Frequent Loss of NISCH Promotes Tumor Proliferation and Invasion in Ovarian Cancer via Inhibiting the FAK Signal Pathway

Jing Li1,2, Xiaoying He3, Ruofan Dong4, Yuan Wang4, Jinjin Yu4, and Haifeng Qiu2,5

Abstract

NISCH encodes the imidazoline receptor Nischarin and is a known tumor suppressor in many human malignancies; however, its roles in ovarian cancer are still largely unknown. Here, we aim to investigate the biologic functions of NISCH in ovarian cancer. We found that NISCH was significantly downregulated, which correlated considerably with advanced tumor stage, poor differentiation, lymph node metastasis, and the serous/mucinous subtypes in a panel of ovarian cancer tissues. Moreover, NISCH gene silencing was mainly the product of promoter hypermethylation, which could be reversed by treatment with 5-aza-dC. In vitro, NISCH overexpression suppressed cell proliferation and colony formation by hindering cell-cycle progression, whereas the opposite was observed in NISCH knockdown counterparts. In vivo, abundant NISCH expression hindered the growth of HO8910 xenografts, whereas NISCH knockdown accelerated the growth of SKOV3 xenografts. In addition, NISCH significantly attenuated cell invasion by inhibiting the phosphorylation of FAK and ERK, which could be neutralized by PF-562271 (a FAK/Pyk2 inhibitor). Accordingly, NISCH knockdown xenografts exhibited increased peritoneal/pelvic metastases that were not present in counterparts treated with PF-562271. Furthermore, NISCH expression in primary ovarian cancer cells predicted a cellular resistance to PF-562271. In conclusion, we showed that NISCH was frequently silenced by promoter hypermethylation in human ovarian cancer. NISCH manipulated cellular proliferation and invasion by arresting cell cycle and inhibiting the FAK signal. Our findings revealed the biologic functions of NISCH in ovarian cancer, and might be useful for treating patients with aberrant expression of NISCH.

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Introduction

Ovarian cancer is the fifth leading cause of cancer-related deaths among the global female population, resulting in the highest mortality among all the gynecologic malignancies (1). In 2014, there were estimated 21,980 new cases of and 14,270 deaths caused by ovarian cancer in the United States (2). Unfortunately, more than 70% of the ovarian cancer patients present with advanced stage disease (stage III/IV) at the initial diagnosis, and are thus given a poor prognosis (3, 4). Despite many advances in surgical techniques and chemotherapy during the past decades, the 5-year survival rates remain as low as 35% and 20% for stages III and IV, respectively (5–7). Similar to other malignancies, ovarian cancer is characterized by rapid proliferation, invasion, and the formation of regional/distant metastasis (8). Thus, a deeper understanding of the mechanisms underlying the initiation and progression of ovarian cancer is urgently needed.

The NISCH gene localizes on human chromosome 3p21.1 and encodes the imidazoline receptor Nischarin, which binds the cytoplasmic tail of α5 integrin to regulate its translocation from the cell membrane to endosomes (9). Recently, several lines of evidence indicate that Nischarin directly interacts with PAK1 (p21-activated kinase1), LIMK (LIM domain kinase), Rac1 (Ras-related C3 botulinum toxin substrate 1), and LKB1 (liver kinase B1), to inhibit cellular migration and invasion (10–13).

NISCH is frequently silenced by loss of heterozygosity (LOH) in human breast cancer, whereas its overexpression is sufficient to attenuate tumor growth and metastasis via α5 integrin signaling pathway inhibition (14). The roles of NISCH in ovarian cancer are largely unknown; therefore, we investigated the role of NISCH in ovarian cancer initiation and progression.

Materials and Methods

Ovarian cancer tissue collection

Two ovarian cancer tissue microarrays (OV952 and OV961) containing 12 normal ovaries, 38 serous, 28 endometrioid, 20 mucinous, and three clear cell ovarian cancers were purchased from Alenabio Biotechnology Company (Xi’an, Shanxi Province, China) for use in immunostaining assays. Another 30 ovarian cancer samples and adjacent normal ovary tissue collected at the Affiliated Hospital of Jiangnan University (Wuxi, Jiangsu Province, China).
Province, China) were used for real-time PCR, quantitative methylation-specific PCR (QMSP), and LOH analysis. All patients were staged and graded following the criteria of International Federation of Gynecology and Obstetrics (FIGO) for ovarian cancer. Patient follow-ups were performed every 6 months for all available cases until July 30, 2014. This study was approved by the ethical committee of the Affiliated Hospital of Jiangnan University and written consents were obtained from all participants.

For primary cultures, 12 freshly obtained ovarian cancer tissues were minced with scalpels before digestion in 0.1% collagenase IA dissolved in DMEM (Gibco) containing 10% FBS (Gibco) for 2 hours at 37°C. After a short centrifugation, cancer cells were suspended with DMEM to a density of approximately 1 x 10^5 cells/mL. For MTT assay, 1 x 10^4 cells per well were plated into the 96-well plates and incubated overnight before the 72-hour treatment with PF-562271.

**Immunohistochemical staining**

All procedures were performed according to previously published protocols (14). Anti-Nischarin mouse monoclonal antibody was purchased from BD Biosciences and used at a dilution of 1:200. Slides were scored on the basis of average staining intensity by two pathologists individually. The criteria were as follows: 0, no expression (negative staining); 1, weak (1%–25% positive); 2, moderate (26%–50% positive); 3, strong (>50% positive).

**Cell culture and reagents**

Human ovarian cancer cell lines were obtained as follows: SKOV3 and IGROV were from the ATCC, A2780 from the European Collection of Cell Cultures, HO8910 from the Cell Bank of the Chinese Academy of Sciences (CBCAS), and HEY was the kind gift from Dr. Chunxiao Zhou (School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC). In September of 2013 and 2014, all working cell lines were authenticated using short tandem repeat DNA profiling at CBCAS. Cell lines were maintained at 37°C in a humidified atmosphere of 5% CO2 with the following media: SKOV3 and HO8910 in DMEM with 10% FBS; IGROV in RPMI-1640 (Gibco) with 10% FBS; and HEY and A2780 in RPMI-1640 with 5% FBS.

**RNA isolation and real-time PCR**

Total RNA was extracted using the TRizol reagent (Invitrogen) and quantified on a Nanodrop 2000 (Thermo Fisher Scientific). Real-time PCR was performed using the SYBR Premix ExTaq II Kit (Takara) and the primers described in Table 1. The relative expression of NISCH was calculated using the \( 2^{-\Delta\Delta Ct} \) method with \( \beta\)-actin as an internal control.

**DNA bisulfite modification and QMSP**

Genomic DNA was extracted from 30 pair-matched ovarian cancer tissues using a DNA Kit (Tiangen Biotech) and treated with bisulfite using the EZ DNA Methylation-Gold Kit (Zymo Research) according to the manufacturer's instructions. The modified genomic DNA then served as template DNA for QMSP reactions with the primers and conditions described in Table 1. Samples were defined to be hypermethylated if the relative level of methylated DNA in which exceeded a set threshold determined by analyzing the overall methylation levels in the 30 pair-matched tissues and considering the best sensitivity and specificity.

**5-aza-dC treatment**

Cells (1 x 10^6 cells/well) were seeded into 6-well plates and cultured overnight before treatment with 5 mmol/L 5-aza-dC (A3656, Sigma-Aldrich) or PBS vehicle for 48 hours. Real-time PCR and Western blotting were then used to assess alterations in Nischarin at the mRNA and protein levels.

**Loss of heterozygosity analysis**

LOH analysis was performed using three microsatellite markers (D3S3026, D3S3561, and D3S3688) with primers described in Table 1. The PCR products were loaded and separated on 7.5% polyacrylamide gels, and the allelic loss was determined in Table 1. The PCR products were loaded and separated on 7.5% polyacrylamide gels, and the allelic loss was determined.

**Western blot analysis**

Total protein was extracted from cells using RIPA (Thermo Fisher Scientific). Equivalent amounts of protein were separated on SDS-PAGE gels (8% for Nischarin and 12% for other proteins) and transferred to polyvinylidene difluoride membranes. After incubation with appropriate primary and secondary antibodies, the specific protein bands were visualized using a commercial ECL Chemiluminescent Kit (Bioyotme). The following antibodies were used: mouse monoclonal anti-Nischarin (1:1,000; BD Biosciences), rabbit monoclonal anti--phospho-ERK1/2Thr202/Tyr204 (1:1,000; Cell Signaling Technology), rabbit monoclonal anti-ERK1/2 (1:1,000; Cell Signaling Technology), rabbit monoclonal anti-phospho-ERK1/2Thr202/Tyr204 (1:1,000; Cell Signaling Technology), rabbit monoclonal anti-CDK4 (1:1,000, Cell Signaling Technology), mouse monoclonal anti-cyclin D1 (1:1,000; Cell Signaling Technology), and mouse monoclonal anti-Ki67.

**Table 1. Primer sequences used in this study**

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<tr>
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<td>C100</td>
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</tbody>
</table>
Establishment of stable cell lines

The NISCH overexpression and NISCH-shRNA vectors were constructed as previously described (14). The full-length clone of human NISCH was amplified by PCR using the following primers: forward, 5’-CTAGGAAATTCGCCACCATGGAACCAGAAACTGATC-TC-3’; reverse, 5’-CTAGGATCTCCCTGAGGGCGCACCAC-CCGAC-C-3’. The 4545 bp PCR product was then cloned between BamHI and EcoRI sites of pcDNA3.1 (Genechem Technology) were transfected with shRNA-NISCH-shRNA vectors were

Cell-cycle analysis

Cell-cycle distribution was determined on a FACS Caliber System (BD Biosciences). In brief, 1 × 10^6 cells were plated into a 60-mm dish and cultured for 24 hours before harvest. Cells were stained in 500 μL staining buffer containing PI (50 μg/mL) and RNase A (20 μg/mL) for 15 minutes. Data were then analyzed in FCS4 Express software (Denovo Software).

Colony formation assay

Ovarian cancer cells with forced overexpression or knockdown of NISCH were seeded into the 6-well plates (100 cells/well) and cultured for 12 days. Resulting colonies were then fixed with 10% cold methanol for 30 minutes and stained in 0.1% crystal violet for 15 minutes. Colonies containing more than 50 cells were then counted using an inverse microscope.

Establishment of the tumor growth and metastasis mouse models

All the animal experiments were permitted and supervised by the Animal Care and Use Committee of Jiangnan University. To investigate tumor growth, 1 × 10^6 SKOV3 or HO8910 cells were s.c. injected into the right posterior flank of 6- to 8-week-old female BALB/c nude mice. Starting at day 10, tumor diameters were measured once a week to calculate tumor volume using the formula: volume = (length × width^2)/2. All mice were sacrificed on day 38 and tumor tissues were collected for IHC analysis.

To determine the effects of NISCH expression on tumor metastasis, 5 × 10^5 SKOV3-luciferase cells with or without NISCH knockdown were injected into the peritoneal cavity of 6- to 8-week-old female BALB/c nude mice. Fifteen mice were randomly assigned into two groups: negative control, NISCH-shRNA, NISCH-shRNA + PF-562271. PF-562271 was given to the mice from days 21 to 38 at a dose of 25 mg·kg⁻¹·d⁻¹. BIoluminescent density was measured once a week and the mice were sacrificed 4 weeks later to collect tumor tissues for IHC analysis.

Statistical analysis

All in vitro experiments were performed in triplicate and repeated at least three times. Statistical analysis was performed using SPSS 17.0 (IBM Software). The χ^2 or t tests were used for categorical or quantitative data, respectively. P values <0.05 were defined as statistically significant.

Results

NISCH was significantly downregulated in human ovarian cancer

We first investigated Nischarin protein expression in two ovarian cancer panels by immunohistochemistry. As expected, Nischarin was significantly downregulated in ovarian cancer tissues when compared with normal controls (P = 0.025, Fig. 1A and B) and was associated with advanced tumor stage (Fig. 1B, P = 0.047), high histologic grade (Fig. 1C, P = 0.029), and lymph node metastasis (Fig. 1D, P = 0.031). A notable loss of Nischarin was also observed in serous and mucinous tumor subtypes (Fig. 1E, P = 0.006 and 0.022, respectively), but not in endometrioid or clear cell ovarian cancer (Fig. 1E, P = 0.198 and 0.351, respectively). Survival data were available for 76 patients at the last follow-up, including 34 alive and 42 deaths. Follow-up periods ranged from 19 to 73 months with a median of 42.0 months. Significantly, Kaplan–Meier survival analysis revealed that high Nischarin expression associated with a much better overall survival (OS; Fig. 1F, P = 0.032).

Consistently, the mRNA level of NISCH in fresh ovarian cancer tumor samples was much lower than that observed in matched normal ovary tissues (Fig. 1G, P < 0.001). As shown in Fig. 1H, the Nischarin expression was notably higher at the mRNA and protein levels in mildly invasive cell lines (SKOV3 and A2780) than in the moderately invasive IGROV cell line, and was lowest in highly invasive cell lines (HEY and HO8910).

NISCH silencing was mediated by promoter hypermethylation in ovarian cancer

The diminished NISCH expression observed in human breast cancer is reportedly due to LOH (14). Through our analysis, we found that NISCH promoter hypermethylation and LOH

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occurred in 36.7% (11/30; Supplementary Fig. S1A and S1B) and 6.7% (2/30; Supplementary Fig. S1C) of ovarian cancer tissues, and were not present in any matched normal tissue (Fig. 2A). Moreover, we found that promoter hypermethylation, but not LOH, associated with NISCH silencing in ovarian cancer (Fig. 2B and C; \( P < 0.001 \) and \( P = 0.2115 \), respectively). After the
demethylation treatment with 5-aza-dC, the mRNA and protein levels of Nischarin were notably restored in HEY cells, but not in A2780 cells (NISCH was hypermethylated in HEY cells, Fig. 2D and E, $P = 0.032$).

Nischarin regulated cellular proliferation by restraining cell-cycle progression

As shown in Fig. 3A, HO8910 cells transfected with pcDNA3.1-NISCH exhibited a significant overexpression of Nischarin (>20-fold, $P < 0.001$). Notably, this overexpression induced a significant G1 phase arrest ($P = 0.039$) and lower cyclin D1 and CDK4 protein levels (Fig. 3B and Supplementary Fig. S2A) when compared with empty vector–negative controls, as well as abrogated the proliferative and colony-forming abilities of HO8910 cells (Fig. 3C and D, $P = 0.018$ and $P = 0.025$, respectively). In vivo, the HO8910 NISCH-overexpressing xenografts grew much slower than controls (Fig. 3E, $P < 0.001$) and exhibited a significantly higher expression of cyclin D1 (Fig. 4F, $P = 0.03$).

In SKOV3 cells, NISCH-shRNA decreased Nischarin protein expression by as much as 85% ($P = 0.005$, Fig. 4A). Consistent with our previous data, NISCH silencing accelerated cell-cycle progression ($P = 0.021$) and upregulated cyclin D1 and CDK4 (Fig. 4B and Supplementary Fig. S2B). Moreover, the cell proliferation and colony formation were significantly enhanced by NISCH silencing (Fig. 4C and D, $P = 0.042$ and 0.029, respectively). In vivo, NISCH-knockdown SKOV3 xenografts progressed much faster than controls (Fig. 4E, $P = 0.012$) and exhibited a significantly higher expression of cyclin D1 (Fig. 4F, $P = 0.03$).

Nischarin attenuated cell invasion by inhibiting FAK signal transduction

In HO8910 NISCH-overexpressing cells, we detected a sharp decline of phospho-FAK and phospho-ERK (Fig. 5A and B, $P = 0.01$ and 0.032, respectively). This finding persuaded us to assess the effects of NISCH expression on cell invasion. As shown in Fig. 5C and D, NISCH overexpression significantly inhibited the invasiveness of HO8910 cells (Fig. 5C and D, $P = 0.029$). In contrast, NISCH-knockdown SKOV3 cells displayed an increased expression of phospho-FAK and phospho-ERK (Fig. 5E and F, $P = 0.016$ and 0.015, respectively) that can be reversed by the treatment with the FAK inhibitor PF-562271 (Fig. 5E and F, $P = 0.009$ and 0.033, respectively). Moreover, cell invasion was also...
enhanced in NISCH-knockdown cells (Fig. 5G and H, P = 0.029), which was neutralized by treatment with PF-562271 (Fig. 5G and H, P = 0.05).

NISCH suppressed tumor metastasis via inhibiting FAK signal in vivo

To investigate the effect of NISCH expression on tumor metastasis, we established a mouse model of ovarian cancer with peritoneal/pelvic cavity metastasis suitable for bioluminescent imaging. As shown in Fig. 6A and B, mice harboring NISCH-shRNA xenografts exhibited a significantly higher bioluminescent signal than negative control counterparts (P = 0.006), which was significantly lowered by treatment with PF-562271 (P = 0.027). Consistently, the expression of phospho-FAK and Ki-67 in metastatic tissues was notably increased following the knockdown of NISCH (Fig. 6C and D, P = 0.023 and 0.02, respectively), as well as with PF-562271 treatment (Fig. 6C and D, P = 0.037 and 0.041, respectively).

Silence of NISCH predicted cellular response to PF-562271

To determine the correlation between NISCH expression and cellular sensitivity to PF-562271, we performed MTT assay with a panel of 12 primary ovarian cancer cell cultures (Table 2). We found that cells with lower mRNA expression of NISCH were
much more sensitive to PF-562271 than those containing higher NISCH (Fig. 6E, $P < 0.001$, $R^2 = 0.7073$).

**Discussion**

During recent decades, several genes have been identified as tumor suppressors that could inhibit the initiation and progression of human cancers (15–17). These genes often manipulate cellular proliferation, cell-cycle progression, migration, invasion, apoptosis, autophagy, and senescence. Unfortunately, epigenetic or genetic alterations (such as promoter hypermethylation, LOH, and mutation) can lead to the inactivation or deletion of these genes, subsequently allowing some normal cells to undergo malignant transformation.

**NISCH** is a known tumor suppressor; thus, we first sought to investigate whether its expression was deregulated in ovarian cancer. As expected, we detected a frequent downregulation of NISCH in a panel of ovarian cancer tissues, which was significantly associated with advanced tumor stage, high histologic grade, lymph node metastasis, and specific tumor subtypes (serous and mucinous). Our findings were supported by Lu and colleagues (18), study, which revealed mild loss of NISCH expression in all the four subtypes of ovarian cancer (Supplementary Fig. S3). Together, these results support an important role for **NISCH** in ovarian cancer.

Furthermore, we demonstrated that the silencing of **NISCH** observed in ovarian cancer is the result of promoter hypermethylation, rather than LOH. Consistently, **NISCH** expression...
was notably restored after the treatment with 5-aza-dC in HEY cells that harbor a heavily methylated NISCH promoter. This result provides evidence that NISCH is mainly silenced by promoter hypermethylation in ovarian cancer. Interestingly, Baranwal and colleagues (14) previously reported that NISCH LOH occurs in 60% (12/20) breast cancer patients and is significantly associated with its silencing. On the basis of these findings, we hypothesized that NISCH silencing might be

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**Figure 5.**
The involvement of FAK signaling in NISCH-mediated cellular invasion. A and B, NISCH overexpression hinders the phosphorylation of FAK and ERK in HO8910 cells ($P = 0.01$ and $0.032$, respectively), and significantly attenuates the invasiveness of HO8910 cells ($P = 0.029$; C and D). E and F, NISCH-down promotes FAK and ERK phosphorylation in SKOV3 cells ($P = 0.016$ and $0.005$, respectively), which can be reversed by the FAK inhibitor PF-562271 ($P = 0.009$ and $0.033$, respectively). G and H, NISCH knockdown enhances the invasiveness of SKOV3 cells ($P = 0.029$), whereas PF-562271 abrogates this effect ($P = 0.05$).
induced by different mechanisms in various cancers, which needs further exploration in the future.

Next, we investigated the functional importance of NISCH silencing in ovarian cancer. In vitro, the forced overexpression of NISCH in HO8910 cells suppressed cell proliferation and led to their arrest in G1 phase, whereas the knockdown of NISCH enhanced cell proliferation and facilitated progression into S phase. In vivo, NISCH expression resulted in delayed tumor growth and a notable downregulation of cyclin D1. Consistent with these results, similar suppressive effects of NISCH on colony formation and xenograft progression have also been reported in breast cancer (14).

Most ovarian cancers present with rapid proliferation and widespread metastasis that preferentially invades the adjacent peritoneal and pelvic tissues. The 5-year survival rate for patients with localized ovarian cancer is more than 90%; however, this drops to 70% and 30% for those with regional and distant metastasis, respectively (2, 3). Therefore, preventing the invasion and metastasis of ovarian cancer seems to be a potent therapeutic strategy. In the present study, we demonstrated that NISCH could suppress ovarian cancer invasion by inhibiting the phosphorylation of FAK. In contrast, NISCH knockdown cells exhibited increased FAK phosphorylation and invasiveness, which could be reversed by the FAK inhibitor PF-562271. In vivo, NISCH

Figure 6. The effects of NISCH expression on metastasis and sensitivity to PF-562271 in ovarian cancer. A, representative bioluminescent images of peritoneal metastasis in mice bearing SKOV3 cells with empty vector, NISCH-shRNA, or a combination of NISCH-shRNA and PF-562271. B, quantification of bioluminescence data in the three groups: NISCH knockdown facilitates peritoneal/pelvic metastasis ($P = 0.006$), which is impaired by the addition of PF-562271 ($P = 0.027$). C and D, NISCH knockdown enhances the expression of p-FAK and Ki-67 ($P = 0.023$ and 0.02, respectively), which is reversed by PF-562271 treatment ($P = 0.037$ and 0.041, respectively). E, NISCH predicts the cellular response to PF-562271 in primary ovarian cancer cells ($P < 0.0001$, $R^2 = 0.8112$).
Table 2. Clinicopathologic characters of patients in primary culture.

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knockdown enhanced the formation of peritoneal/pelvic metastasis, whereas the treatment with PF-562271 was able to neutralize this phenomenon. These findings suggest that NISCH might suppress tumor metastasis by inhibiting FAK-mediated signal transduction in ovarian cancer.

Tumor cell invasion is a hallmark of metastasis and plays an important role during cancer progression (19). In normal epithelial and breast cancer cells, Nischarin (encoded by NISCH) inhibits cellular invasion via selective binding with PAK1 to inhibit its kinase activity (10, 11). Nischarin also could regulate cell invasion by directly binding to the PDZ and kinase domains of LIMK, and negatively modulating the LIMK–cofilin pathway (12). Consistently, we found that NISCH silencing significantly promoted tumor invasion by activating the FAK pathway, as this effect was reversed by treatment with PF-562271. Collectively, these data support that NISCH inhibits tumor invasion by regulating various signal pathways.

Currently, most ovarian cancer patients receive similar systemic treatments that mainly include cytoreductive surgery and platinum-based chemotherapy. However, despite an initial objective response rate of approximately 60%, many patients succumb to their disease due to chemoresistance and tumor relapse (7, 20). In conclusion, we demonstrate that NISCH is frequently silenced by promoter hypermethylation in human ovarian cancer, thus enhancing cell proliferation and invasion by inhibiting FAK-dependent signal transduction. Our results reveal the biologic functions of NISCH in ovarian cancer, which might be useful for treating patients with aberrant NISCH expression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: J. Li, H. Qiu
Development of methodology: J. Li, X. He, R. Dong, Y. Wang, H. Qiu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Li, X. He, Y. Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Dong, Y. Wang, J. Yu, H. Qiu
Writing, review, and/or revision of the manuscript: J. Li, X. He, J. Yu, H. Qiu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Dong, J. Yu
Study supervision: H. Qiu

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Invasion in Ovarian Cancer via Inhibiting the FAK Signal Pathway

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