5 were selected with 400 μ g/mL G418 (Calbiochem Novabiochem, San Diego, CA, USA) for 2 weeks. The cells were then harvested and analyzed for exogenous fibulin-5 expression by Western blotting. Two pcDNA™6.2-GW/EmGFP-miR-based fibulin-5 shRNA plasmids (5'-GAGATAAGGCTCCTCACAGCGGG-3' and 5'-GTATCCAAAGCGGCATATAAGAG-3'; Invitrogen), and one negative control were utilized in this study.

Preparation of Nuclear and Cytosolic Extracts

Nuclear and cytosolic extraction was performed as previously described [24]. Cells were rinsed with cold phosphate-buffered saline (PBS), digested with trypsin and centrifuged for 5 min at 1,200 rpm. The cells were then re-suspended in buffer A containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.5 mM MgCl₂, 10 mM KCl, 1 mM dithiothreitol (DTT), 1 mM phenylmethanesulphonyl fluoride (PMSF) and 1 mM Na₃VO₄ (pH 7.5). The re-suspended cells were incubated on ice for 15 min, passed through a syringe needle 10 times, and centrifuged at 4°C for 10 min at 3,000 rpm. The supernatant (cytosolic fraction) was snap frozen in liquid nitrogen and stored at -80°C with 10% glycerol. The nuclear pellet was washed 2 times with buffer A, re-suspended with a buffer B containing 20 mM HEPES, 1.5 mM MgCl₂, 0.42 M NaCl, 0.2 mM EDTA, 25% glycerol, 1 mM DTT, 1 mM PMSF, and 1 mM Na₃VO₄ (pH 7.5), incubated at 4°C with agitation for 45 min at 500 rpm, and centrifuged at 4°C for 10 min at 13,000 rpm. The supernatant (nuclear fraction) was snap frozen in liquid nitrogen and stored at -80°C.

Cell viability assay

Viability of sub-confluent cells was analyzed by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. The assay was performed in 96-well plates seeded with 4000-6000 cells/well in 200 μ L and incubated for 4 days. On each day, at one time, MTT solution was added [1 g MTT (Sigma M5655) dissolved in 200 mL D-PBS] to each well. The plates were stored at 37°C for 4 hour, and then 100 μ L DMSO buffer was added and incubated in the dark for 10 min. Absorbance was measured on a microplate reader (Labsystems Multiscan MCC/340) at 540 nm. The OD values were normalized with the value on day 0. The experiment was performed in triplicate and repeated three times.

Chromatin immunoprecipitation (ChIP)

ChIP assay was carried out as previously described [25]. Hone 1 cells transfected with vehicle and DDK-fibulin-5 according to the manufacturer's instruction that was described above. The purified DNA was amplified by PCR using FLJ10540 promoter primers (-500~-690) (forward: 5'-

CGTGATCAGAGTGGGAAACA-3' and reverse: 5'-TGGTGCAATCATAGCTCACTG-3'; pre-denaturation for 3 min at 94°C, denaturation at 94°C for 20 sec., annealing at 47°C for 30 sec., and extension at 72°C for 30 sec., for a total of 30 cycles)

Wound-healing assay

Vehicle and fibulin-5-transfectants were used in a “wound healing” assay to characterize cell migration. Cells were seeded uniformly onto 60-mm culture plates with an artificial “wound” carefully created at 0 hour, when a P-10 pipette tip was used to scratch the sub-confluent cell monolayer. Microphotographs were taken at 0 and 24 hour. Quantitative analysis of the percentage of wound healing was calculated using the distance across the wound at 0 and 24 hour, divided by the distance measured at 0 hour for each cell line.

Migration and invasion assays

Migration and invasion assays were conducted with TW01-/Hone1-vehicle, TW01-/Hone1-fibulin-5, TW01-negative, and TW01-sifibulin-5 stable clones using 24-well Transwell chambers. The migration and invasion assays were performed as previously described [3,26]. Briefly, cell migration and invasion were evaluated by counting the number of TW01-/Hone1-vehicle, TW01-/Hone1-fibulin-5, TW01-negative, and TW01-sifibulin-5 stable clone cells that had migrated or invaded by 200× phase-contrast microscopy on 3 independent membranes, then normalized against the vehicle cells to determine the relative ratio.

Statistical analyses

Several clinicopathological factors were evaluated, including gender, age, T, N, M status, and TNM stage. The chi-square test was used to evaluate the correlation between clinicopathological variables and expression of fibulin-5. A p value <0.05 was considered to indicate statistical significance in all analyses. Clinicopathological variables and fibulin-5 expression were taken into account for the analysis of survival, based on the Kaplan-Meier method; statistical significance, defined as having a p value of <0.05 , and was assessed by log-rank test. To determine the effect of particular prognostic factors on survival, a multivariate analysis was performed according to Cox's regression model.

Results

Fibulin-5 was overexpressed in NPC specimens

To determine whether fibulin-5 participates in the pathogenesis of NPC, semi-quantitative RT-PCR and Q-RT-PCR were performed on 6 NPC-tumor and 3 normal tissue samples. *Fibulin-5* expression was high in the tumor specimens and low in normal tissues (Figure 1A). Average expression of *fibulin-5* was 4-fold higher in NPC than in normal tissue samples (see box-plot, Figure 1B). Expression of fibulin-5 in another batch of freshly frozen tumor and normal samples was verified by western blotting, the results of which were

consistent with the observed mRNA expression of *fibulin-5* (Figure 1C).

To determine the potential role of fibulin-5 in NPC, we evaluated fibulin-5 protein expression in surgical specimens by immunohistochemistry. Eighty-four NPC samples and 10 normal tissues were analyzed. Of the 84 NPC samples, 48 (57.1%) showed high expression of fibulin-5, whereas 36 (42.9%) had low expression. All normal tissues showed weak immunoreactivity for fibulin-5. Representative results of fibulin-5 immunostaining in NPC are shown in Figure 2A. In the tumor samples, fibulin-5 expression positively correlated with tumor stage (Figure 2A, b-e) and node stage (Figure 2A, f-g). Fibulin-5 was localized in the nucleus of tumor and normal tissues (Figure 2A, a). Immunohistochemistry with anti-fibulin-5 antibodies from Abnova produced almost the same results. In addition, fibulin-5 immunoreactivity in tumor samples was not noted when anti-fibulin-5 antibody was pre-incubated with protein corresponding to a portion of fibulin-5 (data not shown).

Clinicopathological Factors and Survival of NPC Patients with fibulin-5 Expression

To investigate whether the increased expression of fibulin-5 was associated with various prognostic factors, we classified the patients into groups based on immunohistochemistry (low vs. high fibulin-5 expression). As shown in Table 1, patients with an advanced tumor stage, a higher rate of distant metastasis, and TNM stages III-IV had significantly higher fibulin-5 expression than did patients with stage I-II tumors ($p<0.001$), no distant metastasis ($p=0.042$), and TNM stages I/II ($p<0.001$). No significant difference between high and low levels of fibulin-5 was observed in terms of patient age, gender, or histological type at the time of diagnosis.

A DSS analysis using the Kaplan-Meier method revealed the prognosis of patients with high tumor expression of fibulin-5 was significantly poorer than that of patients with low tumor fibulin-5 expression ($p = 0.003$) (Figure 2B). Univariate analysis showed that DSS in this series was significantly associated with several clinicopathological factors, including T ($p<0.001$), N ($p=0.021$), M ($p=0.001$), TNM stage ($p=0.001$), and fibulin-5 (Table 2). Clinical prognosis, however, was not associated with age, gender, or histological type. For multivariate analysis, however, T stage ($p<0.001$; hazard ratio = 4.349) remained a significant independent prognostic factor of DSS. These results clearly indicated the 5-year survival and clinical prognosis of NPC were associated with fibulin-5 expression in NPC patients.

Fibulin-5 protein was overexpressed in NPC cell lines and distributed in the nucleus

We next examined the expression levels of fibulin-5 in a panel of three human NPC cancer cell lines and one normal human NPC tissue using Western blotting. The data indicated that the protein expression levels of fibulin-5 were higher in NPC cell lines than those of normal tissues (Figure 3A). To determine the subcellular localization of fibulin-5, immunofluorescence of endogenous and exogenous fibulin-5 were studied in Hone1 cell lines. All panels demonstrate the same pattern of fibulin-5 localization, where fibulin-5 was found predominantly in the nucleus of the Hone1 cells, although

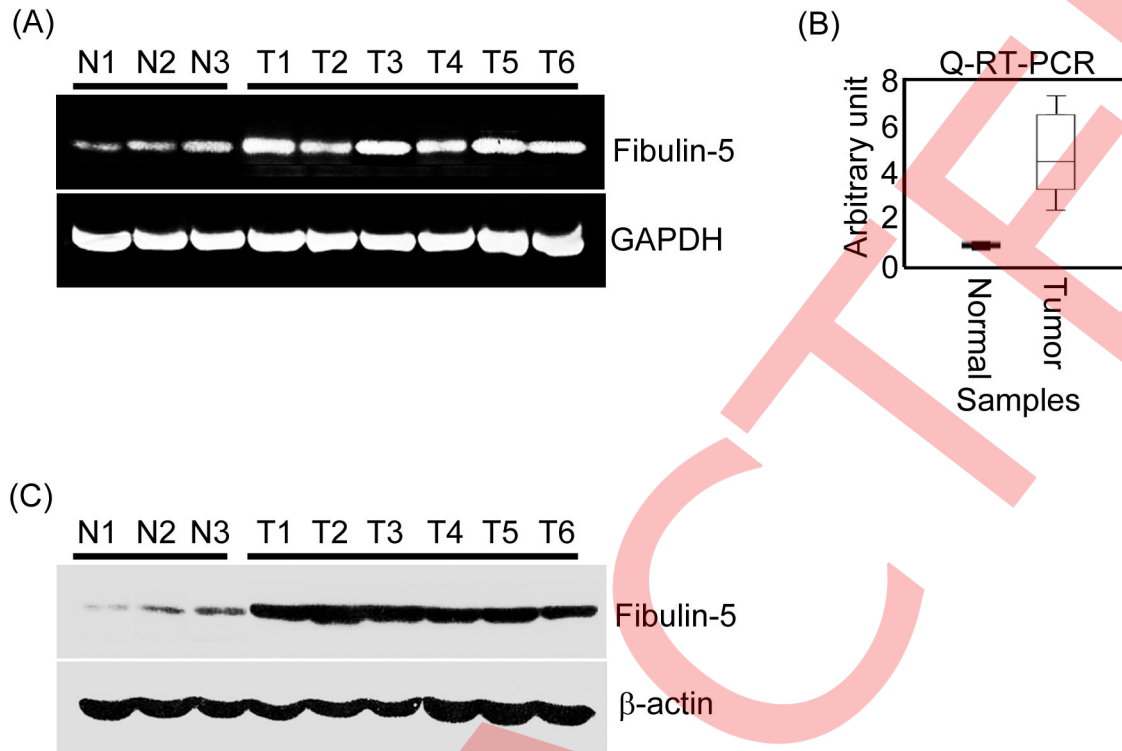


Fig. 1

Figure 1. Overexpression of Fibulin-5 in NPC specimens. Representative (A) RT-PCR and (B) Q-RT-PCR analyses of *fibulin-5* expression in six-NPC samples (T) compared to that in three-normal tissues (N). GAPDH was used as internal control. (C) Western blot analyses of fibulin-5 protein expression level in NPC samples (T) and normal tissues (N) ($p < 0.001$) Total proteins were extracted from respective tissues and probed with antibody against fibulin-5. β -actin was used as a control.

doi: 10.1371/journal.pone.0084218.g001

some was observed in the cytoplasm (Figure 3B). However, this was found almost exclusively in the cytoplasm of other cancer cell lines, such as human epidermoid carcinoma cell line A-431 and the osteosarcoma cell line U2-OS (Figure 3B). We next assessed the distribution of fibulin-5 between the two compartments by fractionation of TW01 and Hone1 cell lysates into nuclear and cytosolic fractions. In each cell line, endogenous fibulin-5 was found in both cytosolic and nuclear fractions but was enriched in the nucleus (Figure 3C). Moreover, the fibulin-5 protein level was also detected in cell culture supernatants by immunoprecipitation (Figure 3C).

Fibulin-5 promoted NPC cell proliferation, migration, and invasion

In order to study the function of fibulin-5, 2 different cell types, Hone1 and TW01, stably expressing DDK-tagged fibulin-5 were established (Figure 4A, and Figure S1). First, fibulin-5-Hone1 stable transfectants could promote cell growth

in low-serum media in comparison to the vehicle control (Figure 4B and Figure S1). Using the same panel, we analyzed whether increased fibulin-5 expression affects metastatic parameters, including migration and invasion. First, the migratory ability of fibulin-5-NPC cells was analyzed in wound healing assays. At 24 hour post-wounding, Hone1-fibulin-5 cells achieved near-complete wound closure, whereas vehicle control cells did not (Figure 4C). There were similar results in TW01 cells (data not shown). We next evaluated fibulin-5-NPC cell invasion through Matrigel and migration through a transwell. The representative fields of cell migration and invasion experiments are shown (Figure 4D). Overexpression of fibulin-5-Hone1 and fibulin-5-TW01 cells led to a significant increase in NPC-cell migration and invasion (Figure 4D, and Figure S1). Quantitatively speaking, the fibulin-5-Hone1 transfectants induced cell migration at a rate that was about 5.5 to 7-fold higher than in vehicle cells; moreover, the invasive ability of the fibulin-5-Hone1 transfectants was approximately

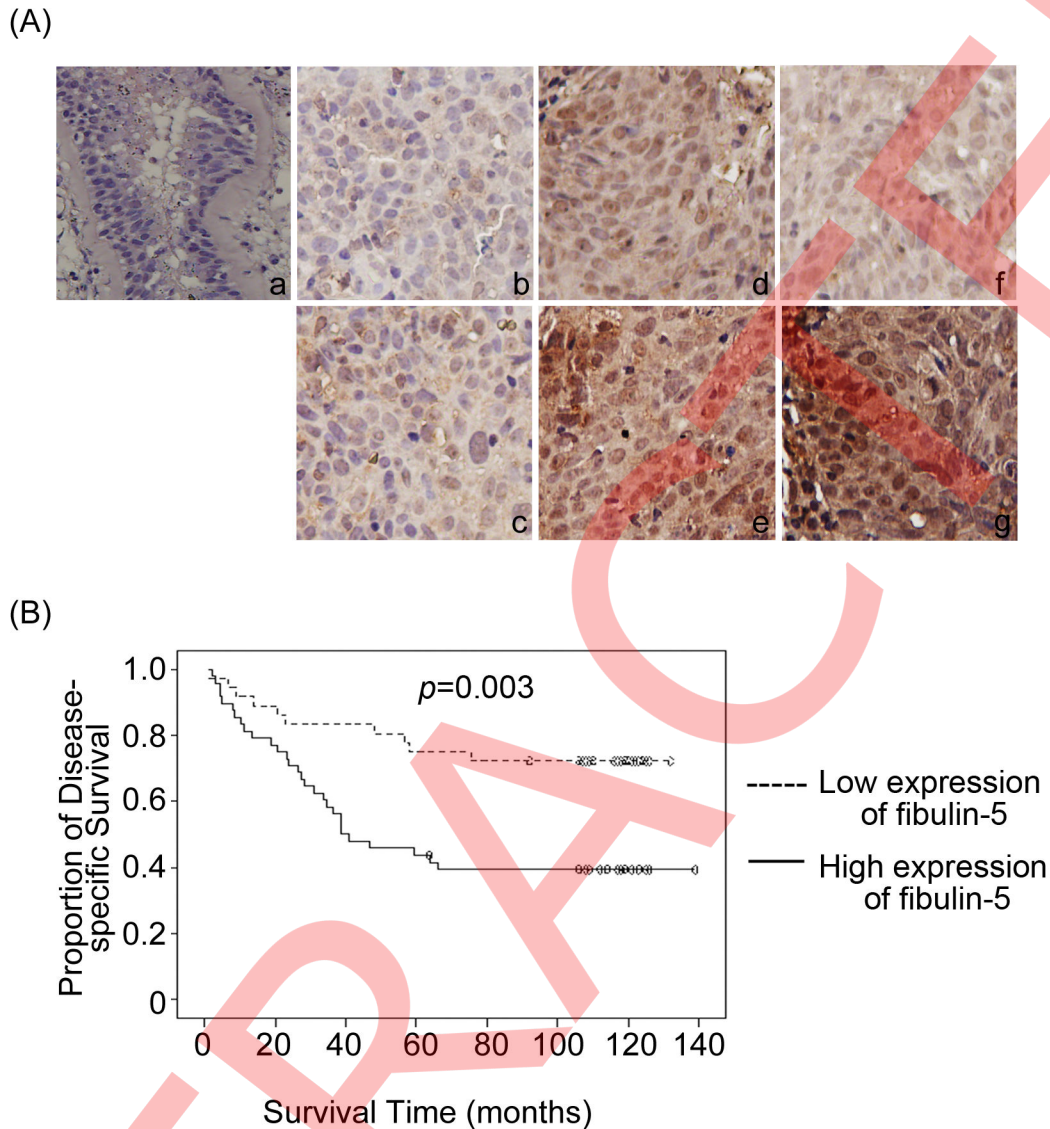


Fig. 2

Figure 2. Immuno-histochemical staining, and disease-specific survival of fibulin-5 in patients with NPC. (A) The intensity of fibulin-5 expression in various tissues by immunohistochemical staining were evaluated in (a) normal tissue and tumor tissues of NPC patients with stage I (b), II (c), III (d), IV (e), lymph node-negative (f), and lymph node-positive (g) samples. Original magnification, 200 \times . (B) Survival of patients with high fibulin-5 expression (solid line) was significantly abbreviated in comparison to those with low expression (dashed line), with a statistically significant difference in survival ($p = 0.003$) by log-rank test.

doi: 10.1371/journal.pone.0084218.g002

7.5 to 8.5-fold higher than that of the vehicle controls (Figure 4D).

Next, to further investigate the possible contributions of matricellular-fibulin-5, recombinant human fibulin-5 was administered to Hone1 cell to determine if increased fibulin-5 protein could confer proliferative and motile advantages to NPC

cancer cells. The data demonstrated that the proliferation, migration and invasion were significantly increased in matricellular-fibulin-5 in a dose-dependent manner in Hone1 cells (Figure 5A-B).

Table 1. Comparisons of fibulin-5 expression between the subgroups of various clinic-pathological parameters.

	Fibulin-5 expression		p-value ^a
	Low (n=36)	High (n=48)	
Age (year)			
< 50	18	23	0.850
≥ 50	18	25	
Gender			
Male	24	38	0.197
Female	12	10	
Histology			
WHO type II	23	25	0.279
WHO type III	13	23	
Tumor size			
T1-T2	34	23	<0.001*
T3-T4	2	35	
Nodal stage			
N0-N1	25	24	0.074
N2-N3	11	24	
Metastasis			
M0	35	40	0.042*
M1	1	8	
AJCC staging			
I-II	23	9	<0.001*
III-IV	13	39	

Abbreviations: NPC nasopharyngeal carcinoma; WHO World Health Organization; AJCC American Joint Committee on Cancer

^a p-value from Chi-square or Fisher exact test. *Statistically significant ($p < 0.05$)

doi: 10.1371/journal.pone.0084218.t001

Fibulin-5-siRNAs suppressed NPC cell proliferation, migration, and invasion

To further confirm the biological functions of fibulin-5, siRNA was used to reduce endogenous fibulin-5 expression in Hone1 and TW01 cells. To examine whether the specific fibulin-5 siRNAs could suppress endogenous fibulin-5 expression in NPC cell lines, siRNAs were transfected into NPC cell lines for 24 hour, followed by Q-RT-PCR and western blot analysis using fibulin-5 Taq-Man probe and antibodies against fibulin-5. The endogenous mRNA and protein expression levels of fibulin-5 in cells transfected with siRNAs targeting fibulin-5 were significantly reduced (Figure 6A, and Figure S2). Knockdown of endogenous fibulin-5 in Hone1 and TW01 cells led to a significant decrease in cell proliferation, wound healing, migration and invasion (Figure 6B-D, and Figure S2). These results strongly suggest fibulin-5 plays an important role in human NPC cancer cell proliferation, migration, and invasion.

Activation of the FLJ10540/AKT pathway was critical for NPC-induced migration and invasion in fibulin-5 transfectants

To unravel the possible fibulin-5-mediated signaling pathway(s) involved in cell metastasis, three well-known signaling pathways, such as MAPK, p38, and PI3K/AKT

Table 2. Disease-specific survival by Cox regression analyses between the subgroups of various clinicopathological parameters.

Parameters	No	No of events	HR (95% CI) ^a	p-value ^b
Age (years)				
<50	41	16	1.635 (0.863-3.096)	0.131
≥ 50	43	23		
Gender				
Male	62	31	0.607 (0.279-1.322)	0.209
Female	22	8		
Histology				
WHO type II	48	23	0.830 (0.438-1.572)	0.567
WHO type III	36	16		
Primary tumor (T)				
T1-T2	57	17	4.688 (2.451-8.966)	<0.001*
T3-T4	27	22		
Nodal status (N)				
N0-N1	49	18	2.097 (1.116-3.941)	0.021*
N2-N3	35	21		
Distance				
M0	75	31	3.766 (1.718-8.258)	0.001*
M1	9	8		
AJCC staging				
Stage I-II	32	7	4.023 (1.770-9.142)	0.001*
Stage III-IV	52	32		
Fibulin-5				
Low expression	36	10	2.883 (1.376-5.831)	0.005*
High expression	48	29		

Abbreviations: HR Hazard Ratio, WHO World Health Organization; AJCC American Joint Committee on Cancer

^a 95% Wald Confidence Limits

^b Associations determined by Cox regression analyses. *Statistically significant ($p < 0.05$)

doi: 10.1371/journal.pone.0084218.t002

pathways were investigated by pharmacological inhibitors. Figure 7A demonstrated that the Hone1 and TW01 cells with fibulin-5-overexpressing had increased levels of cell migration and invasion, which were strongly inhibited by incubation with LY294002, and to a much lesser extent with SB202190 and PD98059. The same was found when PI3K was inhibited with wortmannin, a PI3K inhibitor (data not shown). Moreover, elevated AKT-Ser473 phosphorylation was observed in fibulin-5-Hone1 and fibulin-5-TW01 cells, whereas AKT activation was partially reduced by LY294002 (Figure 7B). Conversely, the activation of AKT was greatly decreased in fibulin-5-depleted cells compared with negative control (Figure 7C).

We previously observed that FLJ10540 not only plays important roles in the oncogenesis of several malignancies, including hepatocellular carcinoma [19], lung cancer [27], and NPC [28], but also enhances the transformation and invasion of cancer cells via AKT activation [19,27]. These observations raise the possibility that fibulin-5/FLJ10540/AKT might be functionally linked or in the same pathway. As shown in Figure 8A, fibulin-5 overexpression enhanced mRNA and protein

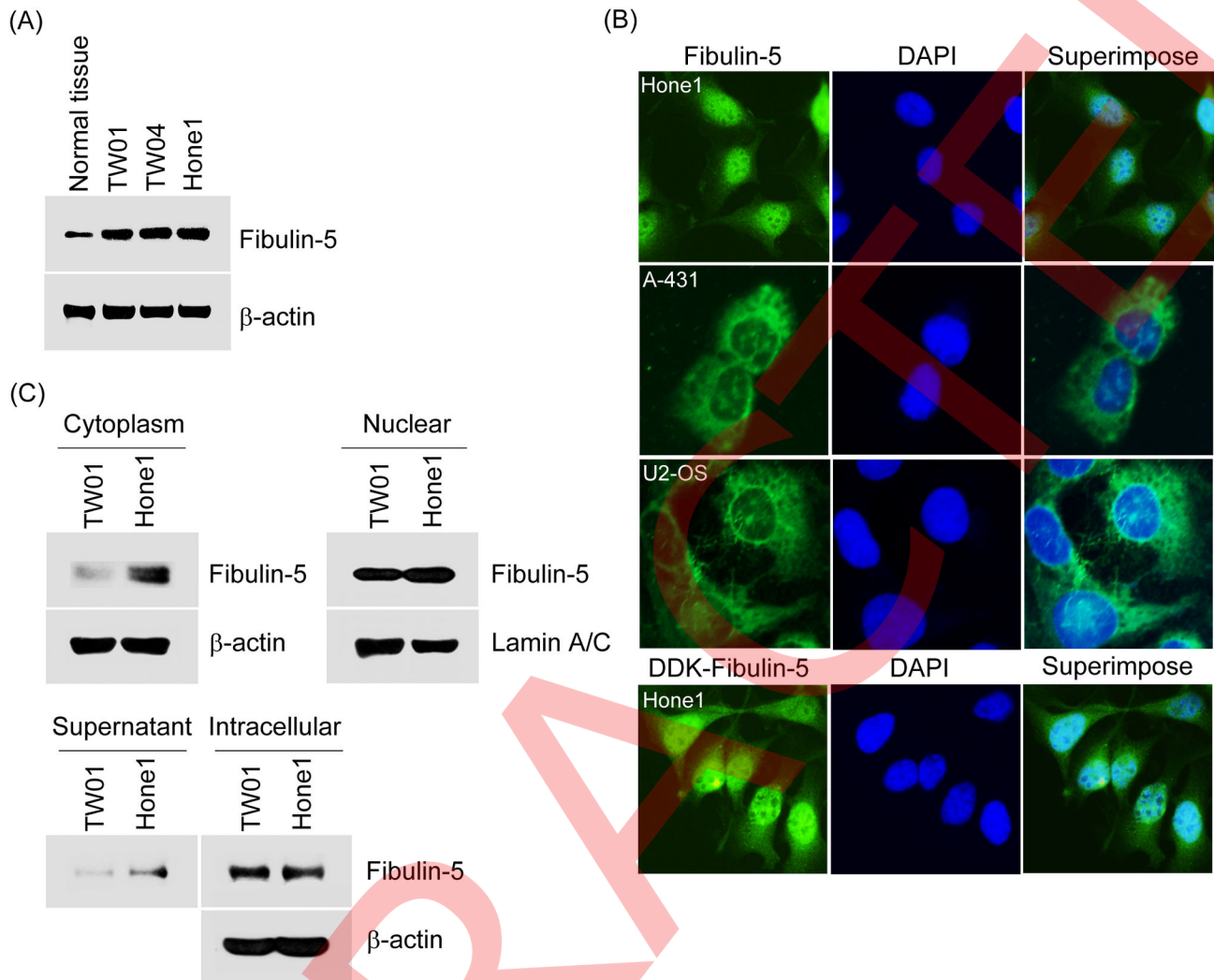


Fig. 3

Figure 3. The distributions of fibulin-5 in NPC and other human cancer cell lines. (A) Immunoblot analysis of total protein from normal tissues, TW01, TW04, and Hone1 cells using monoclonal anti-fibulin-5 antibody. (B) Hone1, A-431 and U2-OS cells were grown on glass cover slips and cultured overnight. Indirect immunofluorescence of endogenous fibulin-5 protein in Hone1 cells was detected by using anti-fibulin-5 antibody. (Bottom panel) Hone1 cells were transfected with DDK-fused fibulin-5 by Plus/Lipofectamine reagents and cultured overnight. Indirect immunofluorescence of highly expressed fibulin-5 protein in Hone1 cells was detected with anti-DDK antibody. The cells were double-stained with DAPI to detect DNA. (C) (Upper panel) Western blot analysis for fibulin-5 of cytoplasm extracts (left) and nuclear extracts (right) of TW01 and Hone1 cells. β -actin and LaminA/C were used as control. (Bottom panel) Immunoprecipitation analysis for fibulin-5 of cell culture supernatants and intra-cellular total cell extracts of TW01 and Hone1 cells.

doi: 10.1371/journal.pone.0084218.g003

expressions of endogenous FLJ10540 in Hone1 cells. A similar result was also observed in that the mRNA expression level of FLJ10540 was increased in overexpressed-fibulin-5-TW01 cells (Figure S3). Conversely, mRNA and protein expressions of FLJ10540 were clearly reduced in the presence of fibulin-5-siRNAs in Hone1 cells (Figure 8B). To further illustrate the role of fibulin-5 in regulating FLJ10540 transcription, we

investigated whether fibulin-5 could regulate FLJ10540 promoter activity. We cotransfected the FLJ10540 promoter-luciferase plasmids into Hone1 cells with DDK-fibulin-5 or vehicle control in a dose-dependent manner. The data demonstrated that a significant activation of the FLJ10540 promoter was increased in DDK-fibulin-5 transfectants compared with vehicle control (Figure 8C, left panel). In

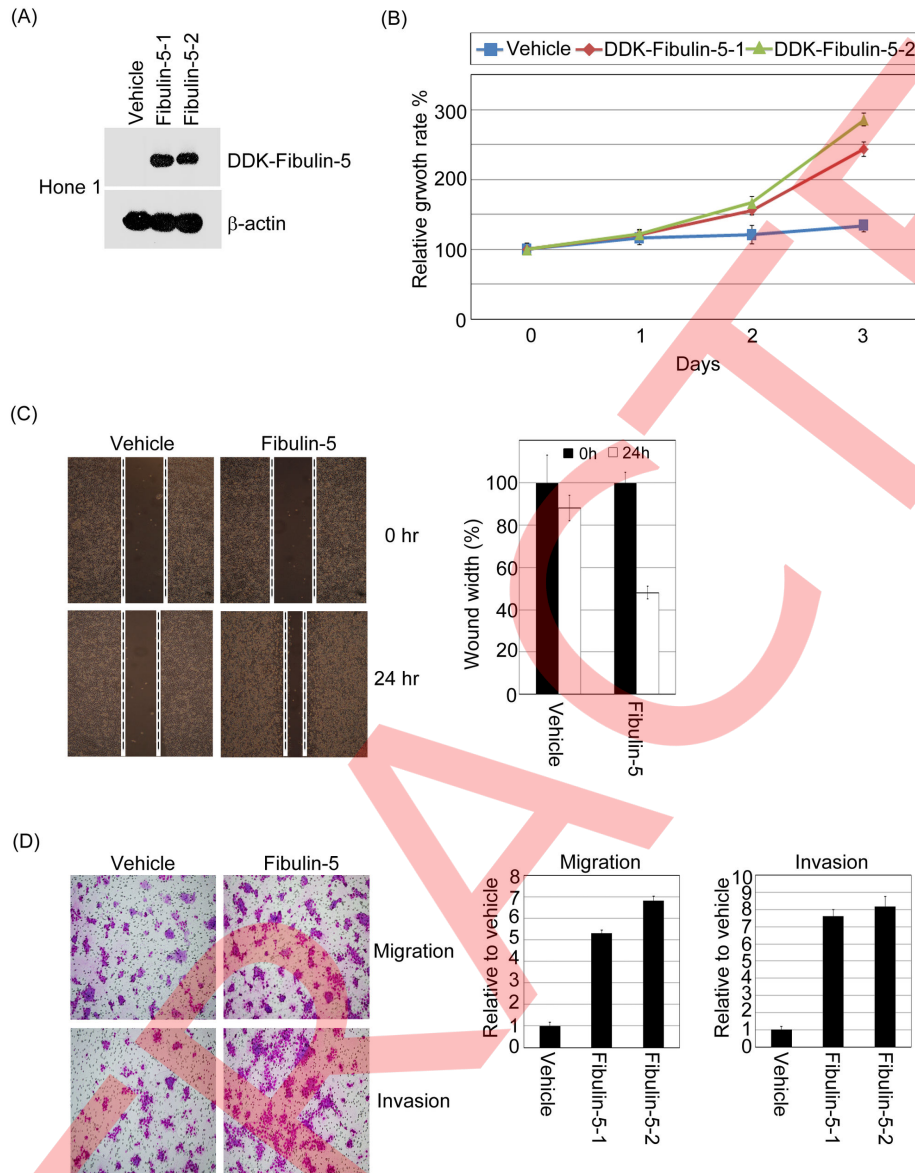


Fig. 4

Figure 4. Exogenous fibulin-5 promotes cell growth, migration, and invasion in NPC cells. (A) DDK-tagged fibulin-5 was stably transfected into Hone1 cells, 2 clones were chosen, and DDK-fibulin-5 expression was determined by western blotting with anti-DDK and anti- β -actin antibodies. (B) Cell viability of Hone1 stable cell lines was measured by MTT assay. The cells were cultured for 0-3 days followed by MTT assay (OD_{570}) to quantitate the cell growth. Data were normalized against the OD_{570} value on day 0 of each treatment. The results represent the mean \pm SD of 3 independent experiments. (C) Wound healing assays with cells expressing empty vector or fibulin-5-Hone1. Representative images captured with a 10 \times objective at the time of wounding or 24 hour after. All experiments were repeated at least three times. The percentage of wound closure corresponds to the distance between wound edges in at least three randomly chosen regions relative to the distance at time 0 hour for each cell. (D) Migration and invasion of vehicle-Hone1 and fibulin-5-Hone1 stable cells (200 \times). For the migration assays, cells (vehicle-Hone1, fibulin-5-Hone1 stable clones) were seeded into the top of a Transwell insert. After 24 hour, the cells on top were scraped, and the cells that had migrated to the bottom were fixed and stained with Giemsa. The relative-fold migration values for the clones were normalized against the vehicle control and are represented diagrammatically. For the invasion assays, cells were seeded after the addition of Matrigel. The relative-fold invasion values for the stable clones were normalized against the vehicle cells and are represented diagrammatically.

doi: 10.1371/journal.pone.0084218.g004

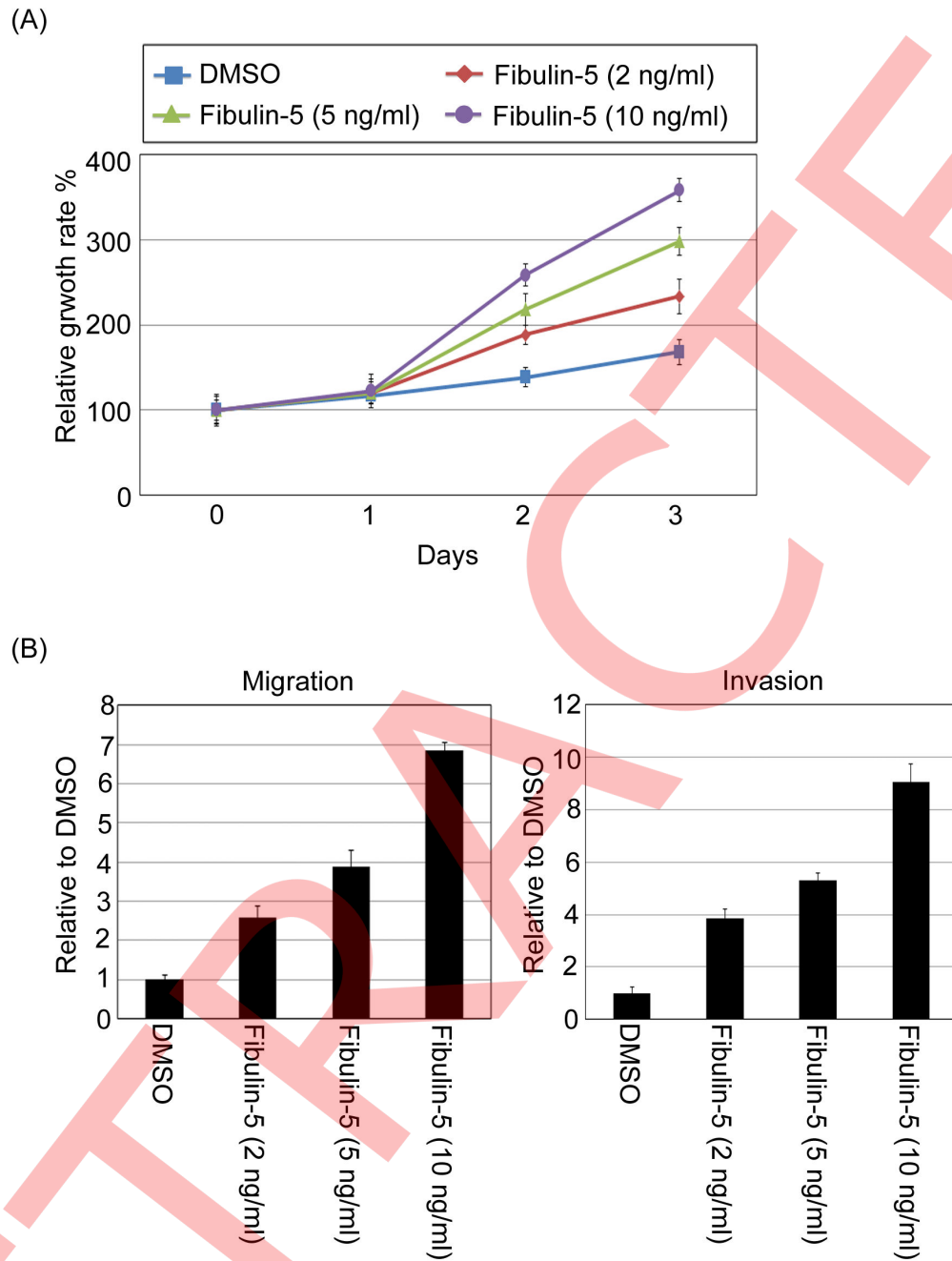


Fig. 5

Figure 5. NPC cells stimulated with fibulin-5 protein encourages cell growth and motility. (A) Hone 1 cells were treated with indicated concentrations of fibulin-5, and cell growth was analyzed on days 0-3 by MTT assay. Data were normalized against the OD_{570} value on day 1 of each treatment. The results represent the mean \pm SD of 3 independent experiments. (B) Migration and invasion of Hone1 cells (200 \times). For the migration assays, Hone1 cells stimulated with indicate concentrations of fibulin-5 protein were seeded into the top of a Transwell insert. After 24 hour, the cells on top were scraped, and the cells that had migrated to the bottom were fixed and stained with Giemsa. The relative-fold migration values for the clones were normalized against the DMSO and are represented diagrammatically.

doi: 10.1371/journal.pone.0084218.g005

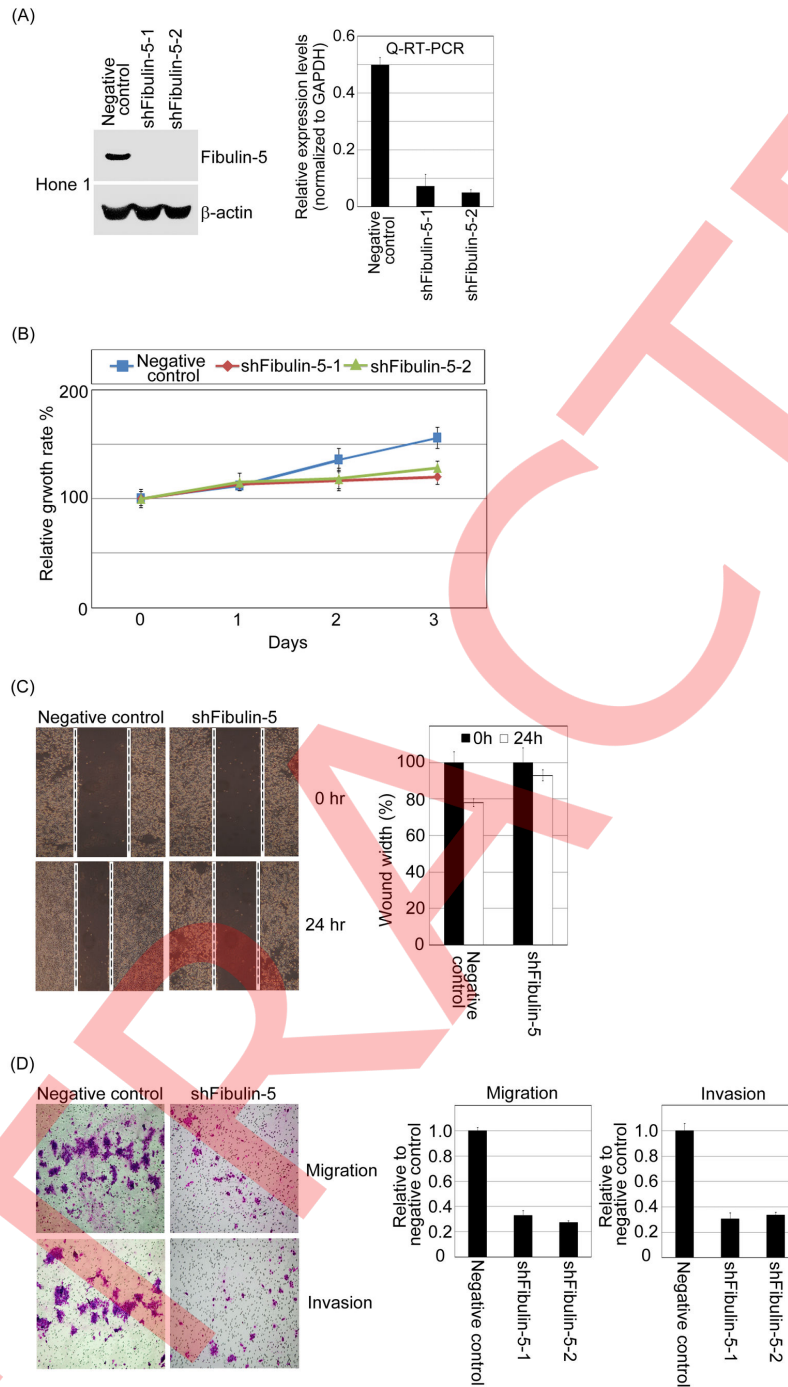


Fig. 6

Figure 6. The proliferation, migratory, and invasive abilities of NPC cells are inhibited by fibulin-5-specific siRNA. (A) A negative control siRNA plus fibulin-5 siRNA was transfected into Hone1 cells for 24 hour. After transfection, western blotting was performed with anti-fibulin-5 and β -actin antibodies. The mRNA expression level of endogenous fibulin-5 was also measured by Q-RT-PCR. (B) Using the same panel, the fibulin-5 transfectants and negative control were seeded into 96-well plates with 5.0%FBS. The cells were cultured for 0-3 days followed by MTT assay to quantitate cell growth. The data were normalized against the value on day 0 of each treatment. The growth curves of Hone1 cells are shown as the mean \pm SD of 3 independent experiments. (C and D) The wound healing, migration, and invasion results of negative control-Hone1 and sifibulin-5-Hone1 stable cells are shown (200 \times). The relative-fold migration and invasion of sifibulin-5-Hone1 cells were normalized against the values for the negative control cells and are represented diagrammatically. The results represent the mean \pm SD of 3 independent experiments. The shFibulin-5-Hone-1 invasion panel of Figure 6D is excluded from this article's CC-BY license. See the accompanying retraction notice for more information.

doi: 10.1371/journal.pone.0084218.g006

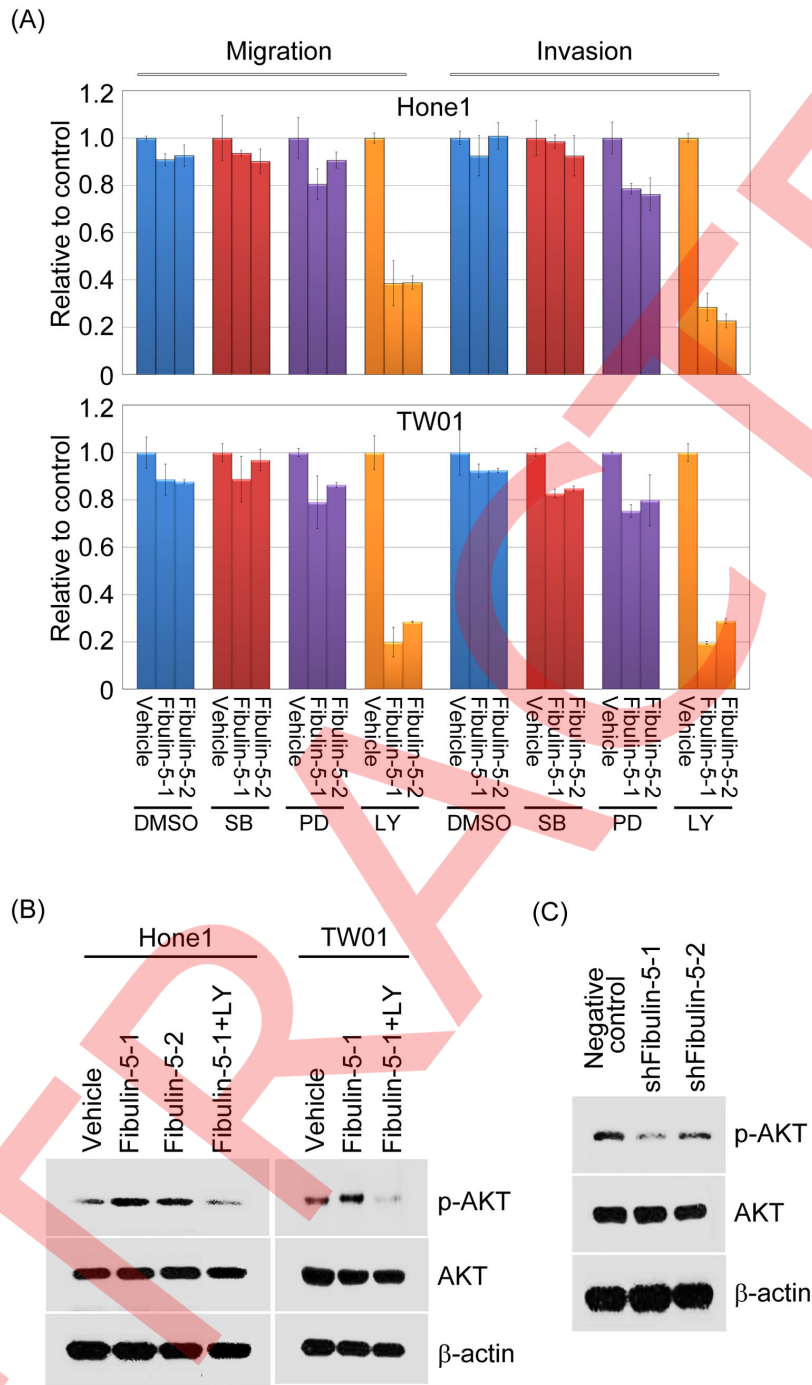


Fig. 7

Figure 7. Fibulin-5 modulates cell migration and invasion through enhanced AKT signaling. (A) Vehicle-Hone1, fibulin-5-Hone1, vehicle-TW01, and fibulin-5-TW01 transfected cells were serum-starved and treated with the indicated inhibitors, SB202190, PD98059, and LY294002 or solvent for 24 hour. The migration and invasion ratios of vehicle-Hone1 and fibulin-5-Hone1 transfected cells were determined as previously described. (B) The fibulin-5 expressing cells of Hone1 and TW01 were serum-starved for 24 hour and treated with or without LY294002 at the final concentration of 10 μM. The Total cell lysates of vehicle-Hone1, fibulin-5-Hone1, vehicle-TW01, and fibulin-5-TW01 transfected cells were immunoblotted for the unphosphorylated and phosphorylated forms of AKT. β-actin was used as the internal loading control. (C) A negative control and fibulin-5 siRNAs were transfected into Hone1 cells for 24 hour and western blotting was performed as in (B).

doi: 10.1371/journal.pone.0084218.g007

contrast, the promoter activity of FLJ10540 was dramatically decreased, when the endogenous fibulin-5 expression was abolished by fibulin-5-siRNAs (Figure 8C, right panel). To investigate the recruitment of fibulin-5 into the FLJ10540 DNA region, ChIP assay was performed in Hone1 cells transfected with DDK-fibulin-5 or control plasmid and immunoprecipitated with α -DDK antibody or control IgG. The results indicated that using α -DDK antibodies were able to specifically immunoprecipitate FLJ10540 promoter in cells transfected with DDK-fibulin-5 vector; however, the control IgG or vehicle transfectants did not show any amplification of FLJ10540 promoter region (Figure 8D). To explore whether fibulin-5-induced FLJ10540 expression could affect cell migration and invasion in human NPC cells, we used siRNA to inhibit endogenous FLJ10540 expression and assayed the motility of fibulin-5-Hone1 and vehicle control cells. The results demonstrated that knockdown of FLJ10540 by FLJ10540-siRNA significantly reduced FLJ10540 protein levels and inhibited the migratory and invasive ability of fibulin-5-Hone1 transfectants (Figure 8E). To confirm the clinical relevance of our *in vitro* observations concerning the regulations between fibulin-5 and FLJ10540, 30 samples with aggressive NPC patients were examined by immunohistochemical staining. The correlation between fibulin-5 and FLJ10540 in each paired IHC scores were analyzed by Spearman's rank tests. The result demonstrated that there were positive correlations between fibulin-5 and FLJ10540 ($\rho = 0.762$, $p < 0.001$) (Table 3).

Next, to determine the effect of FLJ10540 expression on AKT activation, the protein activity of AKT was measured by Western blotting using Hone1/FLJ10540 and Hone1/vehicle transfectants. As shown in the Figure 9A, compared with the vehicle control, FLJ10540 overexpression enhanced the activity of AKT. However, the AKT activation was diminished by AKT inhibitor. Moreover, knock-down endogenous FLJ10540 protein expression by FLJ10540-siRNA resulted in significantly decreased the activity of AKT (Figure 9B). To explore whether FLJ10540-induced AKT activation could affect cell motility in human NPC cells, we used AKT inhibitor to inhibit endogenous AKT activation and assayed the motility of FLJ10540-Hone1 and vehicle control cells. The results illustrated that FLJ10540 seems to promote migration and invasion in NPC cells by increasing AKT activation (Figure 9C). These results support the hypothesis that enhanced NPC cell motility is modulated by fibulin-5-FLJ10540-elicited AKT activation.

Discussion

Fibulin-5 overexpression has been found in many human malignancies, such as fibrosarcoma and breast cancer, and has been suggested as a marker of unfavorable prognoses [8,21]. However, the role of fibulin-5 in invasive behavior and its clinical significance in NPC have not been explored. Here we describe the first systematic survey of fibulin-5 expression in a cohort of primary NPC specimens. Our results indicate fibulin-5 mRNA and protein levels are overexpressed in primary NPC tissues. Overexpression of fibulin-5 is associated with clinically aggressive NPC and correlated with T classification, M classification, and TNM stage. NPC patients with high fibulin-5

expression in tumor cells have a significantly poorer 5-year survival rate than NPC patients with low fibulin-5 expression in univariate analysis. To the best of our knowledge, these results demonstrate that overexpression of fibulin-5 predicts the poor prognosis of NPC and may play an oncogenic role in human NPC.

The ability to migrate and invade the basement membrane into surrounding tissues, blood, and lymphatic vessels is one of the essential hallmarks of cancer and is a prerequisite for local tumor progression and metastatic spread [29]. We have demonstrated that exogenous fibulin-5 in NPC cells or NPC cells stimulated with fibulin-5 protein could regulate NPC cell proliferation, migration and invasion properties in cell culture system. Conversely, the number of cells migrating or invading through the filter was significantly reduced after transfection with fibulin-5-mediated siRNA. Fibulin-5 also plays an important role in cell survival. These results indicate fibulin-5 not only play a key role in the invasion and metastasis of NPC cells but is also consistent with our clinical studies regarding the expression of fibulin-5 in NPC specimens and its correlation with a poor prognosis.

In the present study, we reported that endogenous fibulin-5 as well as exogenous fibulin-5 protein can be mainly found in the nucleus of NPC cultured cells. Two monoclonal antibodies directed against the endogenous fibulin-5 and transfected DDK fused-fibulin-5 consistently showed such a staining pattern. Cell fractionation experiments provided further evidence, for the nuclear localization of fibulin-5 protein, for the most part. It is suggested that the nuclear fibulin-5 may play a transcriptional regulator to modulate the gene expression of fibulin-5 downstream. In contrast, cytoplasmic accumulation of fibulin-5 has been detected in human epidermoid carcinoma cell line and osteosarcoma cell line. In line with its different localization, fibulin-5 might play different roles in human cancer cells.

Fibulin-5 plays multiple roles in diverse cellular processes. It inhibits metastatic colonization of the liver and lung and suppresses MMP-9 and MMP-7 activity [10,30]. The effect of fibulin-5 on tumor progression has also been reported. The mammary epithelial cells stimulated by TGF- β led to overexpression of fibulin-5, production of MMPs, and enhanced growth in fibulin-5-exiting tumor cells [21]. We found that overexpression of fibulin-5 promotes expression and activity of MMP-2 in NPC cells (data not shown). Fibulin-5 plays the role of tumor suppressor or oncogene in cancer cells depending on the related context [8].

To understand the association between fibulin-5 and NPC cell migration and invasion, it would be of great interest to identify the signaling cascades influenced by fibulin-5. For the first time, this study demonstrates that increasing fibulin-5 expression actually promotes AKT activity, whereas fibulin-5 inhibition significantly reduced phosphor-AKT activity and suppressed NPC cell motility. Activated AKT, an important downstream target of PI3K, regulates cell proliferation, metastasis, and prevents apoptosis, and is correlated with a poor prognosis in a variety of human cancers, including NPC [5]. However, the molecular mechanisms by which AKT is activated and control tumor metastasis in NPC have not been

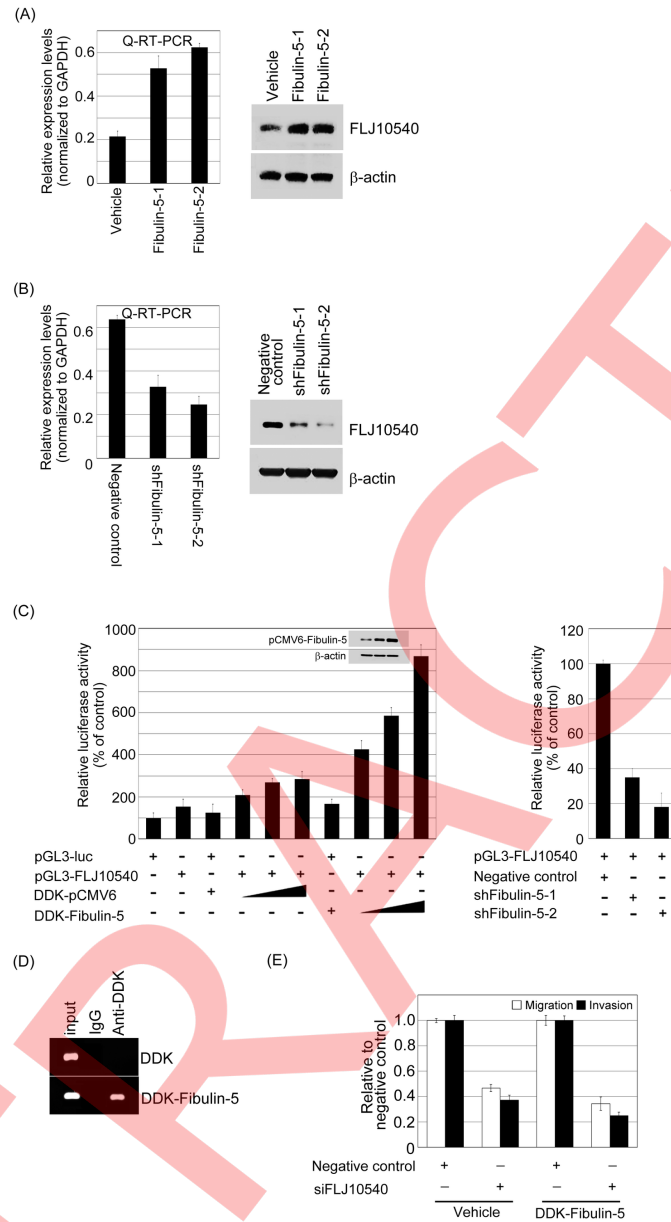


Fig.8

Figure 8. Overexpressed fibulin-5 is up-regulation the expression of FLJ10540, which induced the cell motility in NPC cells. (A) The mRNA and protein expression levels of FLJ10540 were determined by Q-RT-PCR and western blotting in fibulin-5 transfectants. The result of mRNA was normalized against the expression level of GAPDH mRNA in each fibulin-5-stable clones. For protein analyses, the cell lysates (50 μ g) of Hone1/fibulin-5 transfectants was subjected to immunoblot analysis with anti-FLJ10540 and β -actin antibodies. β -actin was used as a control. (B) Fibulin-5 siRNAs decreased the expression levels of FLJ10540 mRNA and protein in fibulin-5-NPC transfectants. The Q-RT-PCR and western blotting were performed as in (B). (C) The luciferase assays were done to detect promoter activities of FLJ10540 in cotransfected with in a dose-dependent manner of DDK-, DDK-fibulin-5-, negative control and sifibulin-5 in Hone1 cells. The luciferase activity in 1 μ g cell lysate was normalized to β -galactosidase activity. Data are representative of three independent experiments done in triplicates. The Western blotting of DDK-fibulin-5 in a dose-dependent manner was shown in the right side of the left panel. (D) ChIP analysis of endogenous FLJ10540 promoter in the presence and absence of fibulin-5 in Hone 1 cells. The protein-DNA complexes were immunoprecipitated with DDK and IgG antibodies, and FLJ10540 promoter element was detected by PCR. (E) Migration and invasion decreased in cells transfected with FLJ10540 siRNA in vehicle-Hone1 and fibulin-5-Hone1 transfectants. The relative-fold migration and invasion values for the stable clones were normalized against the vehicle cells and are represented diagrammatically. All data represent the mean \pm SD of 3 independent experiments.

doi: 10.1371/journal.pone.0084218.g008

Table 3. The correlation between fibulin-5 and FLJ10540 expression in NPC.

		Fibulin-5	FLJ10540
Spearman's rank correlation		1	
Fibulin-5	Sig. (2-tailed)	.	
	Number	30	
	Spearman's rank correlation	0.762**	1
FLJ10540	Sig. (2-tailed)	<0.001	.
	Number	30	30

doi: 10.1371/journal.pone.0084218.t003

defined clearly. Furthermore, for the first time, our data shows that FLJ10540 is not only an upstream regulator of AKT, but that it also contributes to cell migration and invasion following AKT activation in NPC cells. FLJ10540 has several names, including CEP55 [31], and C10orf3 [32]. Several studies have demonstrated that FLJ10540 is not only a cell cycle regulator gene, but is also overexpressed in advanced stages of human cancer tissues, suggesting that it may function as an oncogene in tumor development [31]. Here, we also found that by using overexpressed- and depleted-fibulin-5 approaches to show that increasing fibulin-5 expression upregulates FLJ10540 expression in fibulin-5-transfectants, whereas fibulin-5 inhibition significantly downregulated FLJ10540 expression. However, FLJ10540-Hone1 transfectants did not alter the expression of endogenous fibulin-5, compared to the vehicle control (data not shown). Finally, the immunohistochemical analysis showed a significant correlation that overexpression of fibulin-5 was elevated expression of FLJ10540 in aggressive NPC specimens. This is also the first study to reveal that one of the important upstream regulators of AKT may be modulated by the oncoproteins fibulin-5/FLJ10540 in NPC cancer cells.

Our previous study indicated that fibulin-3 expression negatively correlates with tumor progression and shortened survival in NPC specimens. In this present study, we showed that fibulin-5 expression is correlated with the NPC patients with advanced T stage, TNM stages, and AJCC stage. These results raise the possibility that the protein expression profiles of fibulin-3 and fibulin-5 in NPC specimens may have a negative correlation. By utilizing the Spearman's rank test, we analyzed the relationship between fibulin-3 and fibulin-5 in 40 paired NPC specimens. Our data showed that there was a border-line correlation between fibulin-5 expression and fibulin-3 expression in NPC specimens as expected. Future studies with larger samples are warranted to further investigate the underlying mechanism.

Interestingly, we also found that exogenous fibulin-5 or knockdown endogenous fibulin-5 were unable to affect the proliferation capacity of NPC cells during 24 hour, suggesting that malignant cell developed an endogenous mechanism that rendered them resistant to the effects of fibulin-5. To test this hypothesis, first we examined whether cell cycle related genes were altered in fibulin-5-NPC transfectants. With commercial products of well-established cell-cycle platform by Q-RT-PCR approach, we found that the mRNA expression patterns of p16^{INK4a}, and E2F were upregulated while cyclin D1, and BCL2 were down-regulated in fibulin-5-depleted NPC cells (Figure S4A). These protein expression levels in the NPC specimens with the fibulin-5-low expression were also demonstrated (Figure S4B). Traditionally, in the absence of p16^{INK4a} caused cyclin D1 overactivation in human cancers, including NPC [2] [5] [33] [34]. In addition, the cyclin D/p16^{INK4a}/E2F cascade has been found to be altered in more than 80% of human tumors [35]. Recent work has shown that E2F1 may function as an oncogene or as a tumor suppressor. Untimely expression of E2F1 can cause neoplastic transformation in human cancer cells [36]. However, in other circumstances, the overexpressed E2F1 can lead to apoptosis through p53-dependent and -independent pathways [37] [38]. The nature of this duality is likely to be based on the expression level of E2F in the context of the cell type and transactivation of its target genes [38]. Bcl-2 is an oncogene whose activation could prevent cell apoptosis. EBV-positive NPC cells could induce Bcl-2 expression than EBV-negative NPC cells [39]. In clinical, Bcl-2 is overexpressed in a higher percentage of NPC tumor tissues, and to be a more important factor in NPC development [5]. Taken together, our data illustrated that these molecules may participate in fibulin-5-elicited cancer progression in NPC cell. But, how fibulin-5 regulates these molecules expressions to influence cell proliferation at the same time will be further investigated.

In summary, our findings suggest fibulin-5 up-regulation is a common abnormality in NPC and may play a role in its progression. Fibulin-5 overexpression is correlated with tumor progression and shortened survival. Furthermore, high expression of fibulin-5 in NPC cancer cells contributes to overexpression of FLJ10540 and AKT activity, thus inducing NPC cancer cell migration and invasion. These results strongly suggest that fibulin-5 expression is critical for the invasiveness of malignant NPC cancer cells. Future studies of the physiological targets of fibulin-5 and its potential role in NPC pathogenesis may facilitate the development of novel therapeutic strategies.

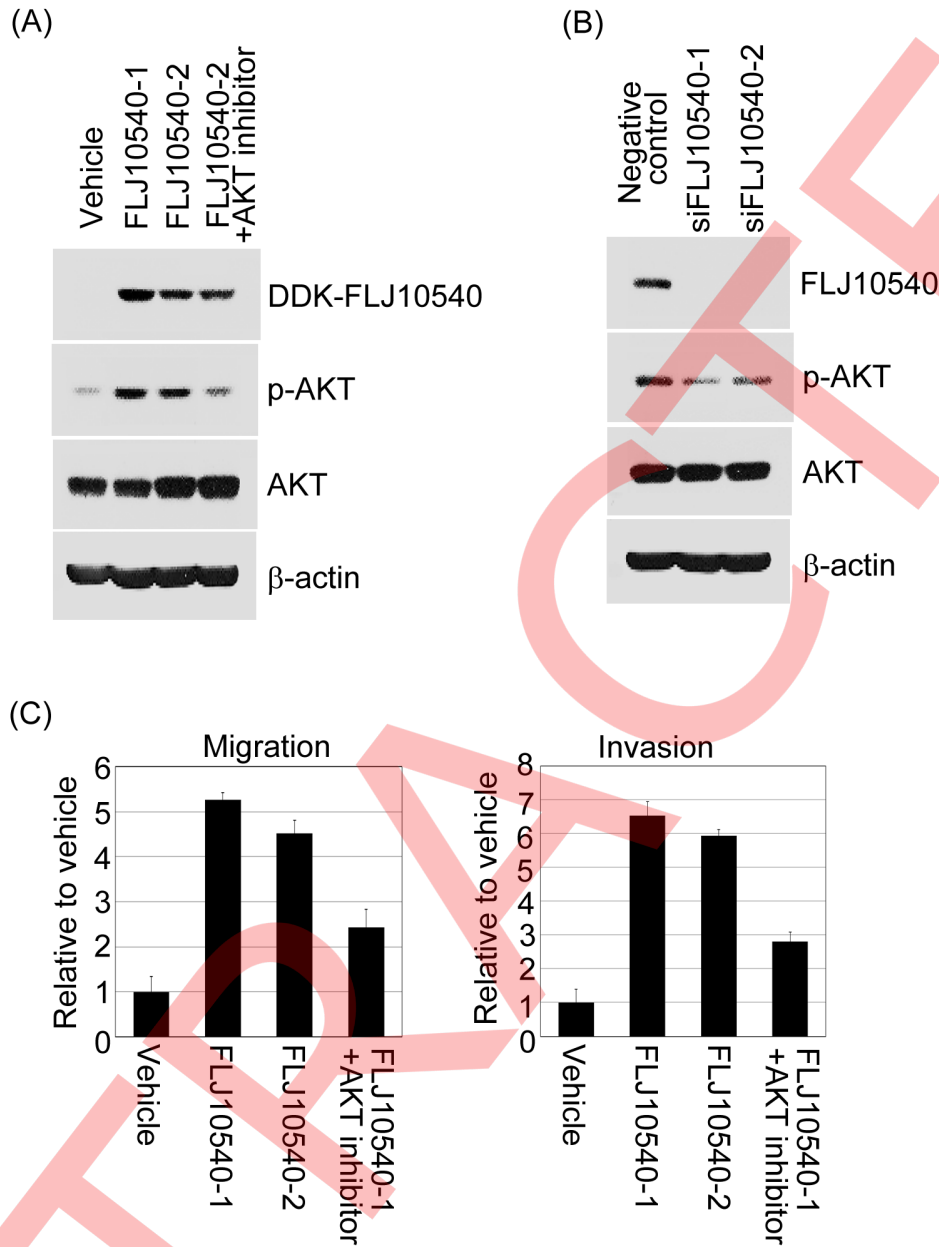


Fig. 9

Figure 9. The activity of AKT induced cell motility is regulated by FLJ10540 in NPC cells. (A) Hone1-expressing FLJ10540 cells were serum-starved for 24 hour and treated with or without AKT inhibitor. The Total cell lysates of vehicle-Hone and FLJ10540-Hone1, transfected cells were immunoblotted for the unphosphorylated and phosphorylated forms of AKT. β -actin was used as the internal loading control. (B) A negative control and FLJ10540 siRNAs were transfected into Hone1 cells for 24 hour and western blotting was performed as in (B). (C) Vehicle-Hone1 and FLJ10540-Hone1 transfected cells were serum-starved and treated with the AKT inhibitor for 24 hour. The migration and invasion ratios of vehicle-Hone1 and fibulin-5-Hone1 transfected cells were determined as previously described.

doi: 10.1371/journal.pone.0084218.g009

Supporting Information

Figure S1. Overexpressed fibulin-5 promotes cell growth, migration, and invasion in TW01-NPC cells. (A) DDK-tagged fibulin-5 was stably transfected into TW01 cells, and DDK-fibulin-5 expression was determined by western blotting with anti-DDK and anti- β -actin antibodies. (B) Cell viability of TW01 stable cell lines was measured by MTT assay. The cells were cultured for 0-3 days followed by MTT assay (OD_{570}) to quantitate the cell growth. Data were normalized against the OD_{570} value on day 0 of each treatment. The results represent the mean \pm SD of 3 independent experiments. (C) Migration and invasion of vehicle-TW01 and fibulin-5-TW01 stable cells are shown (200 \times). For the migration and invasion assays, vehicle-Hone1 and fibulin-5-Hone1 stable clones were seeded into the top of a Transwell insert with or without Matrigel. After 24 h, the cells on top were scraped, and the cells that had migrated to the bottom were fixed and stained with Giemsa. The relative-fold migration values for the clones were normalized against the vehicle control and are represented diagrammatically. The results represent the mean \pm SD of 3 independent experiments. (TIF)

Figure S2. Depleted fibulin-5-TW01-NPC cells suppressed the cell proliferation, migration and invasion. (A) A negative control siRNA plus *fibulin-5* siRNA was transfected into TW01 cells for 24 hour. After transfection, western blotting was performed with anti-fibulin-5 and β -actin antibodies. (B) The *sifibulin-5* transfectants and negative control were seeded into 96-well plates with 5.0% FBS. The cells were cultured for 0–3 days followed by MTT assay (OD_{570}) to quantitate cell growth. The data were normalized against the OD_{570} on day 1 of each treatment. The growth curve of Hone1 cells are shown as the mean \pm SD of 3 independent experiments. (C) The relative-fold migration and invasion of *sifibulin-5*-TW01 cells were

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normalized against the values for the negative control cells and are represented diagrammatically. The results represent the mean \pm SD of 3 independent experiments. (TIF)

Figure S3. Fibulin-5 modulates the FLJ10540 expression in TW01 cells. The mRNA expression level of FLJ10540 was determined by Q-RT-PCR in fibulin-5 transfectants. The result of mRNA was normalized against the expression level of *GAPDH* mRNA in each fibulin-5-stable clones. (TIF)

Figure S4. Fibulin-5 regulates the expression levels of cyclin D1, BCL2, p16INK4a, and E2F in NPC cells. (A and B) The mRNA and protein expression levels of cyclin D1, BCL2, p16INK4a, and E2F were determined by Q-RT-PCR and immunohistochemistry approaches in fibulin-5-depleted NPC cells and tissues. (TIF)

Acknowledgements

The Center for Translational Research in Biomedical Sciences, Kaohsiung Chang Gung Memorial Hospital, for providing the instruments used for this study (CLRPG871342-3), and Chang Gung Medical Foundation Kaohsiung Chang Gung Memorial Hospital Tissue Bank (CLRPG8B0031) for providing the study materials from the Kaohsiung Chang Gung Memorial Hospital Taiwan.

Author Contributions

Conceived and designed the experiments: CFH CHC. Wrote the manuscript: CFH CHC. Literature search and generation of figures: YFY WSW YYZ HTT. Data collection, analysis, and interpretation: CFH LJS LYS SCH TJC FMF CCH TLH CHC.

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