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Original Article

Effect of NSG1 on the biological behavior of esophageal squamous cell carcinoma in a zebrafish transplanted tumor model

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Abstract: Purposes: To investigate the expression of Neuron-Specific Gene family members1 (NSG1) in early esophageal squamous cell carcinoma and whether it is involved in the occurrence and development of esophageal squamous cell carcinoma (ESCC). Methods: The expression of NSG1 in early ESCC and paired normal-appearing tissues was detected by immunohistochemistry (IHC), and the effects of NSG1 on ESCC cell proliferation, tumor angiogenesis, invasion and migration in zebrafish were observed by constructing an ESCC xenograft tumor model of transgenic zebrafish with *NSG1* overexpression/knockout. Results: The IHC results showed that the strong/weak positive rate, negative rate of 65 early ESCC were 87.69%, 9.23% and 3.07%, respectively, and the paired normal-appearing tissues were almost negative. In the vivo experiments of xenotransplantation tumor model showed that ESCC cells in the zebrafish larva in NSG1 overexpression group significantly proliferated and metastasized, while ESCC cells in the knockout group underwent rapid apoptosis. Conclusion: The NSG1 is abnormally overexpressed in cancer tissues, which overexpression/knockout can significantly promote/inhibit the proliferation, invasion, and metastasis of ESCC in a xenograft tumor model of transgenic zebrafish.

Keywords: NSG1, esophageal squamous cell carcinoma, zebrafish, xenograft tumor model

Introduction

Esophageal squamous cell carcinoma (ESCC) has the highest incidence rate in China, making it the sixth leading cause of cancer-related deaths globally [1]. ESCC is characterized by rapid progression, a propensity for metastasis, and high malignancy. Consequently, early diagnosis and prompt radical resection are pivotal for improving the prognosis of ESCC patients. Unfortunately, the elusive early clinical manifestation results in over 80% of patients being diagnosed in the middle or late stages during their first visit. This delay in diagnosis deprives them of optimal surgical intervention, contributing to a 5-year survival rate ranging from 15% to 20% [2]. Therefore, investigating the pathogenesis of ESCC and identifying new molecular targets associated with early diagnosis becomes imperative for enhancing the survival rate and prognosis of ESCC patients.

Neuron-Specific Gene family members 1 (NSG1) is a neuron endosomal protein [3-5], primarily expressed in the brain, skin, prostate, adrenal gland, ovary, and testis, with low or negligible expression in other tissues (BioProject: PRJEB4337) [6]. NSG1 plays an important role in the transport of various receptors, such as neurotensin receptor, axonal cell adhesion molecule L1/NgCAM, and neurotransmitter receptors β-APP, and GluA2, among others, within neurons [7, 8]. Knockout of NSG1 may result in the incorrect localization of these molecules in neuronal cells [8]. Currently, NSG1 research predominantly centers on neuronal mechanisms, with limited research on its role in tumor occurrence and development both domestically and internationally. In a previous study, we found increased NSG1 expression in the serum and cancer tissues of early ESCC patients and less or no expression in adjacent normal tissues. *In vitro* experiments using ESCC

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cell lines indicated that NSG1 overexpression significantly promotes the proliferation, invasion, and migration of ESCC cells [9]. These findings suggest a potential role for NSG1 in the occurrence and development of ESCC *in vitro*. However, whether NSG1 exerts carcinogenic effects on ESCC *in vivo* warrants further investigation.

Zebrafish, a tropical freshwater fish, was established as an exemplary vertebrate model for genetics and developmental biology in the early 1980s [10]. Compared to the mouse model, zebrafish boasts numerous advantages: low cost of breeding and maintenance, prolific reproduction and growth, and optimal optical transparency of embryos and larvae [11-13]. For example, studying the angiogenesis and metastasis of ESCC cells becomes more accessible by inoculating a small number of fluorescent dye-labeled tumor cell lines into transgenic zebrafish with a green, fluorescent vascular system, such as Tag (fli1:eGFI). Moreover, probes facilitate the examination of gene expression in transplanted tumor cells under various experimental conditions using *in situ* hybridization and reverse transcription-polymerase chain reaction [14]. Recently, the transplantation of human cancer cells into zebrafish to construct xenograft tumor models has become a valuable approach for studying important aspects of tumor biology, including growth, invasion, angiogenesis, and metastasis [15]. Successful construction of zebrafish xenotransplantation tumor models has been demonstrated in various cancers, such as acute myeloid leukemia [10], melanoma [12], neuroblastoma [14], and liver tumors [16, 17]. Importantly, the success of these models relies on the regulation of human oncogenes and tumor suppressor genes, with zebrafish tumors exhibiting histopathology and molecular similarities to human cancers [18, 19].

While the genomic similarity between mice and humans surpasses that of zebrafish and humans the complexity of experimental procedures and the opacity of the mouse's entire body hinder the precise location and tracking of dynamic changes, such as tumor cell invasion and metastasis, *in vivo*. In this study, the xenotransplantation tumor model constructed using zebrafish carrying the green fluorescent gene enables the real-time observation and recording of dynamic changes in the survival and proliferation of ESCC tumor cells following the overexpression/knockout of NSG1 in zebrafish. This approach aims to unravel the correlation between NSG1 expression *in vivo* and the occurrence and development of esophageal squamous cell carcinoma.

Materials and methods

Study subject

A total of 65 patients diagnosed with ESCC were enrolled in this study. ESCC diagnoses were confirmed through gastroscopy or esophagoscopy combined with pathological biopsy, and only patients with TNM stages 0, I, and II were included. Informed consent was obtained from all subjects, and the study was approved by the Fujian Provincial Hospital Ethics Review Committee. The zebrafish Tg (fli1:EGFP) was procured from Shanghai Feixi Biotechnology Co., Ltd.; while tumor cells KYSE410 and KYSE-150 were obtained from the Chinese Academy of Sciences Shanghai Branch. Overexpression (pCDH-CMV-MCS-3xflag EF1-mCherry T2A-Puro) and shRNA (hU6-MCS-CMV-mCherry PGK-Puro) empty vectors were purchased from Fuzhou Zaiji Biotechnology Co., Ltd. All experimental procedures involving zebrafish were approved by the Experimental Animal Ethics Committee of Fujian Provincial Hospital.

NSG1 detection by IHC

Tissues were baked overnight at 37°C and then dewaxed with citric acid. Antigen retrieval was performed using a microwave. Subsequently, 50 ul of peroxidase solution was added and incubated at room temperature for 10 min. PBS buffer was used for irrigation 3 times, followed by an additional 10-minute incubation at room temperature. After diluting the NSG1 monoclonal antibody in a specific proportion, 100 ul was applied to cover the entire tissue surface, and incubation occurred overnight at 4°C. Next, 50 μl streptomycin avidin peroxidase solution was added and incubated at room temperature for 10 min, followed by the addition of 100 μl fresh DAB staining solution. Tissues were irrigated immediately after the tissue background turned brown, followed by hematoxylin immersion and sealing with neutral gum.

Construction of ESCC zebrafish xenograft tumor model with NSG1 overexpression/knockout

Acquisition of transgenic zebrafish embryos: Mature and healthy transgenic zebrafish were selected, divided into females and males, and maintained under appropriate conditions with a temperature cycle of 28.5°C and a light cycle of 14 h/dark 10 h. Before the female zebrafish laid eggs, a mature male zebrafish was selected for mixed breeding at a ratio of approximately 1:2. The fertilized eggs were collected, disinfected, and cleaned, then transferred to an incubator containing embryo culture medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$). The eggs were incubated under conditions of light and temperature at 28.5°C. After 6 h, the fertilized eggs were transferred to the embryo culture medium containing 0.0045% PTU. Well-developed embryos were randomly divided into 4 groups, each with 12 embryos; NSG1 overexpression (NO) group, NSG1 overexpression control (NOC) group, NSG1 knockout (NK) group, and NSG1 knockout control (NKC) group.

Preparation before microinjection of esophageal cancer cells: To construct the NSG1 overexpression/knockdown ESCC cell lines, cryopreserved tumor cells KYSE410 and KYSE150 were sub-cultured post-resuscitation. Subsequently, they were transfected with lentivirus containing the NSG1 target gene for overexpression or knockdown. The cells were then placed in RPMI 1640 medium containing 10% Fetal bovine serum and cultured in a cell incubator at 37°C and 5% CO_2 saturation humidity. Simultaneously, zebrafish fertilized eggs were incubated for 24 h, and a 1 g/L Pronase E solution was used to remove the egg membrane for 10 min. Embryos meeting the study criteria were selected under a stereomicroscope.

Under sterile conditions, ESCC cells in the culture bottle were extracted from the medium, washed twice with PBS buffer, digested with 25% trypsin containing EDTA for 1 min, terminated with DMEM medium containing 10% FBS, centrifuged, and resuspended with serum-free DMEM medium. The fluorescent carbocyanine CM-DiI was added (7.5 ul/ml), and incubated at 37°C for 5 min, followed by an additional incubation at 4°C for 15 min. The supernatant was centrifuged and washed with PBS twice, followed by resuspension in a serum-free DMEM medium. The cell density was adjusted to 4×10^8 L^{-1} after observation under the microscope.

Microinjection: Following the method proposed by Yong [20], embryos were incubated with 0.03% phenylthiourea (an inhibitor of zebrafish embryo pigment formation) and microinjected after 48 h. Lightly anesthetized embryos, immobilized with an appropriate amount of tetracaine under a stereomicroscope, were injected with ESCC cells (0.05-0.2 ul) into the embryonic yolk sac development area. Post-injection, zebrafish were rinsed with sterile water and placed in a 28.5°C incubator. Zebrafish not meeting the study criteria were removed six hours after injection.

Observation of laser confocal microscope: 48 h after microinjection, zebrafish embryos from different ESCC cell groups were lightly anesthetized, and placed in a confocal dish, and fluorescent images of tumor cell proliferation in the yolk sac area and metastasis in other parts of the fish were captured.

Statistical analysis

The IHC evaluation was conducted by two experienced pathologists. The criteria for judgment included NSG1 expression on the cell membrane, where buffy granules appeared as positive cells. A count of 500 tumor cells was performed, and tissues with brown-yellow cells < 10% were considered negative, while ≥ 10% were considered positive. Tissues were divided into positive (+) and strong positive (++ or +++) based on color depth. The positive rate of immunohistochemistry was compared using the Chi-square test. All statistical analyses were performed using SPSS 24.0 statistical software package and Microsoft Excel. $P < 0.05$ was considered statistically significant.

Results

Detection of NSG1 expression level in ESCC

The IHC analysis revealed the NSG1 protein expression levels in 65 patients with early ESCC. Among them, 57 cases exhibited intense expression (++/+++), 6 cases showed moderate expression (+), and only 2 cases were nega-

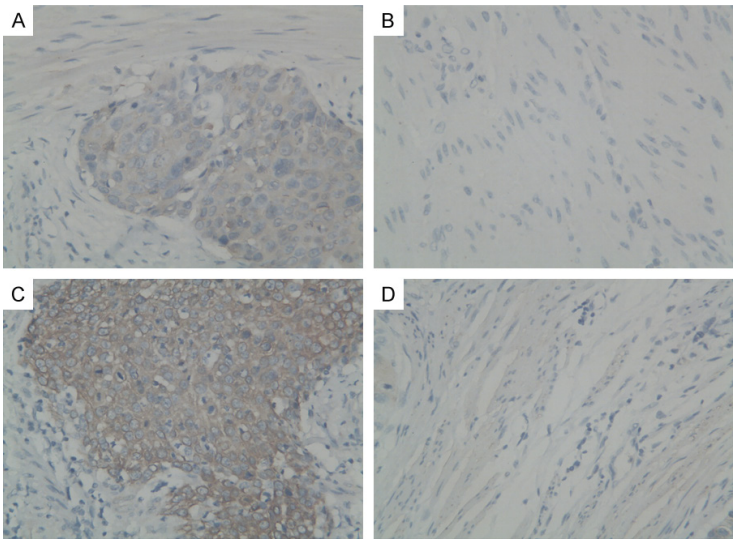

Figure 1. Expression of NSG1 in cancer tissues and paired normal-appearing tissues of early ESCC patients (SP, × 400). A, C: Cancer tissue; B, D: Paired normal-appearing tissue; A: NSG1 expression level is +; C: Expression level is +++; B, D: Expression levels are -.

Table 1. Results of immunohistochemistry in 65 patients with early ESCC

Histological type	Positive cases (+~+++)	Negative cases (-)	*P*
Tumor tissues	63 (96.9%)	2 (3.1%)	< 0.01
Paired paracancerous tissues	4 (6.2%)	61 (95.8%)	

tive (-). In contrast, NSG1 expression in 61 cases of paired normal-appearing tissues was predominantly negative (-), with only 4 cases showing low positive expression (+). The positive rate in cancer tissues was 96.9% (63/65), significantly exceeding that of paired normal-appearing tissues by 6.2% (4/65) ($P < 0.01$), as shown in **Figure 1** and **Table 1**.

Effect of NSG1 overexpression on the biological behavior of tumor cells in zebrafish

Forty-eight hours post-microinjection of esophageal cancer cells into zebrafish, the mortality rates across the four groups ranged from 7% to 13%, consistent with findings reported by Moshal et al [21]. Confocal microscopy observations show widespread cancer cell proliferation throughout the bodies of zebrafish in the overexpression group, as shown in **Figure 2A**, **2B**. Esophageal cancer cells carrying the red fluorescent mCherry gene exhibited extensive proliferation, mainly distributed in the yolk sac and aortic region of zebrafish larvae. In contrast, larvae in the control group demonstrated minimal proliferation and distant metastasis, as shown in **Figure 2C**, **2D**.

Effect of NSG1 knockout on biological behavior of tumor cells in zebrafish

Apart from the observed distribution of red fluorescent tumor cells in the yolk sac area of zebrafish embryos in the knockout group, a few tumor cells were sporadically observed in other regions of the fish, as shown in **Figure 3A**, **3B**. Conversely, the control group exhibited evident proliferation and metastasis of tumor cells, particularly in the aortic area, as shown in **Figure 3C**, **3D**.

Discussion

Our previous study revealed elevated expression of NSG1 autoantibodies in the serum of early ESCC patients, with levels and positive rates significantly higher than those in both the benign esophageal disease group and the healthy control group ($P < 0.01$) [22]. This observation prompted our suspicion that NSG1 is not exclusively highly expressed in neurons but also in tumor tissues, particularly ESCC tissues. To validate this hypothesis, we employed IHC to assess NSG1 protein expression in cancer tissues obtained from 65 patients with early ESCC. The results demonstrated intense NSG1 expression in 57 early cancer tissues, with only 2 cases exhibiting negativity, resulting in a positive rate of 96.9%. This confirmed our hypothesis that NSG1 is abnormally overexpressed in early ESCC tissues.

To further explore whether NSG1 is involved in ESCC pathogenesis, our research group conducted *in vitro* cell experiments, constructing human ESCC cell lines with NSG1 overexpression. Comparatively, NSG1 overexpression significantly promoted the proliferation, invasion, and migration of ESCC cell lines, while NSG1

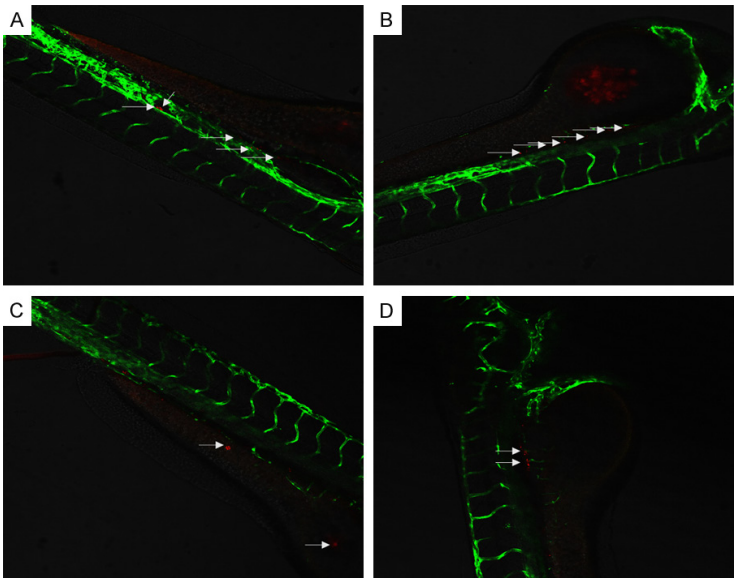

Figure 2. Confocal image of the proliferation, invasion, and metastasis of esophageal cancer cells in zebrafish larvae. (A, B) is the NSG1 overexpression group, (C, D) is the control group, where (A, C) is the tail of the larva body, and (B, D) corresponds to the yolk sac of the larva.

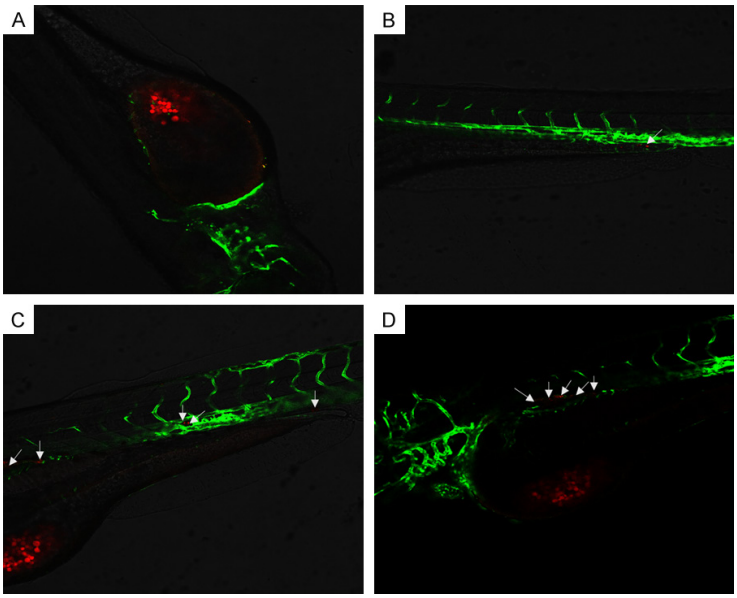

Figure 3. Confocal image of the proliferation, invasion, and metastasis of esophageal cancer cells in zebrafish larvae. (A, B) is the NSG1 knockout group, (C, D) is the control group, wherein (A, C) is the tail of the larva body, and (B, D) is the yolk sac of the larva.

gene knockout weakened the tumor cell characteristics [9]. These findings suggest that NSG1 may play a role in the initiation and progression of ESCC. The potential carcinogenic effects of NSH1 on ESCC *in vivo* warrant further investigation.

Given the high genetic similarity between zebrafish and humans, zebrafish serve as a valuable model organism for *in vivo* studies to a certain extent. Zebrafish, native to tropical areas, boast a small body size, rapid reproduction, and minimal growth environment requirements, providing cost and time advantages for research. Additionally, the high optical permeability of zebrafish larvae lays a foundation for observing the *in vivo* biological characteristics of tumors, such as the proliferation and metastasis of ESCC cells within the fish. For example, Buckley et al, identified Pyrazinib's ability to enhance radiosensitivity in oesophageal adenocarcinoma *in-vivo* in zebrafish, offering insights into an isogenic model of OAC radio resistance [23]. In this study, the Tg (fli1:EGFP) zebrafish with green fluorescent vascular endothelial cells were selected to explore the effect of NSG1 abnormal expression on the occurrence and development mechanism of ESCC.

In the process of constructing the xenotransplantation tumor model, we first constructed the ESCC cell line with NSG1 overexpression/knockout using lentivirus. Embryos within 48 h of fertilization were carefully selected for microinjection, with 300-500 ESCC cells injected into the yolk sac area. The subsequent growth of the embryos was observed at regular intervals. The results of the model construction demonstrated an overall survival rate of nearly 90%, indicating the successful construction of the transgenic zebrafish Tg (fli1:EGFP) xenograft tumor model required for subsequent research in this study.

Given that ESCC cell lines KYSE410/KYSE150 specifically express the mCherry red fluores-

cent protein, the vascular pattern of green fluorescence in transparent zebrafish larvae, and the number and distribution of red fluorescent tumor cells after proliferation and metastasis, were observed under a laser confocal microscope. A comparable study observed the proliferation and metastasis of red tumor cells, revealing that retinoblastoma metastasis occurs in the early stages, and certain antiangiogenic drugs can interfere with tumor invasiveness and metastasis [24]. In our study, a notable difference was observed between the NO and NOC groups. ESCC cell kyse410 in the NO group exhibited significant proliferation and migration, as evidenced by widely distributed red fluorescent tumor cells in zebrafish larvae. Conversely, the proliferation and migration ability of kyse150 cells in the KN group was significantly reduced compared to the KNC group, indicating a limited presence of red fluorescent cancer cells in the yolk sac development area. These findings indicate that the expression of NSG1 in cancer tissues significantly promotes the biological behavior of cancer cells *in vivo*. It can be inferred that NSG1 may also play a role in *in vivo* the carcinogenesis mechanism of ESCC.

Our current study has unveiled novel insights into NSG1, revealing its expression not only in neurons but also its abnormal overexpression in early ESCC tissues. The establishment of a transgenic zebrafish xenograft tumor model has further substantiated that NSG1 overexpression significantly amplifies the proliferation, invasion, and migration of ESCC cells, while NSG1 knockout exerts an inhibitory effect on the biological behavior of tumor cells. While our study has yielded compelling findings, the precise mechanism by which NSG1 promotes development and metastasis in the ESCC xenograft tumor model remains elusive. A significant limitation of this modeling approach is the inherent challenge of precisely controlling both the timing and location of cancer initiation. However, in comparison with mice, zebrafish present distinct advantages. Its short growth cycle and transparent embryos enable the comprehensive observation of the entire development process, including tumor proliferation, metastasis, and angiogenesis from embryos to mature individuals, underscoring the extensive potential applications of the zebrafish model.

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Disclosure of conflict of interest

None.

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