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Original Article

NRSN2 promotes melanoma cell proliferation through PI3K/Akt/GLI pathway

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Abstract: Neurensin-2 (NRSN2), a small neuronal membrane protein, is mainly localized in small vesicles and reported in nervous system. It remains largely unknown to the role of NRSN2 in cancer progression. In current study, we screened the published database and found that NRSN2 is highly expressed in melanoma. To confirm it, the levels of NRSN2 in eighteen fresh Melanoma and adjacent tissues were detected. We found that NRSN2 is significantly expressed in melanoma tissues. We further investigated the cellular function of NRSN2 through gain and loss of function experiments, which suggests that NRSN2 promotes cell growth. Furthermore, our data obtained from western blot and CCK-8 assays show that NRSN2 could activate glioma-associated (GLI) and Akt, thereby affecting cells viabilities. In conclusion, our findings demonstrate NRSN2 is highly expressed in melanoma tissues and the overexpression NRSN2 promotes melanoma cells proliferation partly through PI3K/Akt/GLI pathway.

Keywords: NRSN2, proliferation, PI3K/Akt, GLI

Introduction

The cancer statistics from 2014 revealed that malignant melanoma is the primary cause of death among skin cancers, and its incidence has been rising for past three decades [1]. Though the disease is mostly treated by surgical resection at the beginning stage, the patients are poorly survived because of highly metastatic, aggressive characters resulted in resistance to conventional therapies [2, 3]. Therefore it is urgent to better understand the potential molecular mechanisms in melanoma progression.

NRSN2, a 21983 Da neuronal membrane protein composed of 204 amino acids, is characterized showing high sequence homology to Neurensin-1 [4]. Previous study have revealed that NRSN2 is mainly located in small vesicles and may be involved in organelle transport and in conduction of nerve signals [5, 6]. To our knowledge, NRSN2 was mainly reported in nervous system. Factors in nerves have been found could exert some effects on cancer. The inverse role of NRSN2 was investigated in hepatacellular carcinoma (HCC) and non-small cell lung cancer (NSCLC) from few reports [7, 8]. Since NRSN2 is found to be deleted in human breast cancer. Ma et al. propose that NRSN2 may be relevant to other epithelial malignancies as a tumor suppressor [4]. Melanoma, as a neuroendocrine tumor, undergoes an epithelial to neuroendocrine differentiation [9, 10]. Therefore, we suspect that NRSN2 may play a role in melanoma progression. In current study, we found that NRSN2 does heightened in melanoma and promotes cell proliferation partly through PI3K/Akt/GLI signaling.

Materials and methods

Ethic statement

Eighteen fresh specimens and adjacent tissues were collected from patients with pathologically and clinically confirmed melanoma in Henan Provincial People's Hospital. The written informed consents were signed by all patients in advance. The present study was approved by the Ethics Committee of the hospital.

Cell culture

The melanoma cell lines, A375 and C8161, and a human keratinocyte cell line, HACAT, were

Table 1. Sequence of primers and sh RNAs

Name	Sequence (5'-3')
NRSN2 primer	F: GATGGCAAGTGGTATGGGGTC
	R: CGAGGAC-AGGCTGATCTTCC
GAPDH primer	F: AGCCTCAAGATCATCAGCAATGCC
	R: TGTGGTCATGAGTCCTTCCACGAT
GLI primer	F: CTTTCTCCACACCCTGCTG
	R: GGCTGCGAGGCTAAAGAGTC
Si-GLI	AGCAGCAACTGTATAAGTGATTCAAGAGATCACTTATACAGTTGCTGCT
Sh-#1	CCGGGCAGGATGAGTGAAGACGTTTCTCGAGAAACGTCTTCACTCATCCTGCTTTTTTG
Sh-#2	CCGGCTCTTCCAAGATACCAGCATTCTCGAGAATGCTGGTATCTTGGAAGAGTTTTTTG

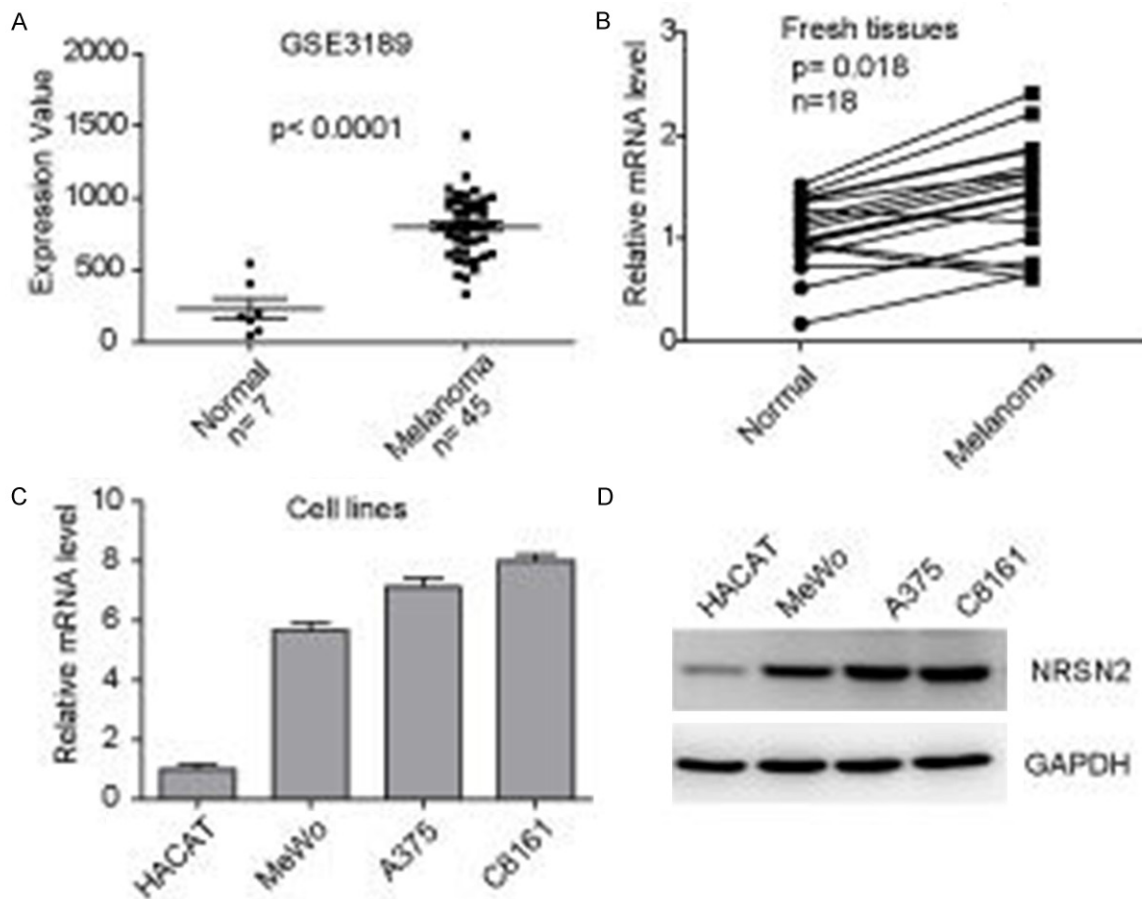

Figure 1. Expression of NRSN2 in melanoma. A: The expression of NRSN2 was dramatically elevated in melanoma tissues compared with normal tissues. The result was rooted in the previously published microarray data, GSE3189. B: The mRNA levels of NRSN2 were elevated in our fresh melanoma tissues compared with adjacent tissues. C and D: The mRNA and protein levels of NRSN2 in HACAT and melanoma cell lines (MeWo, A375, C8161) were performed by quantitative PCR and western blot.

purchased from the American Type Culture Collection (Manassas, USA) and culture cultured in RMPI 1640 and DMEM respectively containing 10% FBS (GIBCO, USA) 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

Quantitative real-time PCR

Total RNA was extracted from melanoma and adjacent tissues or cells using TRIzol (Invitrogen) according to the manufacturer's instruction. RNA was reversely transcribed SuperScript

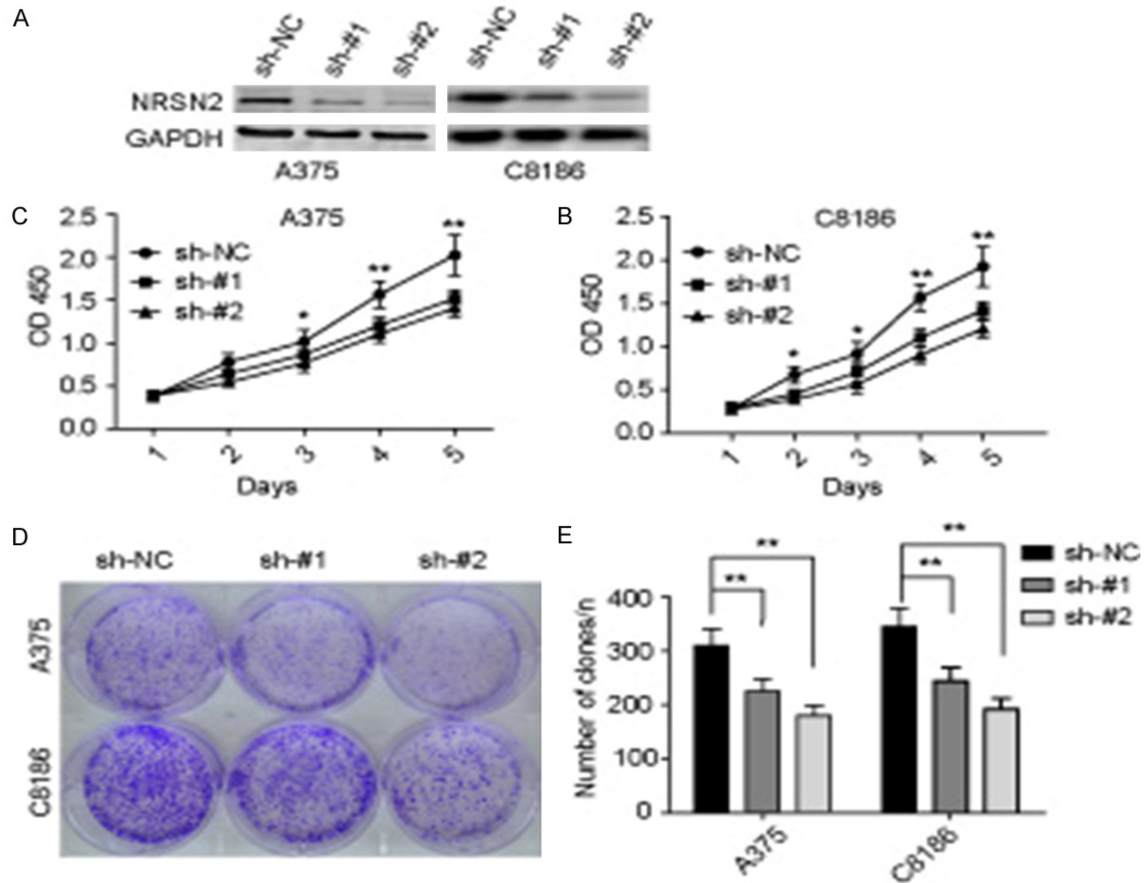

Figure 2. Silencing NRSN2 inhibits melanoma cell viability and proliferation. A: The level of NRSN2 in A375 and C8186 was significantly reduced after shRNA-mediated silencing. B: The specific shRNAs, sh-#1 and sh-#2, were employed in interfering NRSN2 in C8186 cells, which significantly inhibited the viability of cells compared with negative control group. C: Silencing NRSN2 in A375 cells significantly inhibited cell viability compared with negative control group. D: The image show the ability of clone formation dramatically decreased when NRSN2 interfered especially in A375. E: Statistical analysis of clone formation assay (**, P<0.01).

Reverse Transcriptase III (Invitrogen). Quantitative real-time PCR was performed by SYBR green Supermix (ABI) 7500 PCR system. Primers in this study were described in **Table 1**, and the relative expression of NRSN2 was analyzed by the comparative cycle threshold method, which was normalized to GAPDH.

Western blots

Cells were lysed in RIPA lysis buffer (P0013B, Beyotime, China); all the procedures were following the manufacturer's instruction. Subsequently the cell lysates were boiled in 1× SDS-PAGE loading buffer for 10 min, and resolved by 10% SDS-PAGE. After separating, the proteins were transferred onto nitrocellulose membrane, which was blocked in TBS buffer containing 5% BSA (Sangon, China). The following antibodies were used for probing with membranes: NRSN2 (Proteintech, USA), GAPDH (Proteintech, USA), p-Akt (CST, USA), Akt (CST, USA), GLI (CST, USA), and then followed by horseradish peroxidase (HRP)-linked secondary antibodies (CST, USA). Immobilon Western Chemiluminsecent HRP Substrate kit (Millinpore Corporation, Germany) was used for detection.

Construct stable cell lines

To generate stable silenced NRSN2 and GLI cell lines, vectors containing shRNAs and siRNA were purchased from Sigma-Aldrich. Aim was to express NRSN2 and GLI, vectors containing ORF were obtained from Genecopoeia. According to manufactures protocols, we transfected

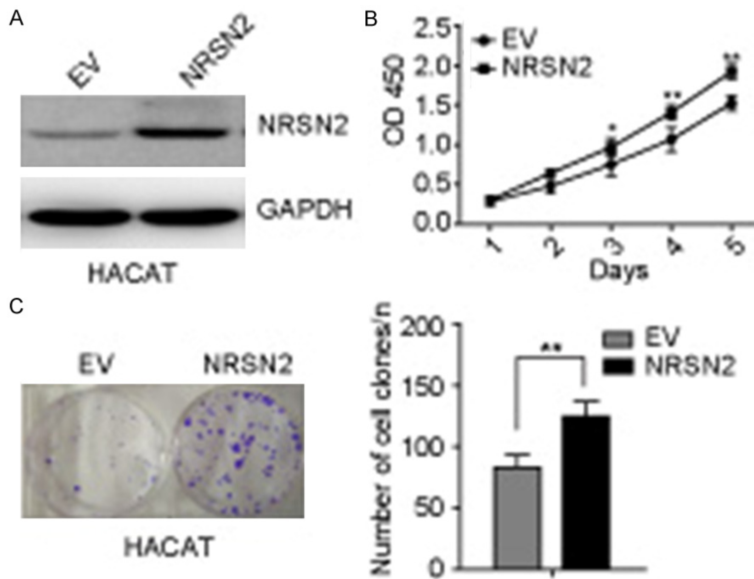

Figure 3. Elevating the level of NRSN2 promotes cell viability and proliferation. A: NRSN2 levels were examined in HACAT cells after overexpression. B: The viability of HACAT cells which stable overexpressing NRSN2 were analyzed by CCK-8 cell viability assays, and significantly elevated when compared with control group (*, P<0.05; **, P<0.01). C: The ability of clone formation significantly increased when NRSN2 over-expressed in HACAT (**, P<0.01).

cells by lipofectamine 2000 Reagent (Invitrogen, USA) with these vectors. The supernatant media containing virus was collected by centrifugation for removing cellular contaminant. The resulting viruses were used to infect indicated cell lines, and then stably transfected cells were selected by administration of 2 μg/ml puromycin for 2 weeks. The sequence of shRNAs, siRNA and GLI were listed in **Table 1**.

Cell viability and colony-formation assay

Cells were trypsin digested and then seeded into six-well plates at 400 cells per well and incubated at 37°C in a humidified incubator with 5% CO_2 for 12 days. The medium was replaced at 3-day intervals. At the end of incubated period, the cultures were fixed with 4% paraformaldehyde and stained with crystal violet. Cells were seeded into a 96-well plate at 1×10^3 cells/well with 100 μL cultured medium and cultured at 37°C, 5% CO_2. The cells viabilities were quantified by addition cell counting Kit-8 (C0038, Beyotime). Following incubation, the absorbance at 450 nm was measured by Power Wave XS microplate reader (BIO-TEK). All experiments were performed in triplicate.

Results

NRSN2 is highly expressed in melanoma

We screened the published database, GSE3189, and found that NRSN2 was commonly elevated in cohorts of patients (**Figure 1A**). To confirm this phenomenon, we firstly detected the levels of NRSN2 mRNA in our collected fresh melanoma and adjacent tissues through qPCR, and found that the NRSN2 were significantly expressed in eighteen fresh melanoma tissues (**Figure 1B**). Then the mRNA and protein levels of NRSN2 in three melanoma cells were further examined. As shown in **Figure 1C** and **1D**, the results indicated that the expression of NRSN2 does enhanced in melanoma.

Silencing NRSN2 inhibits melanoma cell viability and proliferation

The elevated NRSN2 reminds us to investigate the cellular function of NRSN2 in melanoma cell lines. We selected two different shRNAs to silence NRSN2 in A375 and C8186 cells, and thus analyzing protein levels of NRSN2 (**Figure 2A**) and cells viability. The results revealed that the cell viability was significantly reduced after silencing NRSN2 (**Figure 2B** and **2C**). The CCK-8 cell viability assays disclosed the pivotal role of NRSN2 in cell viability. Convincingly, we further found knockdown NRSN2 also inhibited the ability of cell proliferation, as shown in the cell clone formation assays (**Figure 2D** and **2E**).

Elevating the level of NRSN2 promotes cell viability and proliferation

Considering knockdown NRSN2 effects in melanoma cell lines, we constructed the expression vector of NRSN2 stably transfected into HACAT cells, and found that cells viability and ability to proliferate elevated by over-expressing the levels of NRSN2, as **Figure 3** indicates. Taken together, NRSN2 does play a key role in cells viability and proliferation.

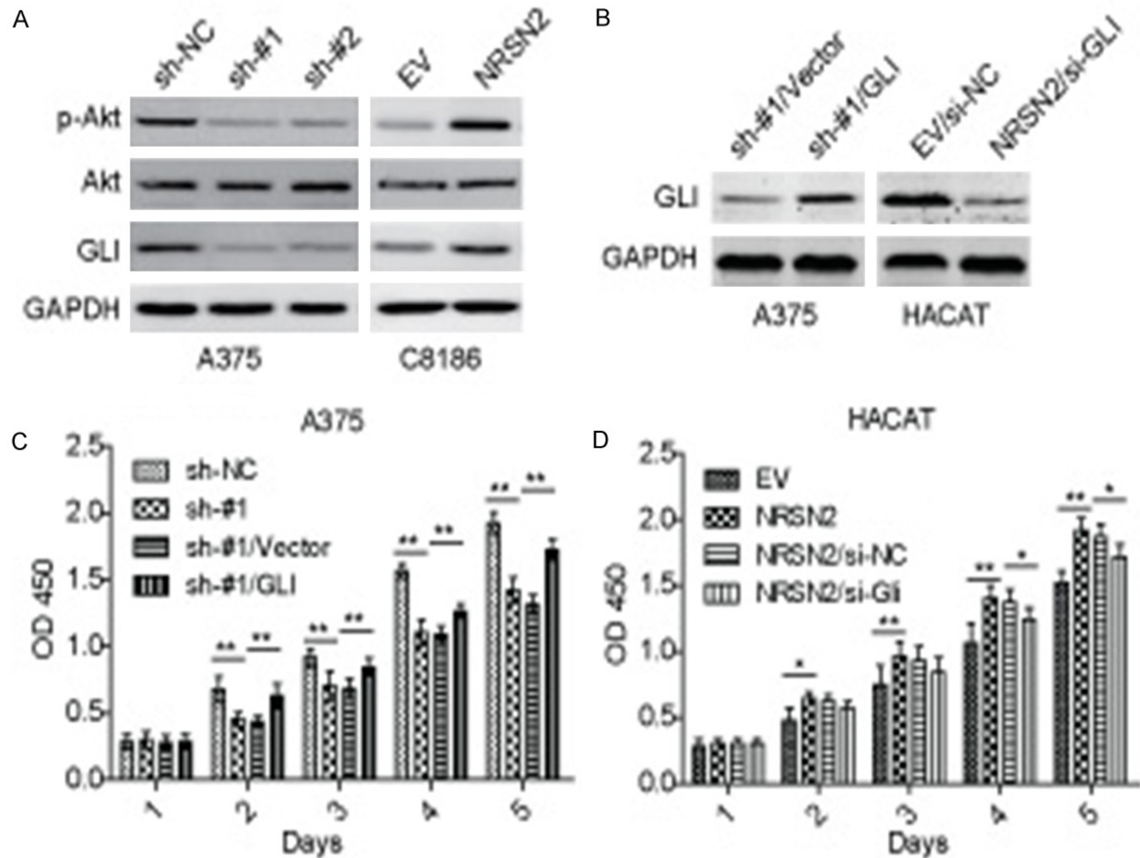

Figure 4. NRSN2 promotes the viability of cells through activating GLI and Akt. A: The level of GLI and the phosphorylated Akt were positively correlated with NRSN2. B: The protein levels of GLI were examined in A375 and HACAT, which co-transfected by sh-#1/GLI and NRSN2/si-GLI. C: A375 cells viability were significantly increased after knockdown of NRSN2, meanwhile over-expressing GLI (**, P<0.01). D: The viability of HACAT cells were moderate decreased after knockdown of GLI, meanwhile over-expressing NRSN2 (*, P<0.05; **, P<0.01).

NRSN2 promotes cells proliferation through activating GLI and Akt

GLI as a transcriptional factor activates Hh signaling required in epithelial repair and regeneration [11, 12]. That implies GLI may play a role in Melanoma progression as well as PI3K/Akt, which proved in previous study. Therefore, we detected the expression of GLI and phosphorylated Akt after NRSN2 silencing and overexpression in A375 and C8186 cells (**Figure 4A**). The reduced GLI and phosphorylated Akt inspired us to further investigation. Then the recombinant expression vectors sh-#1 and GLI were constructed and co-transfected in A375 cells. NRSN2 and si-GLI were in HACAT cells. We found silencing SNRN2 and over-expressing GLI significantly promotes cells viability in A375 cells (**Figure 4C**), while HACAT cells viability reduced (**Figure 4D**). These clues indicate that NRSN2 might regulate cell proliferation partly through activating GLI and Akt.

Discussion

The biological function and associated mechanism of NRSN2 is firstly reported in melanoma. Previously, it has been reported that NRSN2 as a tumor suppressive gene in shRNAs screen experiment for HCC proliferation [8]. While Zhang et al. suggest that NRSN2 promotes NC-CSLC cell growth [7]. Our study shows that membrane protein NRSN2 does heightened in melanoma and could promote cell proliferation partly through PI3K/Akt/GLI signaling.

As we known, there are very a few studies of NRSN2 in cancer, not to mention melanoma. The reverse role of NRSN2 in HCC and NCCSLC also remind us to enlarge the understanding of the function of NRSN2 in cancer. In this study,

NRSN2 in our collected melanoma and adjacent tissues is highly expressed, which result is consistent with the database. In the further studies, we found that silencing NRSN2 significantly reduced cell viability and proliferation, and to treat HACAT cells with EV over-expressed NRSN2 could promote cell growth. These findings indicated that NRSN2 play an oncogenic role in melanoma progression.

Recent evidence supports PI3K/Akt pathway is affected by miRNA in melanoma [13, 14], and exhibits a targeted role in medical treatment [15, 16]. Evidently, PI3K/Akt is a commonly activated pathway and regulates melanoma cell growth, recurrence, and metastasis. PI3k/Akt positively regulates Shh pathway by controlling PKA-mediated GLI inactivation in embryonic development and Hh-dependent tumors [17]. Shh-GLI signaling also regulates the proliferation and survival of human melanomas [18]. In additional, GLI factors (GLI1, GLI2, GLI3) in vertebrates are the terminal effectors of the Shh signaling [19]. GLI2 have been shown to mediated melanoma invasion and metastasis [20]. Stecca B et al. have demonstrated that enogenous Akt signaling regulate the transcriptional activity of GLI1 in melanoma [21]. GLI transcriptional factors have been proved to be positively regulated by PI3K-Akt in breast, gastric, and renal cell carcinoma [22-24]. In the current study, the protein levels of GLI and phosphorylated Akt were positively associated with NRSN2 in A375 and C8186 cells, and silencing NRSN2 and over-expressing GLI enhanced the viability of A375 cells, while the result was reverse in HACAT cells co-transfected with NRSN2/si-GLI. These clues have reminded us that NRSN2 may promote the cell proliferation through the linking PI3K/Akt pathway and GLI factors. Although the elevated pAkt level was accompanied by GLI, which is consistent with reports, the crosstalk between PI3K/Akt and Hh signaling in melanoma deserves further investigation.

Taken together, NRSN2, a membrane protein, is expressed in neural cell and play an oncogenic role in melanoma through PI3K/Akt/GLI signaling, which could be a novel target for melanoma treatment.

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Disclosure of conflict of interest

None.

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