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ORIGINAL PAPER

NAV2 facilitates invasion of cutaneous melanoma cells by targeting SNAI2 through the GSK-3β/β-catenin pathway

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Abstract

Previous studies have identified neuron navigator 2(NAV2) as an oncogene in several human tumors. However, the NAV2 gene expression changes and its role in the pathogenesis of cutaneous melanoma have not been clearly illustrated. Further investigations of NAV2 in cutaneous melanoma may provide new mechanistic insight and treatment strategy for this disease. Through immunohistochemistry assay and bioinformatics analysis, we found that melanoma tissues showed an upregulated expression of NAV2 which correlated with poor prognosis of cutaneous melanoma. To investigate the effect of NAV2 on the proliferation and invasion of melanoma, shNAV2 and NAV2-cDNA were transfected into melanoma cell lines. NAV2 overexpression significantly promoted melanoma cell proliferation, migration and invasion, while NAV2 silencing effectively inhibited this process. The potential underlying mechanisms were investigated using bioinformatics analysis, qRT-PCR, and western blot. Results showed that NAV2-mediated invasion of melanoma cells was driven by enhanced epithelial–mesenchymal transition, which was resulted from SNAI2 upregulation via the GSK-3β/β-catenin pathway. This study suggested that NAV2 could induce melanoma proliferation and invasion by epithelial–mesenchymal transition through the GSK-3β/β-catenin-SNAI2 pathway. Our findings on the pathological mechanisms of NAV2-associated cutaneous melanoma may contribute to the development of potential therapeutic strategy for melanoma.

Keywords NAV2 · Melanoma · SNAI2 · Epithelial-to-mesenchymal transition · Bioinformatics analysis

Abbreviations

NAV	Neuron navigator
SKCM	Skin cutaneous melanoma
EMT	Epithelial–mesenchymal transition
SNAI2	Snail homolog 2
GO	Gene ontology
KEGG	Kyoto encyclopedia of genes and genomes

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Introduction

Skin cutaneous melanoma (SKCM) is the most malignant cutaneous tumors with approximately 87,000 new cases each year in the United States. It is estimated that 9,700 patients will result in death due to metastasis. Meanwhile, the morbidity of cutaneous melanoma is increasing faster than that of any other cutaneous cancers [20]. While early stage melanomas are usually able to be cured by surgical resection, metastatic melanomas are still fatal as they often demonstrate resistance to chemotherapeutic drugs. Hence, it is necessary to find potential biological markers that allow early diagnosis and effective therapeutic approaches to improve the outcome of SKCM patients.

The neuron navigator (NAV) proteins are a family of proteins which were found highly conserved in vertebrates. In humans, three different isoforms (NAV1, NAV2, NAV3) have been identified and were originally demonstrated to be involved in cell migration and outgrowth [14, 22]. Several findings indicated that human cancers such as uterine sarcoma and colorectal cancer tend to have more unfavourable prognoses when they possess elevated expression of

NAV2 [4, 11]. NAV2 contains several functional domains, including one cytoskeletal interacting domain (CSID), four coiled-coil (CC) domains, one AAA (ATPase associated with a variety of cellular activities) domain and one calponin homology (CH) domain [15]. These functional domains play key roles in numerous cellular processes, including the regulation of cytoskeletal remodeling, microtubule dynamics and cell migration, which affect cell matrix and cell–cell adhesion, and ultimately facilitate tumor invasion and metastasis [2, 4, 16, 18, 23, 24]. Epithelial–mesenchymal transition (EMT) is a complicated phenomenon involving various cellular processes, such as sustenance of the tight junctions, microtubule dynamics and cytoskeletal remodeling [10]. The snail homolog 2 (SNAI2) protein belongs to a large super family of zinc finger transcription factors which play crucial roles in the regulation of protein transcription, cell migration, chromatin remodeling and many other processes [17]. SNAI2 was identified to accelerate tumor development and metastasis via EMT, which involved the deficiency of cell adhesion and polarity and the enhancement of migration and invasion properties, mainly through the GSK-3β/β-catenin pathway which could regulate SNAI2 ubiquitylation and degradation. In line with its key role in tumor metastasis, SNAI2 expression was increased in multiple kinds of tumors, including the skin cutaneous melanoma [5, 12, 13, 19, 21]. However, the interaction between NAV2 and SNAI2 in SKCM is still unknown. Here, this study is the first to suggest that NAV2 was overexpressed in melanoma tissues and related to poor prognosis of melanoma patients. And we demonstrated that NAV2 promoted skin cutaneous melanoma malignancy by targeting SNAI2 via the GSK-3β/β-catenin pathway further.

Methods and materials

Reagents and cell lines

Human skin cutaneous melanoma A875 and A375 cell lines were acquired from the China Center for Type Culture Collection (Wuhan, China). Each cell line was pre-validated by isoenzyme analyses and short tandem repeat (STR) profiling prior to commencement of experiments as well as randomly throughout the study. Additionally, all cells were declared free of intraspecies cross-contamination and free of mycoplasma contamination. Both cell lines were cultured for no more than 20 passages (2 months) in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Life Technologies, USA).

Tissue microarray and immunohistochemistry

Alenabio Biotechnology Co. (Xi'an, China) supplied a human melanoma tissue microarray. And we also collected 32 normal skin tissues removed at Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. Meanwhile, the patient consent was obtained from the subjects. Immunohistochemistry (IHC) staining was performed as described previously [27]. Briefly, this involved observation of five random 40 × 10 fields for every section. IHC findings were arbitrarily grouped into five categories based on the proportion of positively staining cells as follows: score 0, no positive cells; score 1, < 25% of the specimen made out of positive cells; score 2, 25–50% of the specimen made out of positive cells; score 3, 50–75% of the specimen made out of positive cells; score 4, > 75% of the specimen made out of positive cells. The intensity of the staining was also arbitrarily quantified such as: score 0, no staining; score 1, pale yellow staining; score 2, buffy staining; score 3, strong brown staining. A final score for each section was derived from the product of both these parameters. The following scale was used to assess the final score of each section: score 0, negative (−); score 1–4, weak positive (+); score 5–8, middle positive (++); score 9–12, strong positive (+++).

In vitro EMT model

TGF-β1 (PeproTech, New Jersey, USA) was used to provoke EMT. After allowing cells to achieve 80–90% confluence, the expanded cells were subjected to 24-h serum starvation in 0.2% FBS. Following this, all cells were incubated for 3 days in 5 ng/ml of TGF-β1 in a 5% CO2 environment at 37 °C [6]. A phase-contrast microscope (Model CKX41, Olympus, Tokyo, Japan) was used to examine cell morphological changes.

Plasmid constructs and transfection

NAV2 and SNAI2 overexpression vector complementary DNA (cDNA) was constructed by the Goodbio Company (Wuhan, China) through insertion of human NAV2 and SNAI2 cDNA into the pcDNA3.1 vector. The A875 and A375 cells were transfected with these cDNA constructs with Lipofectamine 3000 (Invitrogen, USA) in compliance with the manufacturer's protocols. Controls were cells transfected with empty vectors. NAV2-targeting siRNA was obtained from Shanghai Genechem Co., Ltd (Guangzhou, China) and applied to transfect both human melanoma cell lines. Target sequence of the NAV2-siRNA was 5′-AAG GACTCCAGCTCTATGGAA-3′. The shSNAI2 target sequence was 5′-GGAATATGTGAGCCTGGGCGCC-3′. Negative controls were cells infected with scrambled siRNA.

Cell migration and invasion assays

Human melanoma cells were also used for scratch wound migration assays as documented in previous literature [27]. Cells were allowed to grow in a 35-mm dish until confluence was achieved. The cell layer was then gently scratched with a 100-µl pipette tip. Photos at 0 and 24 h after the wound was inflicted were captured to document cell migration. Growth factor-reduced matrigel invasion chambers (BD Biosciences, USA) were used to perform invasion assays based on the manufacturer's protocols. Cells were incubated for 24 h before undergoing fixation to allow quantification under a microscope.

shRNA rescue experiment

Stable NAV2-shRNA-transfected cells were left to expand in six-well plates before undergoing re-transfection with NAV2-cDNA for 72 h. All cells were then exposed to geneticin (Dalian Meilum Biotechnology Co., LTD, Dalian, China) (500 µg/ml) to facilitate selection of cells with the geneticin-resistant gene which were in turn used to produce stable monoclonal cell lines. Blank controls were cells that were not transfected while negative controls were cells transfected with an empty lentivirus vector. NAV2 expression and EMT markers of EMT were detected by western blot and quantitative real-time PCR (qRT-PCR).

Cell counting kit-8 assay

The rate of cell proliferation was examined using the Cell counting kit-8 (CCK8) assay (Dojindo Molecular Technologies, Inc., Shanghai, China). Cells were seeded at a density of 5×10^3/well in 96-well plates and incubated in 100 µl complete culture medium. Cell proliferation was assessed on days 1, 2, 3, and 4 through 2-h incubation with 10 µl CCK8. The optical density of cells was measured at a 450 nm wavelength. Blank samples were wells with CCK8 and medium alone, without cells.

Western blot assay

Cells first underwent lysis before being electrophoresed by sodium dodecylsulfate(SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred onto a NC membrane (Millipore, USA). The electroblotted membranes were incubated with primary and secondary antibodies, while an enhanced chemiluminescent reagent (Thermo Scientific, Thermo Fisher Scientific, USA) was used to detect immune complexes. The provenience of antibodies was listed as follows: NAV2 (Abcam, UK), GAPDH, SNAI2, N-cadherin, vimentin, E-cadherin, β-catenin (Proteintech, Wuhan, China), GSK-3β, p-GSK-3β (Cell Signaling Technology, Beverly, MA, USA).

RNA extraction and qRT-PCR

Reverse transcription reaction and real-time quantitative PCR were carried out as documented in previous literature [27]. TRIZOL reagent (Invitrogen, Thermo Fisher Scientific, USA) was used to extract total RNA from cells. The PrimeScript™ RT reagent kit (Takara, Japan) was used to synthesize complementary DNA (cDNA). qRT-PCR using the SYBR® Premix Ex Taq™ Kit (Takara, Japan) was then performed. All primer sequences were supplied by TSINGKE Biological Technology Company Limited (Wuhan, China) (Online Table 1). GADPH was used as the standard housekeeping gene to normalize cell cycle time (Ct) values of the amplified genes. Fold changes were derived with the relative quantification ($2^{-\Delta\Delta Ct}$) method. The formula is $2^{-\Delta\Delta Ct} = 2^{\text{Control group (Ct value of Target gene} - \text{Ct value of GAPDH)} - \text{experiment group (Ct value of Target gene} - \text{Ct value of GAPDH)}}$. All experiments took place in triplicates.

Bioinformatics analysis

The association between NAV2 expression and OS in SKCM patients was assessed with data in GSE8401 from R2. The R2 web-based application (http://r2.amc.nl) was used to examine the genes correlated with NAV2, the GO analysis and KEGG pathway analysis of these genes. The protein–protein interaction (PPI) network analyses of genes positively correlated with NAV2 were conducted using the online analysis tool STRING (https://string-db.org/) and Cytoscape software.

Statistical analysis

Quantitative data are depicted as the mean ± SD (standard deviation). The differences between the two groups were evaluated using Student's *t*-test, and the differences between three or more groups were evaluated using one-way ANOVA test. Linear correlation between two quantitative variables was analyzed using Pearson's correlation test. Survival rates between subgroups were assessed with Kaplan–Meier and log-rank analyses. Statistical significance was achieved when the *p* value was less than 0.05. All statistical analyses were carried out with the GraphPad Prism 7.0 statistical software.

Results

NAV2 is overexpressed and associated with unfavorable prognosis in SKCM patients

Using immunohistochemistry, we explored NAV2 expression in tissue microarrays and specimens of normal tissue. Final results showed that the NAV2 immunostaining intensity of melanoma tissues was stronger than that of

normal tissues (Fig. 1a, b). We then further studied the gene expression of NAV2 in expression profiling data (GSE3184 and GSE8401) downloaded from R2. The NAV2 level of melanoma was higher than that of normal skin and nevus in GSE3184. Meanwhile, the results of GSE8401 suggested that human metastatic melanoma tissue expressed a significantly elevated mRNA level of NAV2 in contrast to primary melanoma tissues (Fig. 1c, d). Following this, the melanoma patients were then grouped into two groups depending on their mRNA levels of NAV2. The Kaplan–Meier analysis showed that survival time of the melanoma patients with high NAV2 mRNA levels was shorter than that of patients with low NAV2 mRNA levels both in TCGA database and GSE8401 (Fig. 1e, f). These results revealed that NAV2 was overexpressed in SKCM patients and the high mRNA levels of NAV2 correlated with a poor prognosis in SKCM patients.

To further acknowledge the biological functions of the molecular mechanisms linking NAV2 to SKCM, we screened genes correlated with NAV2 by analyzing GSE8401 data in R2 (Online Table 2). The biological functions of genes found to be correlated to NAV2 were then explored with Gene Ontology (GO) analysis. There were a total of 973 clusters of GO terms that were significantly enriched. These were then enumerated according to their functional annotations. Primary clusters were related to regulation of cell adhesion, cell migration, cell proliferation, apoptotic process, cell activation, cell death and

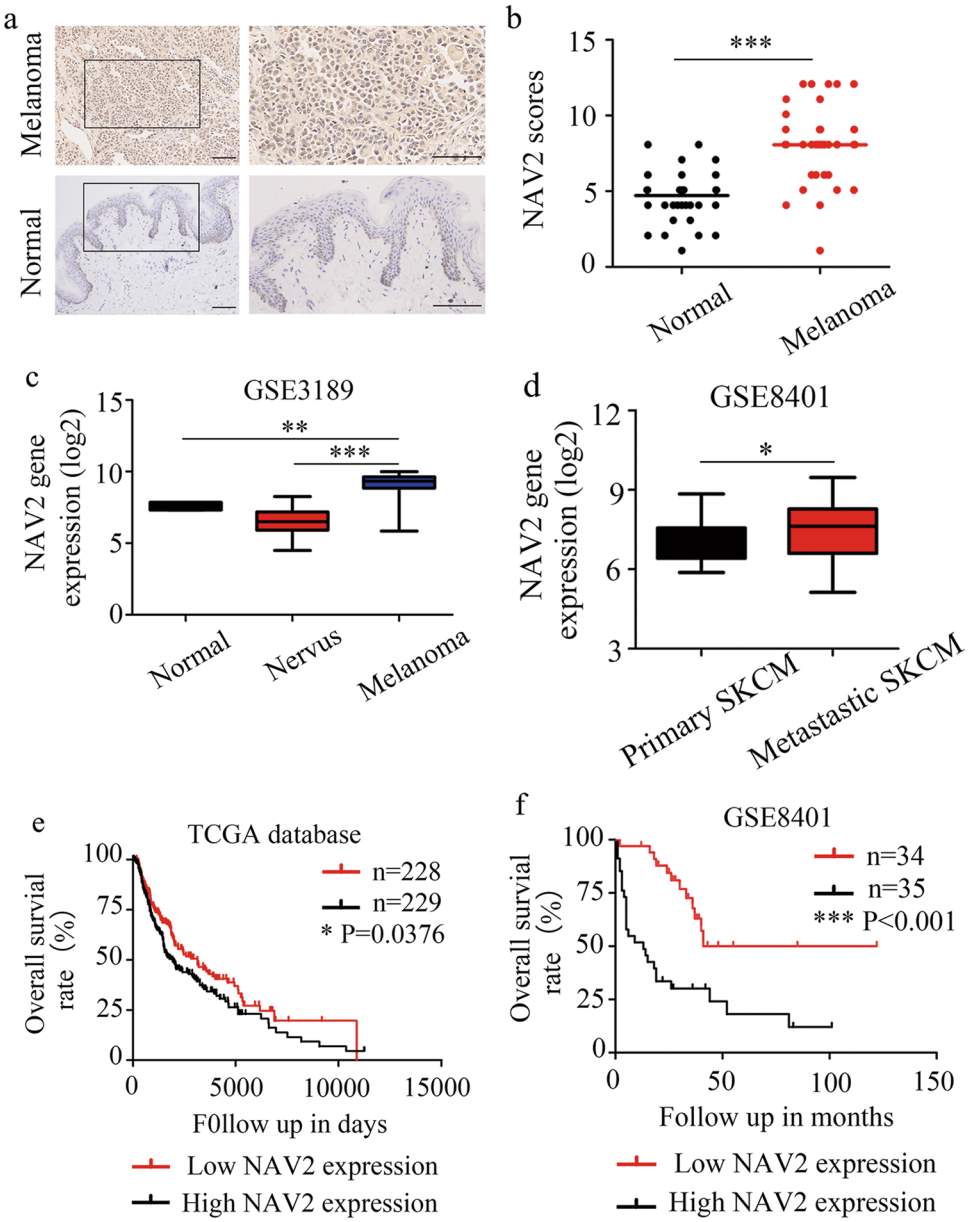


Fig. 1 NAV2 is overexpressed and associated with unfavorable prognosis in SKCM patients. **a** The expression of NAV2 was detected using immunohistochemical staining in melanoma tissues and normal tissues (the left panel was at a 100× magnification and the right panel was at a 200× magnification). Scale bar: 100 μm. **b** The immunostaining scores of melanoma tissues ($n=24$) and normal tissues ($n=24$) were displayed as dot blots. **c** The expression file of GSE3189 showed different mRNA levels of NAV2 in normal skin ($n=7$), nevi samples ($n=18$) and melanoma samples ($n=45$). **d** The mRNA expression of NAV2 was presented in primary SKCM patients ($n=31$) and metastastic SKCM patients ($n=52$) from GSE8401. **e** The OS analysis of SKCM patients with high NAV2 expression and low NAV2 expression from TCGA database. **f** The OS analysis of SKCM patients with high NAV2 expression and low NAV2 expression from GSE8401. *$p<0.05$, **$p<0.01$, ***$p<0.001$

protein complex assembly (Online Fig. 1). Furthermore, we studied the pathways in which the positively correlated genes were involved using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Online Fig. 1). In accordance with the results of GO analysis, these genes were found to be involved in 126 pathways regulating cell adhesion, tube formation, cell proliferation and apoptosis, and so on. Overall, these data suggested that NAV2 might be engaged in several important biological processes in SKCM.

NAV2 promotes invasion and proliferation of SKCM cells by facilitating EMT in vitro

To illustrate the function of NAV2 in SKCM cell proliferation and invasion, A375 and A875 cells were transfected with NAV2-cDNA. NAV2 expression levels were assessed via western blotting (Fig. 2a). Scratch wound migration assays further indicated that SKCM cells had an augmented ability to migrate in contrast to control cells (Fig. 2b). Transwell assays suggested that NAV2 overexpression significantly enhanced cell motility and invasive capabilities (Fig. 2c). Cell growth rates in NAV2-cDNA-transfected cells were markedly elevated in contrast to those of control cells, as demonstrated by CCK8 assays (Fig. 2d). We then sought to identify NAV2 function by first silencing its expression with short hairpin RNA (shRNA). We confirmed that silencing took place by western blot analysis (Fig. 2a). Silencing NAV2 in melanoma cells markedly diminished the migration, invasion and cell growth rate (Fig. 2b, c, d).

Further experiments aimed at investigating how NAV2 controlled melanoma cell invasion and migration. A375 and A875 cells which initially had epithelial morphology developed an elongated fibroblast-like morphology upon exposure to TGF-β1-induced EMT (Fig. 2e). Conversely, TGF-β1-treated NAV2 knockdown cells mostly retained their primary epithelial morphology (Fig. 2e). EMT markers (including vimentin, E-cadherin and N-cadherin) were detected in A375 and A875 cells through qRT-PCR and western blotting. Knockdown of NAV2 increased the expression of E-cadherin but decreased the expression of N-cadherin and vimentin in melanoma cells (Fig. 2f, g). Ulteriorly, TGF-β1-induced EMT attenuated epithelial marker E-cadherin expression, while augmenting mesenchymal marker (N-cadherin and vimentin) expressions (Fig. 2f, g). However, melanoma cells transfected with shNAV2 did not undergo these changes in cell markers in response to TGF-β1 as seen in non-transfected cells (Fig. 2f, g). These alterations in EMT markers were able to be reversed in the NAV2 rescue experiments (Fig. 2f, g). All of these findings suggested that NAV2 facilitated the invasion and proliferation by promoting EMT of melanoma cells in vitro.

SNAI2 takes part in NAV2-regulated SKCM cell invasion and proliferation

A previous study showed that NAV2 regulated cytoskeletal remodeling, microtubule dynamics and cell migration, which are also EMT-related cellular events. Meanwhile, SNAI2 is a key downstream regulator of EMT [1, 5, 19]. We then sought to determine the correlation between NAV2 and EMT regulators (SNAI2, SNAI1, TWIST1 and ZEB1) in cutaneous melanoma data from GSE8401. Moderate positive correlation was significantly shown between NAV2 and SNAI2 when compared with the correlation between NAV2 and the other EMT regulators (Fig. 3a). Kaplan–Meier survival analysis performed on from GSE8401 data revealed that higher SNAI2 expressions in melanoma patients conferred a shorter overall survival period (Fig. 3b). qRT-PCR and western blot also demonstrated that SNAI2 was downregulated when NAV2 was silenced in melanoma cell lines (Fig. 3c, d). Furthermore, the expression of SNAI2 was recovered in the NAV2 rescue experiment (Fig. 3c, d). The mRNA levels of the other EMT regulators such as SNAI1, TWIST1, and ZEB1 were not changed across different groups (Fig. 3c).

Thus, we tested the possibility that SNAI2 would be associated with NAV2-regulated melanoma cell invasion and proliferation. After knockdown of SNAI2 in melanoma cells, the invasive and proliferative cell capabilities were abolished (Fig. 3e, Fig. 3f, Fig. 3g, h). We further detected changes in EMT marker expression and found that SNAI2 knockdown reduced N-cadherin and vimentin expression but increased E-cadherin expression (Fig. 3i). In conclusion, this indicates that NAV2 may function to enhance EMT through upregulation of SNAI2 expression.

NAV2 promotes SNAI2-induced EMT via modulating the GSK-3β/β-catenin pathway

The GSK-3β/β-catenin signal pathway functions in tumorigenesis, cellular differentiation and proliferation as well as tumor chemoresistance [8, 28, 30]. Previous studies suggest that downstream SNAI2 expression is modulated by β-catenin and that attenuated β-catenin levels repress SNAI2 expression, culminating in cell invasion [26]. STRING and Cytoscape tools were used to analyze the PPI network of genes positively correlated with NAV2 in GSE8401. The PPI network is shown in Fig. 4a. CTNNB1 (also called β-catenin) was one of the hub genes whose connective degree ranked first (Fig. 4b). It was also observed to have a high combined score with SNAI2 in GSE8401 (Fig. 4c). Therefore, we hypothesized that NAV2-induced SNAI2 upregulation is mediated by GSK-3β/β-catenin signaling. Subsequently, we examined the expressions of SNAI2, GSK-3β, β-catenin and EMT markers through western blotting (Fig. 4d). shRNA-mediated silencing of NAV2 and SNAI2 increased E-cadherin expression while

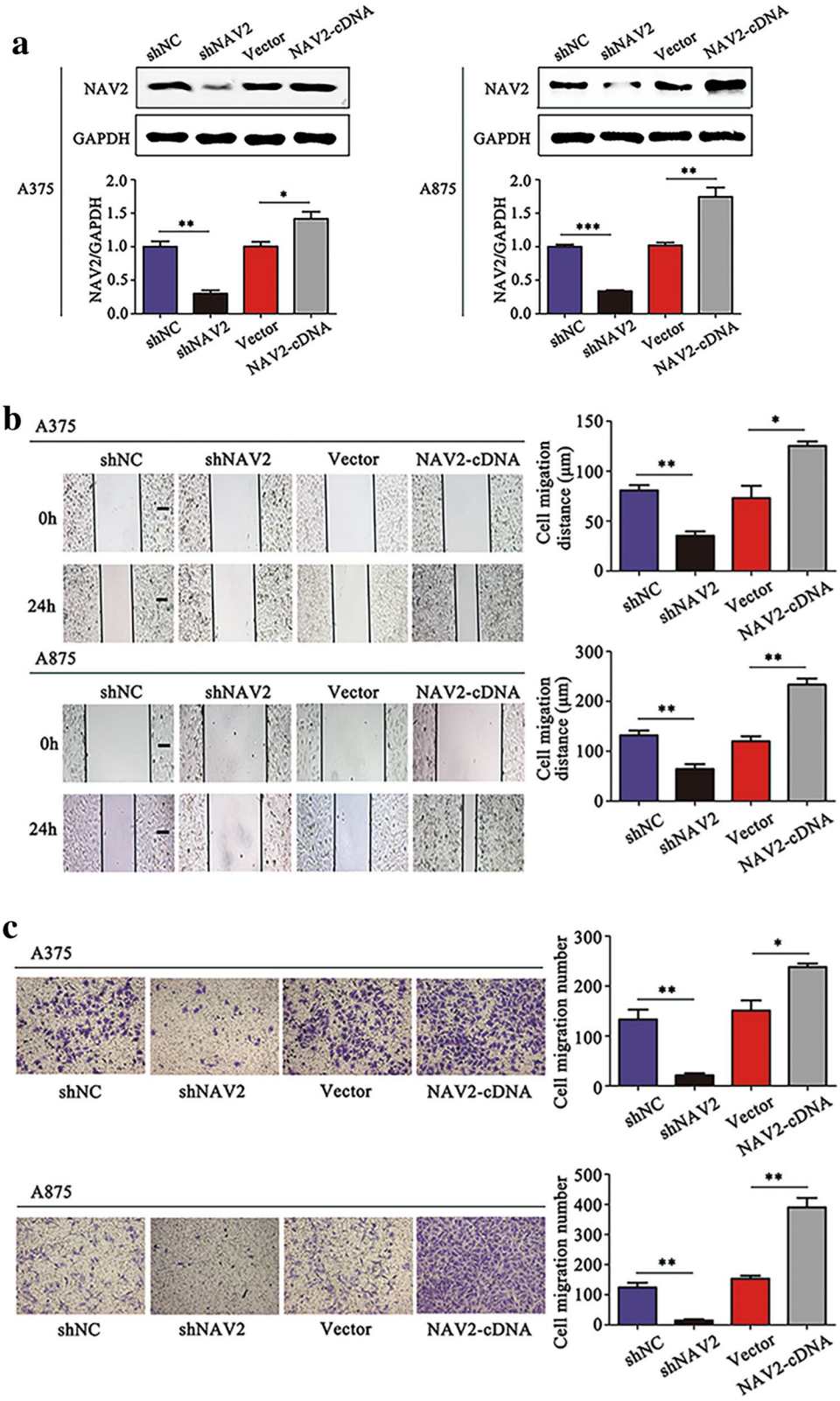
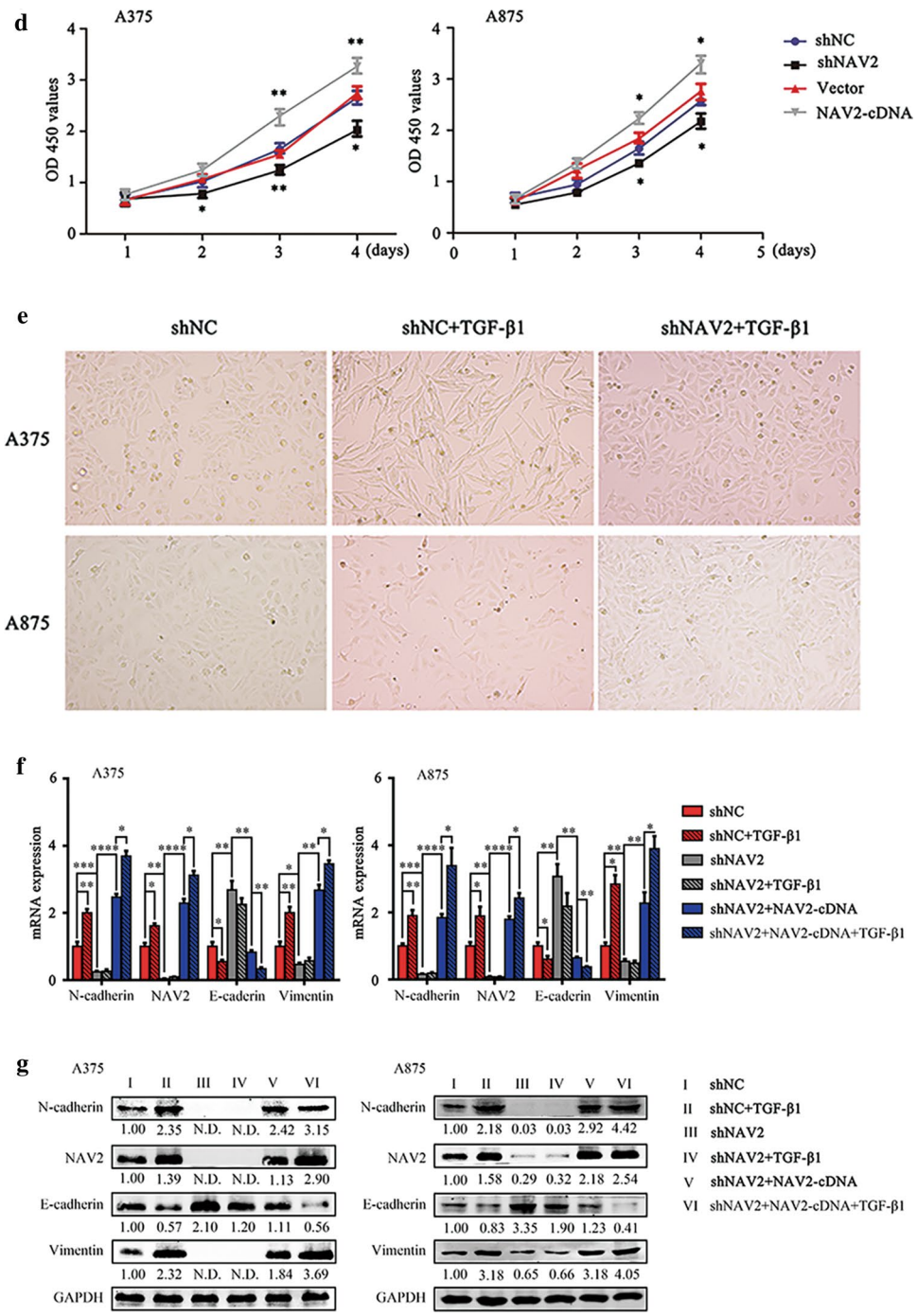


Fig. 2 NAV2 promotes invasion, proliferation of SKCM cells by facilitating EMT in vitro. **a** The expressions of NAV2 were validated in melanoma cells transfected with shNAV2 and NAV2-cDNA. The represented bands were displayed in upper panels. The statistical histograms of the relative gray intensity of bands were displayed on lower panels. **b** The migration of melanoma cells were validated by the wound-healing assay. Left panel: representative image. Right panel: statistical histograms. Scale bar: 100 μm. **c** The results of transwell assay showed that knockdown of NAV2 weakened the invasion of melanoma cells and overexpression of NAV2 enhanced the invasion of melanoma cells. Left panel: representative image. Right panel: statistical histograms. **d** The results of CCK8 assay suggested that knockdown of NAV2 restrained melanoma cell proliferation while upregulation of NAV2 accelerated melanoma cell proliferation. **e** The melanoma cells had the epithelial morphology. When added with TGF-β1 (5 ng/ml), the melanoma cell of control group appeared to be an elongated fibroblast-like morphology, but the TGF-β1-treated NAV2 knockdown cells mostly retained their primary morphology. Magnification: 100×. **f** qRT-PCR was applied to test the mRNA expression of NAV2 and EMT markers (N-cadherin, vimentin and E-cadherin) in different melanoma cell groups. **g** The protein levels of NAV2 and EMT markers were validated using western blot in different groups of melanoma cells. The Arabic numerals represent the relative gray intensity. *N.D.* not detected. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$

decreasing vimentin and N-cadherin expressions. Furthermore, while GSK-3β expression did not change significantly, SNAI2, phosphorylated GSK-3β (p-GSK-3β) and β-catenin expressions were markedly attenuated when NAV2 was knocked down. The above changes were restored during the NAV2 rescue experiment. Conversely, silencing of SNAI2 did not significantly change expressions of β-catenin, GSK-3β or p-GSK-3β. In conclusion, our systematic experiments reveal

Fig. 2 (continued)



that NAV2 might to be a positive regulator of SNAI2 by acting through the GSK-3β/β-catenin signal pathway and subsequently promotes melanoma growth, migration and invasion.

Discussion

This study detected the functions of NAV2 in the progression of SKCM and demonstrated that NAV2 played a significant part in SKCM cell proliferation, migration and invasion by facilitating EMT. In contrast to normal tissues and skin nevi, we demonstrated the overexpression of NAV2 in melanoma tissues. NAV2 expression levels were increased in metastatic melanoma patients in comparison with non-metastatic melanoma patients. Patients with melanoma that had elevated high NAV2 expression levels experienced shorter overall survival times in contrast to patients with lower NAV2 expression levels. These results suggested that NAV2 expression levels correlated with clinical metastasis and patient survival.

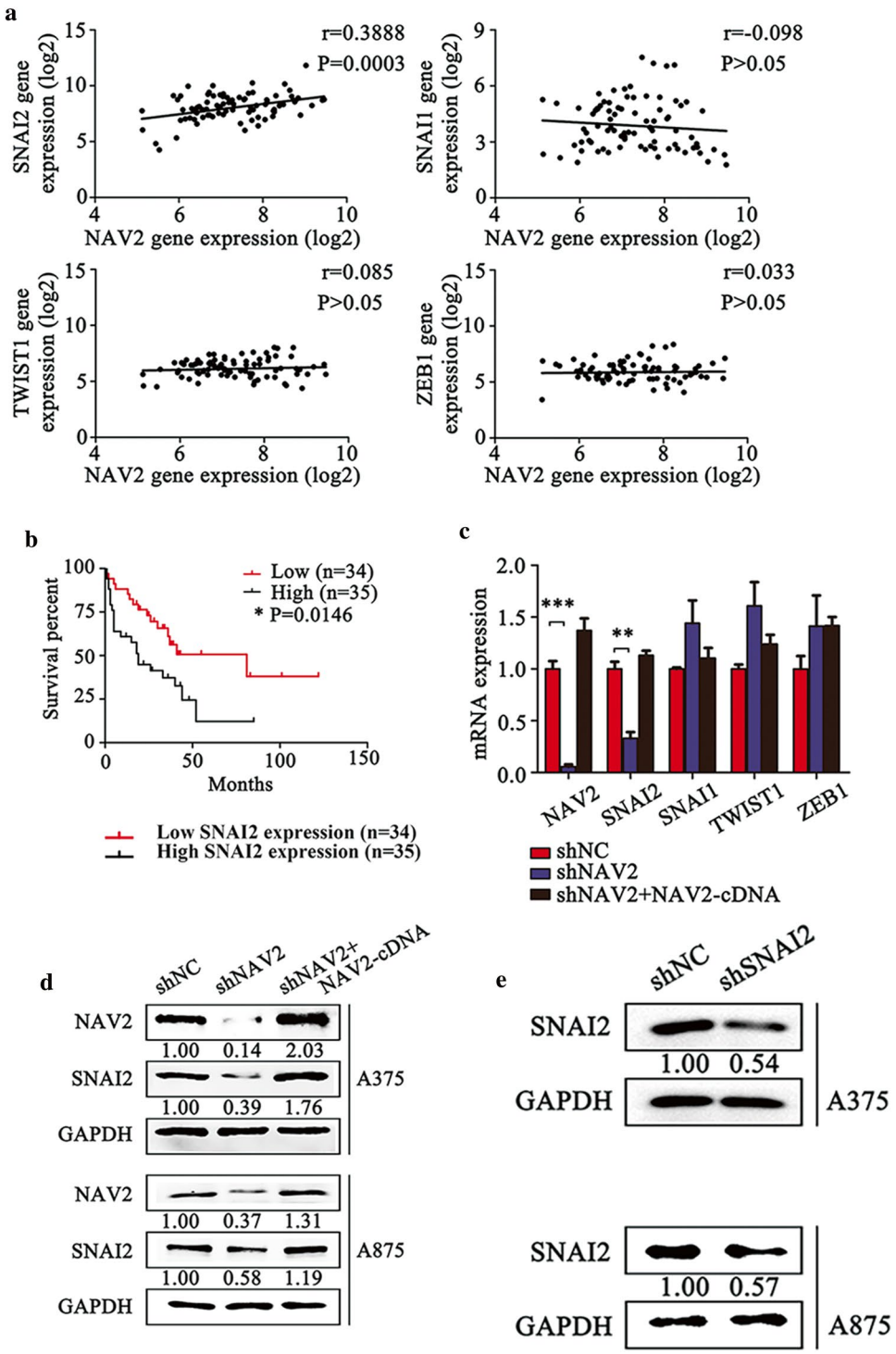
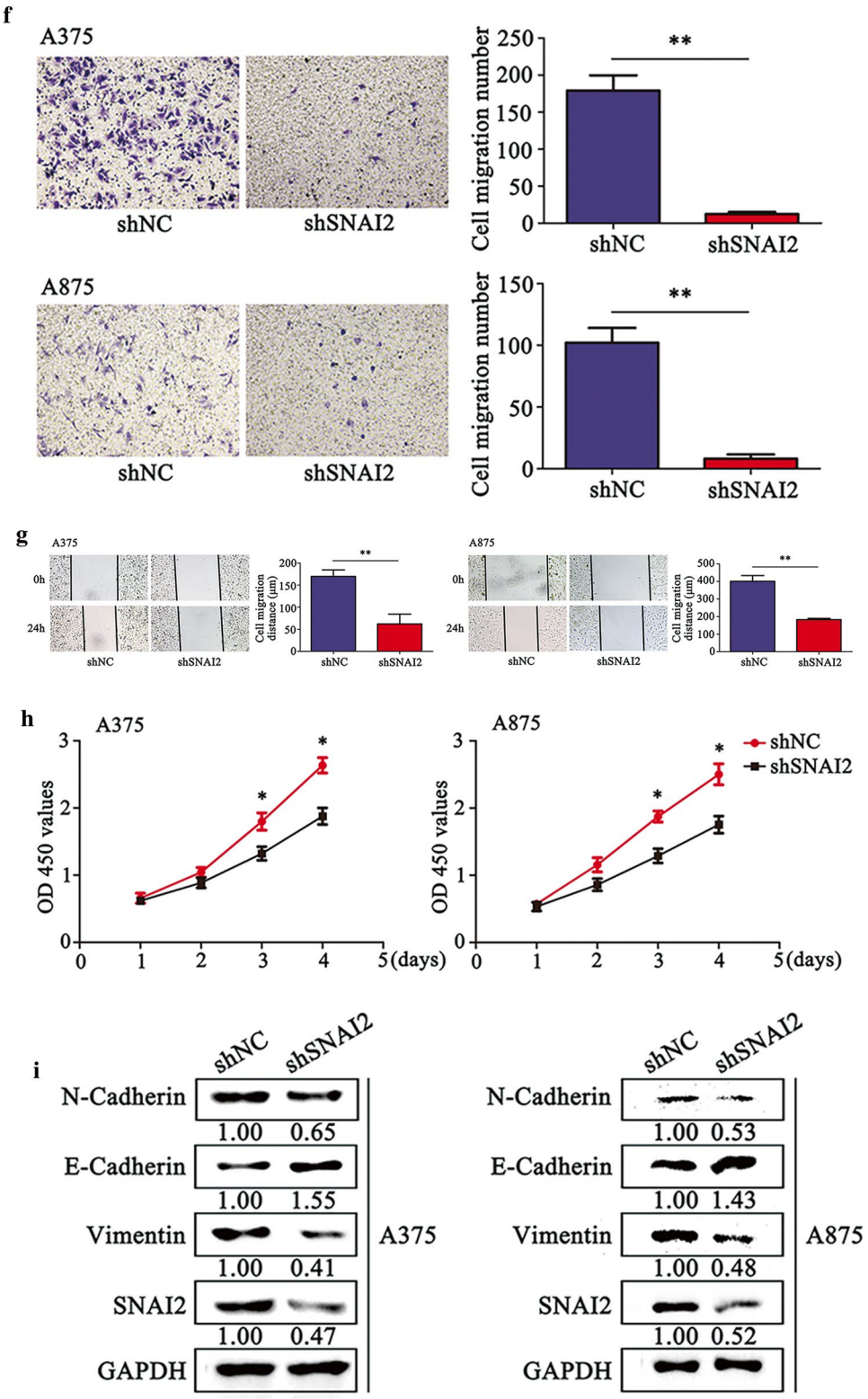


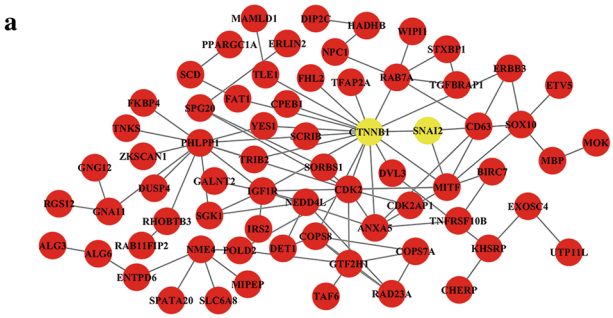
Fig. 3 SNAI2 takes part in NAV2-regulated SKCM cell invasion and proliferation. **a** The correlation between NAV2 mRNA level and EMT regulator (SNAI2, SNAI1, TWIST1 and ZEB1) mRNA levels was determined by Pearson correlation analysis using data from GSE8401. **b** The OS analysis of SKCM patients with high SNAI2 expression and low SNAI2 expression obtained from GSE8401. **c** The mRNA expression of NAV2 and EMT regulators (SNAI2, SNAI1, TWIST1 and ZEB1) was evaluated in different melanoma cell groups by qRT-PCR. **d** The expressions of NAV2 were validated in melanoma cells treated with shNAV2 and NAV2-cDNA by western blot. **e** The protein levels of SNAI2 were tested in melanoma cells transfected with shNC and shSNAI2 using western blot. **f** The results of transwell assay showed that knockdown of SNAI2 weakened the invasion of melanoma cells. Left panel: representative images. Right panel: statistical histograms. **g** The wound-healing assay demonstrated that knockdown of SNAI2 restrained the migration of melanoma cells. The data were presented by representative images and statistical histograms. **h** The results of CCK8 assay suggested knockdown of NAV2 inhibited the proliferation of melanoma cells. **i** Silencing NAV2 reduced N-cadherin and vimentin expression but increased E-cadherin expression. $*p<0.05$, $**p<0.01$, $***p<0.001$

Subsequently, the GO analysis and pathways analysis of the genes correlated with NAV2 revealed that NAV2 participated in multiple important biological processes such as cell migration, cell adhesion and cell proliferation. NAV2 has been reported to be essential for facilitating cytoskeleton rearrangement, axis formation, cellular motility, cell differentiation, cell cycle regulation and vesicle-mediated transport [18, 23, 24]. Meanwhile, we found that suppressing NAV2 expression inhibited in vitro melanoma cell growth, invasion and migration. Furthermore, NAV2 expression was found to be strongly connected with changes of EMT markers. To gain more in-depth knowledge about the molecular mechanism of these changes, we established a cellular EMT model using

Fig. 3 (continued)



TGF-β1, and observed for alterations of EMT markers in this model. NAV2-knockdown blocked changes in the EMT markers of cells exposed to TGF-β1. Additionally, we discovered that NAV2 was moderately related to SNAI2, a known EMT regulator. SNAI2 itself was also known to be an indicator of poor prognosis in SKCM patients. Therefore, we hypothesized that knockdown of SNAI2 could also suppress the in vitro melanoma cell



b

Protein	Connective Degree
CTNNB1	17
PHLPP1	12
CDK2	9
IGF1R	8
MITF	8
GTF2H1	6
ANXA5	6
RAB7A	6
COPS8	5
SOX10	5
NME4	5
RAD23A	4
NEDD4L	4
COPS7A	3
SPG20	3
POLD2	3
ERBB3	3
SNAI2	3
SGK1	3
SEC31A	3

c

Node1	Node2	Combined Score
COPS8	COPS7A	0.999
NEDD4L	SGK1	0.994
IRS2	IGF1R	0.993
CTNNB1	DVL3	0.987
CTNNB1	MITF	0.979
CTNNB1	IGF1R	0.972
RABL5	IFT81	0.965
TLE1	CTNNB1	0.962
CTNNB1	SNAI2	0.956
GNG12	GNA11	0.956
RAD23A	GTF2H1	0.951
ALG3	ALG6	0.943
UTP11L	EXOSC4	0.932
HPS4	HPS5	0.931
GLT25D2	PLOD3	0.924
GTF2H1	CDK2	0.922
COPS8	RAD23A	0.92
GTF2H1	TAF6	0.916
EXOSC4	KHSRP	0.914
NME4	POLD2	0.913

d

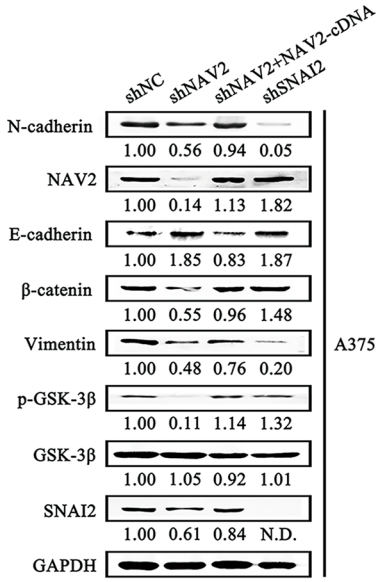


Fig. 4 NAV2 promotes SNAI2-induced EMT via modulating the GSK-3β/β-catenin pathway. **a** The PPI network was performed to analyze genes positively correlated with NAV2 in GSE8401. The nodes indicated the genes and lines represented the interactions. **b** The connective degrees showed that CTNNB1 (namely β-catenin) was one of the hub genes. **c** The combined scores manifested that CTNNB1 had a high combined score with SNAI2. **d** Western blot was used to detect the protein levels of EMT markers and regulators of the pathway involved in EMT. The Arabic numerals represent the relative gray intensity

proliferation, invasion and migration. Indeed, silencing SNAI2 resulted in decreased expressions of mesenchymal markers (N-cadherin and vimentin) but increased expression of the epithelial marker E-cadherin. Additionally, we further discovered that NAV2 might facilitate melanoma cell EMT by upregulating SNAI2 through activation of the GSK-3β/β-catenin signaling pathway. GSK-3β signaling has been reported to regulate EMT by regulating β-catenin degradation [29, 30]. Several lines of evidence suggest that transcriptional activator β-catenin regulates SNAI2 expression [3, 7, 25]. Although β-catenin is mostly located in the cell membrane, which is related to cell–cell adhesion complexes, a small part of this molecule is located in the cytoplasm, which is modified following GSK-3β phosphorylation and succeeding proteasome degradation [9]. Dephosphorylated GSK-3β is the active form of the molecule which stimulates β-catenin degradation. Thus, inactivated GSK-3β inhibits β-catenin degradation, resulting in an accumulation of β-catenin. Accumulated β-catenin then serves to upregulate SNAI2 expression that promotes EMT [3, 7, 25]. In this report, we explored how NAV2 affected the GSK-3β/β-catenin signaling pathway. NAV2 knockdown reduced the expression of phosphorylated GSK-3β, β-catenin and SNAI2 in contrast to their respective expressions in the control groups. Reversal of alterations in phosphorylated GSK-3β, β-catenin and SNAI2 expressions was achieved in NAV2 rescue experiments. There were no visible differences of total level of GSK-3β in different groups. In addition, EMT-associated marker expressions were quantified with western blotting. Changes in their respective expressions concurred with previous experiment findings. In conclusion, the above

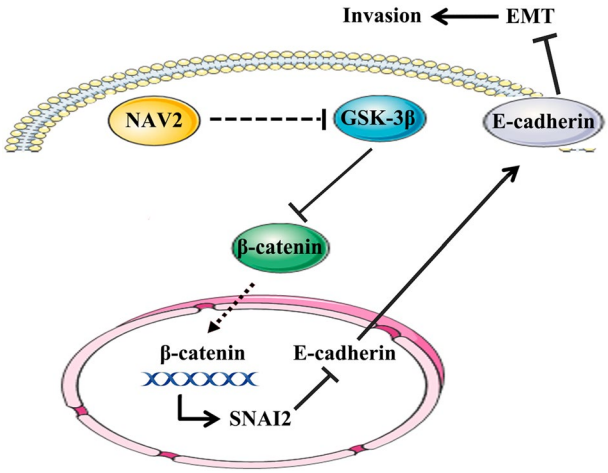


Fig. 5 Graphical presentation

data indicated that NAV2 could affect GSK-3β/β-catenin signaling pathway activation and EMT of SKCM cells through regulating the expression of SNAI2.

Notably, there are some limitations of our study. First, the association between NAV2 expression levels and prognosis of melanoma need to be confirmed in further larger melanoma patients. Second, we did not illustrate the mechanism by which NAV2 affects the expression of phosphorylated GSK-3β, which needs to be investigated in further mechanistic studies. Furthermore, in this study, although we provided evidence that NAV2 participated in the regulation of EMT in melanoma pathogenesis, further studies especially those including animal models are warranted to elucidate its accurate function in the future.

In summary, we provided evidence that NAV2 could affect GSK-3β/β-catenin signaling pathway activation and EMT of SKCM cells through regulating the expression of SNAI2 (Fig. 5). These results put forward a model for how NAV2 could promote SKCM invasion, which might contribute to the development of novel therapeutic strategy for the treatment of SKCM.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in this study involving human participants were approved by the Ethics Committee of Tongji hospital and in accordance with the declaration of Helsinki.

Informed consent Informed consent was obtained from all individual participants included in the study.

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