

Therapeutic targeting Netrin-1-positive invasive bladder cancer cells with oridonin

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Research

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Abstract

Background

Small compound oridonin acts as an effective anti-tumor agent used for a wide variety of human malignancies, while the antitumor efficacy and molecular mechanism of oridonin against bladder cancer remains unclear.

Methods

Four independent cohorts of bladder cancer were employed to assess the correlation between netrin-1 expression and progression and prognosis of bladder cancer. Clinical potential of netrin-1 as a biomarker and oridonin-mediated netrin-1-targeting anti-tumor mechanism were investigated by QRT-PCR, Western blot and ELISA. Regulatory mechanisms of Netrin-1 were investigated by luciferase reporter assay and Western blot. The EJ-inoculated nude mice xenograft model was used for the *in vivo* study.

Results

High expression of netrin-1 was significantly correlated with a poor overall survival in three independent bladder cancer cohorts. Urinary netrin-1 level was significantly elevated in bladder cancer patients correlating with tumor invasion and grade. Netrin-1 promoted cell proliferation, tumorsphere-forming, migration and invasion in bladder cancer cells. Oridonin treatment strikingly suppressed these malignant phenotypes and induced cell death in TCC cell lines and murine xenografts. Oridonin markedly decreased netrin-1 through inhibiting NF- κ B transcriptional activity and shortening the half-life of *NTN1* mRNA via inducing *IRE1 α* , resulting in repression of its downstream signaling.

Conclusions

Together, these results strongly suggest that *netrin-1* promotes the progression of bladder cancer, and acts as a potential urinary biomarker for the diagnosis as well as the prediction of the tumor progression. Oridonin exerts its strong anti-tumor activity against the invasive bladder cancer by suppressing *netrin-1* mRNA production and stability.

Introduction

Transitional cell carcinoma (TCC) is the most predominant pathologic subtype of bladder cancer, which accounts for ~ 90% of the tumors. The patients with the invasive diseases display a high risk of the progression and commonly the poor prognosis. To date, *TP53* mutation [1], p27Kip1 [1, 2], surviving [3], EGFRs [4], Integrin-linked kinase [5] and several microRNAs [6] have been identified to be related to the progression and the unfavorable outcome of the muscle-invasive tumors. Owing to the heterogeneity of

the property of bladder cancer, the treatment of the invasive and the advanced TCCs remains a clinical challenge.

NTN1 (Netrin-1) is a secreted extracellular matrix protein that plays a fundamental role in axon guidance of the developing brain, through binding to deleted in colorectal cancer (DCC) and UNC5 (UNC5A, UNC5B, UNC5C and UNC5D) receptors [7]. DCC and UNC5 have been considered to be the dependence receptors that transduce the distinct signals, dependent on their availability of *netrin-1* [8, 9]. Recently, the aberrant expressions of *netrin-1* and its receptors DCC and UNC5 have been documented to promote tumor progression and metastasis. Overexpression of *netrin-1* have been described in the metastatic breast cancer, non-small-cell lung cancer, aggressive neuroblastoma, pancreatic adenocarcinoma, glioblastoma and colorectal cancers [10–15], indicating that *netrin-1* might be a potential target for cancer therapy. Nevertheless, the precise role of *netrin-1* in the progression of bladder cancer remains to be elucidated.

Oridonin, a cell-permeable diterpenoid compound purified from the herb *Rabdosia rubescens*, has been shown to possess the wide pharmacological activities against inflammation, bacteria and tumors. Recent studies have demonstrated that oridonin functions as an effective anti-tumor agent in a wide variety of human malignancies [16]. Oridonin-mediated anti-tumor activity was implicated in cell cycle arrest, inhibition of cell growth, induction of senescence, apoptosis and autophagy [16]. However, it has not yet investigated whether oridonin could effectively inhibit the malignant behaviors of bladder cancer cells and also its potential target(s) in bladder cancer is unknown.

In this study, we have addressed the clinical and the functional significance of *netrin-1* in TCC of bladder and elucidated the molecular mechanisms behind the anti-tumor activity of oridonin targeting *netrin-1* against the invasive bladder cancer.

Materials And Methods

Tumor tissue samples

Thirty-five freshly-frozen primary bladder tumors were obtained from First Hospital of China Medical University (CMU) with an informed consent. The present study was approved by the Institutional Research Ethics Committee. The clinical parameters of these 35 TCCs were described in Supplemental Table 1. Human TCC tumor tissue array containing 65 primary TCCs and 22 paired normal tissues was provided by Shanghai Biochip (Shanghai, China). The clinical parameters of these samples were summarized in Supplemental Table 2. Two independent open-access datasets of bladder cancer were used: The Cancer Genome Atlas (TCGA) dataset (n = 406) (<http://www.oncolnc.org/>; <https://portal.gdc.cancer.gov>) and the TumorBladder-Hoglund-308 dataset (n = 308) in the R2 platform (<http://hgserver1.amc.nl/cgi-bin/r2/main.cg>).

Cell culture and reagents

Human TCC-derived T24, EJ and J82 cells were cultured in RPMI 1640 medium supplemented with heat-inactivated 10% FBS. The reagents, expression plasmids and siRNAs used in this study were described in detail in Supplementary Materials.

Enzyme-linked immunosorbent assay (ELISA)

Urine samples were preoperatively obtained from 30 primary TCC patients who did not receive any neoadjuvant therapies. Among them, the urine samples were collected again from 10 patients with muscle-invasive tumors 4 weeks after the radical cystectomy. Ten age- and the gender-matched non-tumor patients (ureteral calculus: 8; benign prostatic hyperplasia: 2) and 10 normal individuals were recruited as control. ELISA was performed as described in Supplementary Material.

Western blot analysis

For western blot analysis, whole cell lysates were prepared by lysing cells with 1 × SDS sample buffer. Protein concentrations were quantitated by means of Protein Assay Reagent (Bio-Rad). Equal amounts of proteins were resolved by SDS-PAGE, blotted onto the Immobilon-P membranes (Millipore), and probed by the indicated antibodies. The membranes were developed with an enhanced chemiluminescence (ECL) detection system (GE Healthcare). The antibodies used in this study were as follows: anti-*netrin-1* (1:500, Abcam), anti-cleaved caspase-3 (1:1000, Cell Signaling), anti-PARP (1:1000, Cell Signaling), anti-LC3 (1:500, ABGent), anti-phospho-IkBα (1:1000, Cell Signaling), anti-IkBα (1:1000, Cell Signaling), anti-p50 (1:1000, Cell Signaling), anti-phospho-p65 (Ser-536) (1:1000, Cell Signaling), anti-p65 (1:1000, Cell Signaling), anti-phospho-Src (Try-416) (1:1000, Cell Signaling), anti-Src (1:1000, Cell Signaling), anti-phospho-Akt (Ser-473) (1:2000, Cell Signaling), anti-Akt (1:1000, Cell Signaling) and anti-actin (1:4000, Sigma) antibodies.

In vivo tumor xenograft study

The 6-week-old female BALB/cA Jcl-nu/nu mice (CLEA Japan) were used in our current studies, which were fed and housed according to the institutional guidelines for laboratory animal research. EJ cells (1.0×10^6 cells) in the exponential growth phase were subcutaneously inoculated together with a Matrigel Basement Membrane Matrix (1:1 in dilution, BD Biosciences) into the right flank of the mice. After tumor volume reached 50 mm³, the mice were randomly separated into two groups (6 mice/group) and treated with vehicle or with oridonin (15 mg/kg/day) by the intraperitoneal injection daily for 20 days. Tumor volume was estimated based on the following formula: tumor volume (mm³) = (length × width²) / 2. On day 21, the mice were sacrificed and xenograft tumors were excised and weighted.

Other assays

Quantitative RT-PCR, immunohistochemical staining, WST-8 cell proliferation assay, flow cytometry analysis, migration/invasion assay, sphere formation assay, luciferase reporter assay, colony formation assay and monodansylcadaverine (MDC) vital staining were conducted as described in Supplementary Material.

Statistical analysis

Statistical analysis was performed using SPSS Statistics ver.19 (IBM). Survival distributions were compared using the log-rank test. Mann-Whitney U Test was used to explore the differences in mRNA expressions of *netrin-1* and *UNC5* family between the non-invasive and the invasive samples. Cox regression analysis was used for the impact of the several risk factors on survival. Two-tailed Student's *t*-test was used to explore the differences between the other experiment groups. Statistical significance was declared if *P*-value was < 0.05.

Results

High expression of Netrin-1 predicts a poor prognosis of TCC patients

Expression of *netrin-1* protein was assessed by IHC staining in human TCC tumor tissue array containing 65 primary TCCs and paired normal tissues. Compared to normal adjacent tissues, *netrin-1* expression level was significantly higher in TCC tumors (Fig. 1A-B), and significantly associated with the high tumor stage (Supplementary Table 1), consistent with our previous observations on a cohort of 160 primary TCCs [17]. At mRNA level, *netrin-1* expression levels were remarkably higher in the high-grade (Mann-Whitney U Test, low-grade vs. high-grade, *P* = 0.027, Fig. 1C) and the muscle-invasive subsets (Mann-Whitney U Test, non-invasive vs. invasive, *P* = 0.035, Fig. 1C) in an independent cohort of 35 primary TCCs, however no big difference in the expression levels of *UNC5* family members was observed between 2 subsets in those samples (Supplementary Fig. 1). Kaplan–Meier survival analysis showed that the high expression level of *netrin-1* is significantly correlated to a poor overall survival (OS) (*P* = 0.009, Fig. 1C).

The correlation of *netrin-1* expression to TCC was further validated in 2 independent open access datasets: the TCGA bladder cancer RNA sequencing dataset (*n* = 406) and the R2 bladder cancer microarray expression dataset (Hoglund-308-custom-ilmnht12v3, *n* = 308). In accordance with our observations, *netrin-1* expression levels were obviously higher in the high-grade as well as the invasive TCCs and predicted a poor OS in both datasets (the TCGA dataset, *P* = 0.006, Fig. 1D; Hoglund-308-custom-ilmnht12v3, *P* = 0.012, Fig. 1E). Moreover, the univariate and the multivariate cox regression analyses demonstrated that *netrin-1* expression level is independently associated with a poor outcome in our and the TCGA cohorts (Table 1). Further clinical microarray data analysis showed that there exists a clear correlation between *netrin-1* and the oncogenic signals such as Wnt/ β -catenin and apoptosis inhibition in human bladder cancer (Fig. 1F,G and Supplementary Fig. 2), indicating that *netrin-1* might be a universal biomarker of bladder cancer with the malignant behaviors.

Consistent with the previous observations, *netrin-1* expression level was markedly higher in the invasive TCC-derived T24 cells and their highly malignant subline EJ cells [18, 19], whereas J82 cells expressed *netrin-1* at extremely low level, which were in line with their growth capabilities (Supplementary Fig. 3A-D). ELISA assays further revealed that these TCC cells secrete *netrin-1* in an autocrine manner, which is in

proportion to their growth capabilities (Supplementary Fig. 3E). In contrast, *UNC5A* and *UNC5D* were consistently expressed at various levels, while *DCC*, *UNC5B* and *UNC5C* were mildly expressed or undetectable among the indicated 3 TCC cells (Supplementary Fig. 3B-C). These results indicate that a high autocrine expression of *netrin-1* might contribute to the acquisition of more malignant and aggressive behaviors of the invasive TCC through its receptors *UNC5A* and *UNC5D*.

Urinary Netrin-1 is a potential diagnostic biomarker of TCC

To ask its clinical utility, urinary *netrin-1* was measured by ELISA. Compared to normal individuals and the patients with non-tumor urologic diseases, TCC patients showed a significantly higher urinary *netrin-1* level (Fig. 2A), which were further elevated in the patients with the invasive and the high-grade tumors (Fig. 2B-C). Moreover, urinary *netrin-1* concentrations of 10 patients with the invasive TCC remarkably reduced 4 weeks after the operation relative to their preoperative ones (Fig. 2D). These results indicate the potential of urinary *netrin-1* as a diagnostic biomarker of TCC.

Netrin-1 promotes the malignant behaviors of TCC cells

Enforced expression of *netrin-1* significantly promoted cell proliferation of J82 cells (Fig. 3A and B). The migration and the invasion abilities of T24 cells were also enhanced by forced expression of *netrin-1* (Fig. 3C and D). In support of these observations, siRNA-mediated knockdown of *netrin-1* remarkably inhibited cell proliferation, migration and invasion of T24 cells (Fig. 3E-H). Additionally, the anchorage-independent growth of T24 cells and their highly malignant subline EJ cells was also attenuated by *netrin-1* silencing (Fig. 3I). Moreover, T24 cells stably expressing *netrin-1* formed a larger number of tumor spheres than the control cells under the sphere-forming conditions (Fig. 3J).

Notably, *netrin-1* overexpression induced the activating phosphorylation of Src and Akt in T24 cells (Fig. 3K). Similarly, the phosphorylation level of I κ B α , a key inhibitor of NF- κ B, was also up-regulated (Fig. 3H), which might trigger its proteolytic degradation and thereby activating NF- κ B dimer [20]. These observations were supported by the results obtained from *netrin-1*-knockdown cells (Fig. 3L).

Oridonin has a strong antitumor activity against TCC cells expressing Netrin-1

We tested the anti-tumor effectiveness of the nature small compound oridonin on the invasive TCC. As shown in Fig. 4A-C, oridonin treatment inhibited cell growth and induced cell death in a dose- and a time-dependent manner in the indicated 3 TCC cells. Additionally, the migration and the invasion abilities of T24 cells were reduced in response to oridonin. FACS analysis and MDC vital staining demonstrated that oridonin induces cell death through apoptosis and autophagy in T24 and EJ cells, whereas autophagy-mediated cell death was largely observed in J82 cells (Supplementary Fig. 4A-C). These results were supported by oridonin-mediated induction of cleavage of caspase-3 as well as poly(ADP-ribose) polymerase (PARP) and the increase in the amount of LC3-II, which act as the molecular markers of apoptosis and autophagy, respectively (Fig. 4D) [21]. In *netrin-1* highly expressing T24 and EJ cells, *netrin-*

1 was strongly down-regulated in response to oridonin in a time-dependent manner. Under these experimental conditions, oridonin exposure caused down-regulation of *UNC5A* and *UNC5D* in T24 cells, whereas the transient up-regulation of these gene expressions were detectable in oridonin-treated EJ cells, which was also observed in J82 cells with an extremely low *netrin-1* level (Fig. 4E and Supplementary Fig. 4D-E). As expected, T24 cells stably expressing *netrin-1* acquired the resistance to oridonin-induced cell death (Fig. 4F and G).

The anti-tumor effectiveness of oridonin was further confirmed in a mouse model with EJ xenografts. Compared to the control group, tumor growth in oridonin-treated mice was markedly reduced as indicated by the significant decreases in both tumor volume and average tumor weight (Fig. 4H and I). While, their average body weights did not show the significant difference (Supplementary Fig. 5A). The expression levels of *netrin-1* and *UNC5A* but not of *UNC5D* were remarkably lower in oridonin-treated tumors than in the control ones (Fig. 4J and Supplementary Fig. 5B), indicating that oridonin inhibits *netrin-1*-mediated pro-oncogenic signaling *in vivo*. Of note, *UNC5D* expression level was robustly up-regulated in 1 oridonin-treated tumor xenograft, raising a possibility that, distinct from *UNC5A*, *UNC5D* might play a role in oridonin-mediated tumor cell death in TCC.

Oridonin suppresses Netrin-1 at mRNA level

It has been reported that *netrin-1* is a direct transcriptional target of NF- κ B, whose up-regulation in response to NF- κ B activation contributes to the development and the progression of colorectal cancer [12, 22]. Indeed, *netrin-1* was up-regulated in T24 and J82 cells treated with TNF α , a classic NF- κ B inducer, in a time-dependent manner (Fig. 5A). While, TNF α -mediated stimulation of *netrin-1* was largely attenuated in the presence of PDTC, a potent NF- κ B inhibitor (Fig. 5B). Therefore, it is likely that *netrin-1* might be transcriptionally up-regulated through the activation of NF- κ B in TCC cells. Intriguingly, the reporter assay demonstrated that oridonin significantly inhibits TNF α -mediated transactivation activity of NF- κ B (Fig. 5C). In addition, p65 subunit-mediated *netrin-1* induction was suppressed by oridonin (Fig. 5D) and *netrin-1* promoter activity was down-regulated by oridonin (Fig. 5E). These results were consistent with the previous observations showing that the sequence-specific binding of NF- κ B to *netrin-1* promoter is prohibited in the presence of oridonin [23]. We have also found that the phosphorylation of Src, Akt and I κ B α was robustly inhibited in oridonin-treated T24 cells with the high *netrin-1* expression but not in oridonin-exposed J82 cells with the extremely low *netrin-1* level (Fig. 5F and Supplementary Fig. 6). These observations indicate that oridonin represses NF- κ B-mediated transcriptional up-regulation of *netrin-1*.

Meanwhile, oridonin treatment sharply shortened the half-life of *netrin-1* mRNA from approximately 12.6 hours to 1.4 hours in T24 cells (Fig. 6A), indicating that oridonin promotes a rapid degradation of *netrin-1* mRNA. Previous study has shown that, the endoribonuclease, *IRE1 α* , specifically recognizes and efficiently degrades *netrin-1* mRNA [24]. To explore the molecular mechanisms behind oridonin-mediated degradation of *netrin-1* mRNA in TCC cells, we sought to examine the possible involvement of *IRE1 α* in this phenomenon. As expected, knockdown of the endogenous *IRE1 α* increased the amount of *netrin-1* protein (Supplementary Fig. 7). Additionally, we have found that oridonin, as an effective inducer of ER-stress [25] induces the expression of *IRE1 α* and thereby reducing the amount of *netrin-1* (Fig. 6B). On the

other hand, knockdown of *IRE1a* partially attenuated the inhibitory effect of oridonin on *netrin-1* mRNA expression (Fig. 6C). These results suggest that oridonin might reduce the stability of *netrin-1* mRNA at least in part by activating RNA hydrolase *IRE1a* in TCC cells (Fig. 6D).

Discussion

More and more attention has been paid to the role of netrin-1 and its receptors in tumorigenesis and as a possible new target for tumor therapy. In this study, we have provided an evidence that *netrin-1* expression levels are tightly linked to the aggressive properties and poor outcome of TCC of the bladder. Analyses of 3 independent TCC cohorts, in which *netrin-1* expression level was examined by 3 different approaches, demonstrated that *netrin-1* is an independent indicator for the prediction of the tumor progression and poor prognosis of TCC patients. In support of these observations, *netrin-1* was highly expressed in TCC-derived cells with the high invasive potential. More importantly, urinary *netrin-1* levels were significantly elevated in patients with TCC and positively associated with the tumor invasiveness and grade, indicating that *netrin-1* might be a promising non-invasive biomarker for the diagnosis and also for the prediction of tumor progression as well as prognosis of TCC. Indeed, several recent studies have described that the evaluated urinary and serum *netrin-1* levels might act as a biomarker for monitoring the tumor progression in medulloblastoma and advanced non-small cell lung cancer [26, 27]. Although the diagnostic utility of the urinary *netrin-1* in bladder cancer should be further confirmed by much more larger sample size, our present study strongly suggests that *netrin-1* might be a potential biomarker and an attractive therapeutic target for the highly malignant bladder cancer.

Based on our present results, *netrin-1* might play a critical oncogenic role in TCC. Clinical microarray data analysis revealed that *netrin-1* correlated to Wnt / β -catenin as well as apoptosis-promoting signals in human bladder cancer, suggesting that *netrin-1* might participate in the regulation of the malignant behaviors of numerous bladder cancer cells. Our *in vitro* results showed that the increased expression level of *netrin-1* potentiates the aggressive behaviors of TCC cells through the activation of oncogenic Src, Akt and NF- κ B signaling cascades, which in turn up-regulates *netrin-1*, forming a positive feedback loop that enforces the invasive proliferation of TCC cells (Fig. 5H). Of note, ectopic expression of *netrin-1* stimulated the tumor sphere formation of T24 cells, indicating that *netrin-1* contributes to the gain of cancer cell stemness in TCC cells. Consistent with these findings, it has been reported that *netrin-1* regulates cell reprogramming as well as pluripotency maintenance [28], and thereby promoting the gain of cancer cell stemness and invasiveness in glioblastoma [29], indicative of the significance of the targeting *netrin-1* in anti-cancer therapy.

Another important finding of this study is that the natural small compound oridonin has a strong anti-tumor activity against TCC through down-regulation of *netrin-1* mRNA. These results indicate that oridonin markedly reduces *netrin-1* by suppressing NF- κ B-mediated *netrin-1* transactivation and promoting the endoribonuclease IRE1 α -dependent *netrin-1* mRNA degradation, and thereby prohibiting *netrin-1* downstream signaling to trigger apoptotic as well as autophagic cell death (Fig. 6D). Notably, the current main approach to target *netrin-1* signaling is to block the interaction between *netrin-1* and its

receptors by taking advantage of a soluble recombinant DCC ectodomain fragment [10, 12, 15] or *netrin-1*-interfering antibody [30, 31], whose effectiveness has been experimentally evaluated in several tumor cells and animal models. It is worth pointing out that our present findings uncover the novel inhibitory pharmacological effect of oridonin on mRNA production and stability of *netrin-1*.

Conclusions

In summary, our current results provided an evidence that *netrin-1* might be used as a biomarker and a potential therapeutic target for the invasive bladder cancer. Additionally, oridonin might be a novel promising therapeutic candidate for the invasive TCC as well as the other tumors with the increased *netrin-1* expression.

Declarations

Ethics approval and consent to participate

This study was approved by ethical committees of the China Medical University. Written informed consent was obtained from all patients and data was analyzed anonymously. All Animal experiments complied with the national guidelines for the care and use of laboratory animals.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Study design: MY, YZ and YL; Data collection: MY, DS, HX, JA, JY and AN; Data analysis: YL, BW, SL, FY, KO, YW and CK; Manuscript preparation: HI, MY, TM, HN and YZ. All authors read and approved the final manuscript.

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Table

Please see the supplementary files section to view the table.

Figures

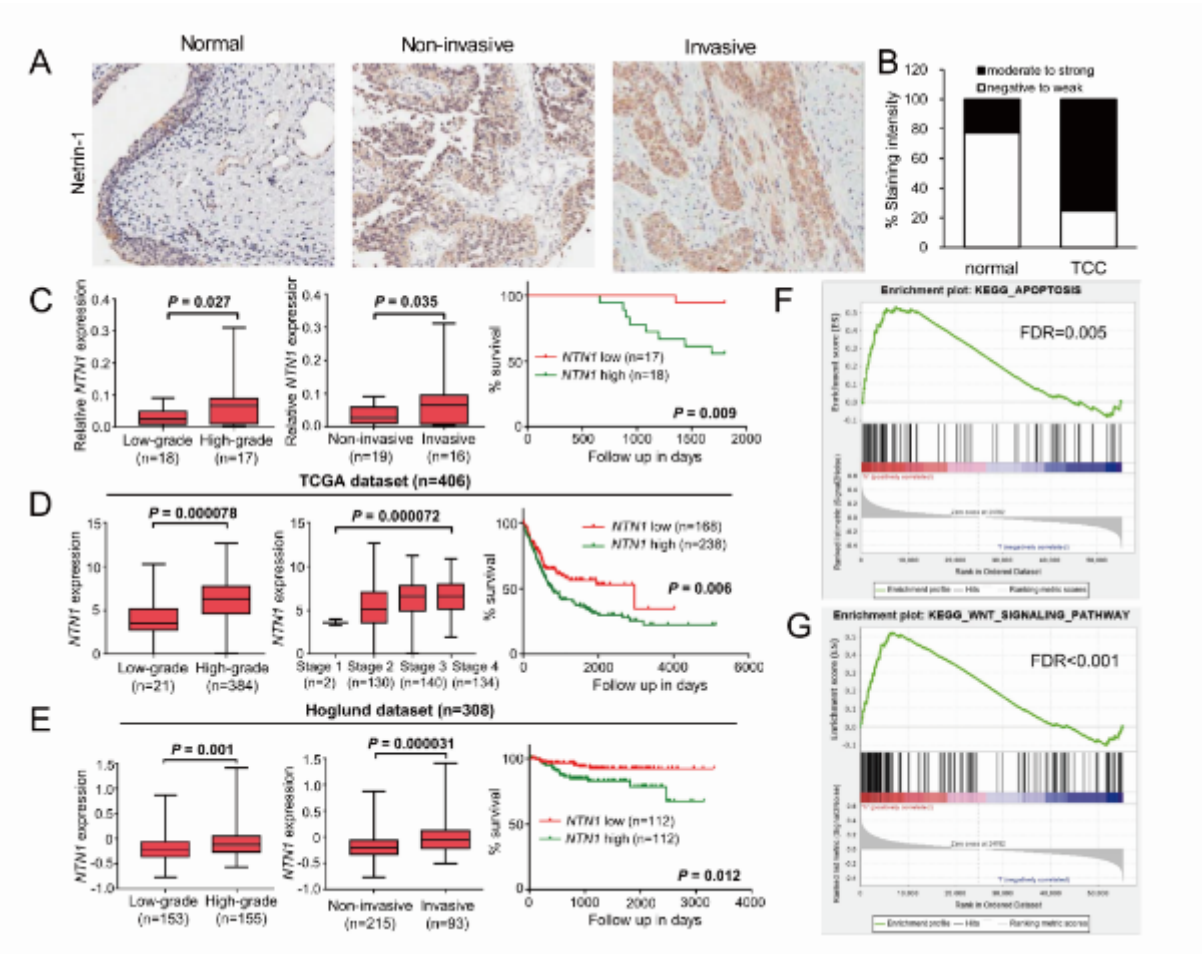


Figure 2

High expression of Netrin-1 predicts a poor prognosis of TCC. (A) Representative IHC images of netrin-1 expression in normal bladder tissues, non-invasive and invasive TCCs (400× magnification). Expression of netrin-1 was examined by IHC staining in human TCC tumor tissue array containing 65 primary TCCs and 22 paired normal tissues. (B) Netrin-1 is highly expressed in TCC tumors. Expression levels of netrin-1 in (A) were evaluated and the samples were classified into netrin-1-negative to -weak and netrin-1-moderate to -strong groups according to their scores. (C) Netrin-1 is highly expressed in high-grade and invasive TCCs and associated with poor outcome. Expression levels of netrin-1 were measured by quantitative RT-PCR in a cohort of 35 primary TCCs. Mann-Whitney U Test was used to explore the differences of netrin-1 expression levels between low-grade and high-grade (left) or non-invasive and invasive (middle) tumors. Kaplan–Meier survival curves were drawn for netrin-1-low versus netrin-1-high subgroups (right). Survival distributions were compared using the log-rank test. (D) Statistical analyses of the TCGA bladder cancer dataset (n = 406). The optimal netrin-1 expression cutoff determined in a recent study [29] was adopted in our analysis. (E) Statistical analyses of the bladder cancer dataset Hoglund-308-custom-ilmnht12v3 from the R2 platform (n = 308). Survival curves were drawn using the median expression value for netrin-1 as a cutoff. (F&G) Gene set enrichment analysis (GSEA) comparing netrin-1-high with netrin-1-low bladder tumors in the TCGA microarray data sets (n = 406), using the indicated Wnt and apoptosis gene signatures.

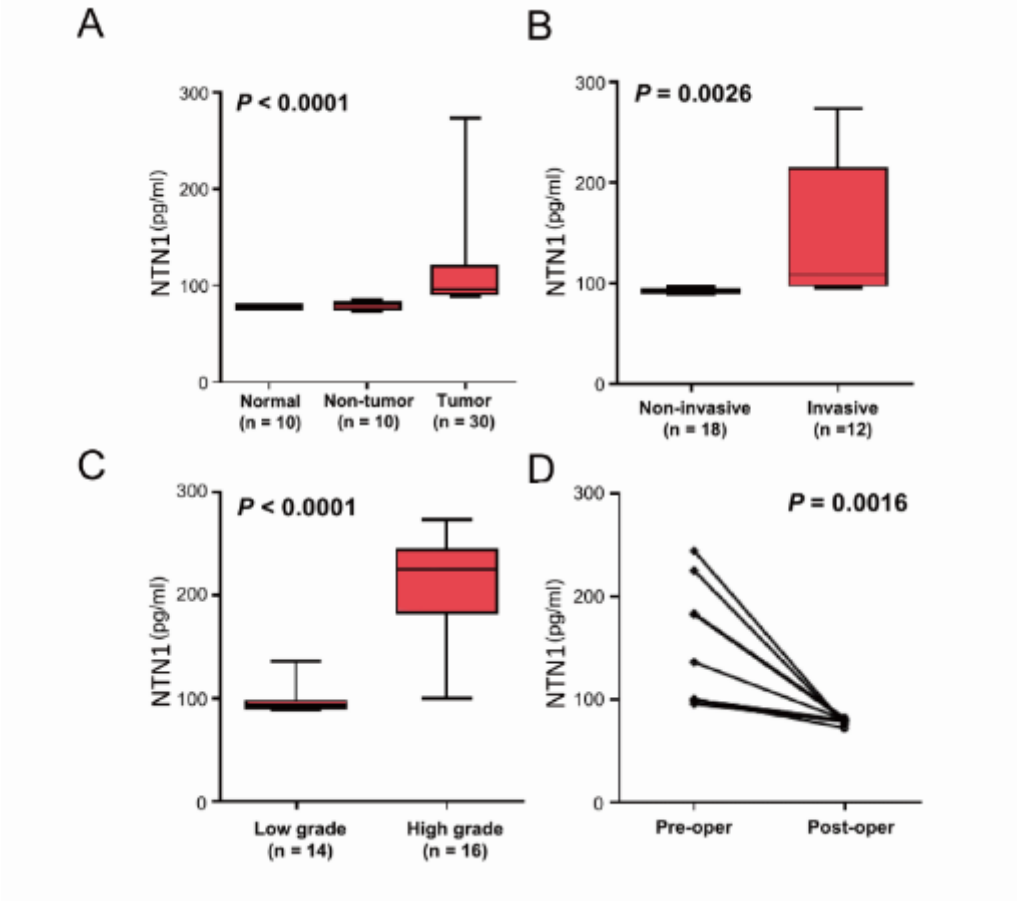


Figure 4

The potential utility of Netrin-1 as a urinary biomarker of TCC. (A) Urinary netrin-1 levels were quantified by ELISA and compared between normal controls (n = 10), age- and gender-matched non-tumor patients (n = 10) and TCC patients (n = 30). Data were shown in box plot format (median, 25–75%). (B) Comparison of urinary netrin-1 levels between non-invasive (n = 18) and invasive (n = 12) TCCs. (C) Comparison of urinary netrin-1 levels between low-grade (n = 14) and high-grade (n = 16) TCCs. (D) Comparison of preoperative (Pre-oper) and postoperative (Post-oper, 4 weeks) urinary netrin-1 levels in 10 patients with invasive TCC.

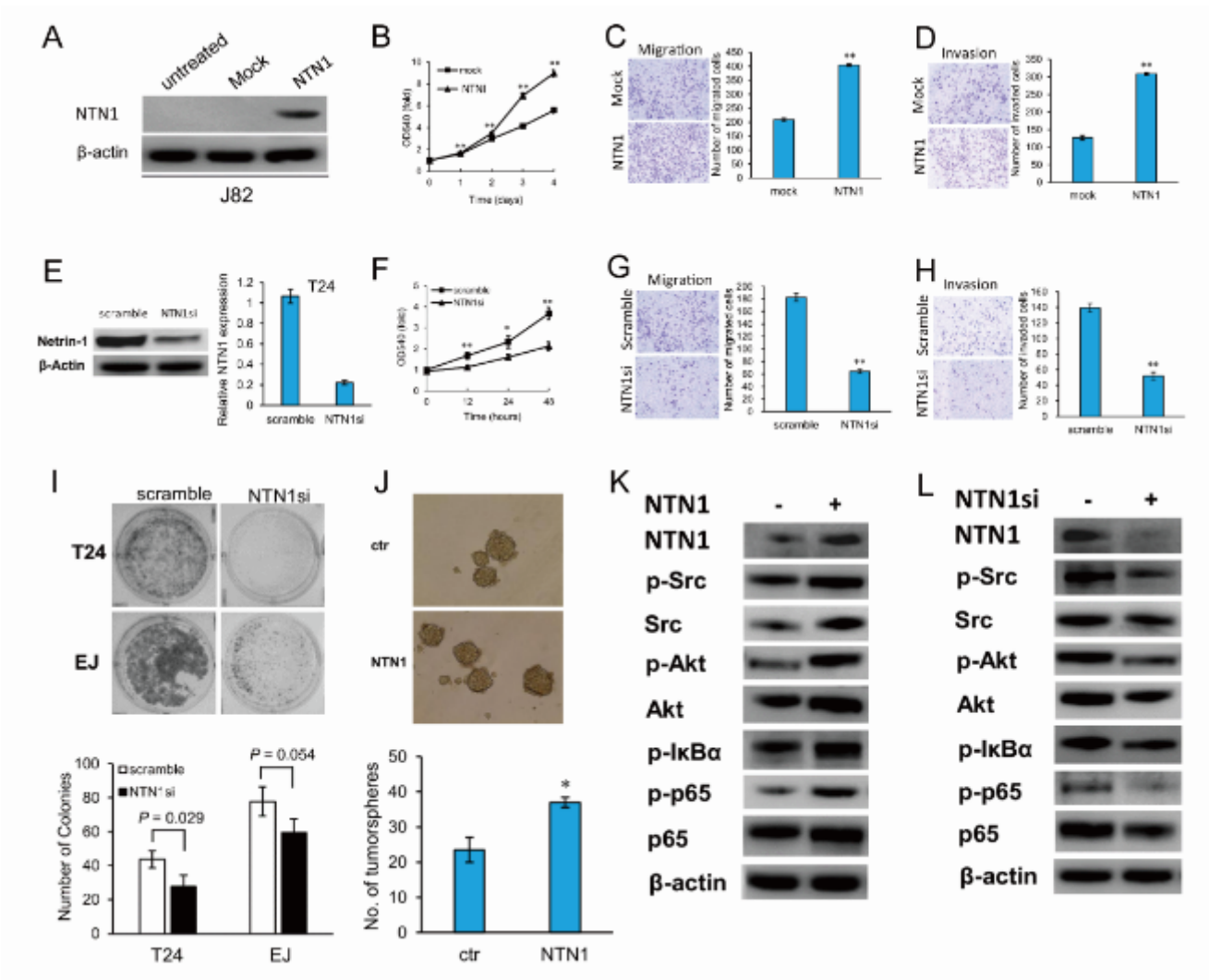


Figure 6

Netrin-1 promotes the malignant behaviors of TCC cells. (A) Western blot analysis of netrin-1 overexpressing J82 cells. (B) Cell proliferation assay of netrin-1 overexpressing J82 cells. J82 cells were transfected with mock or with netrin-1 expression plasmid. Cell proliferation assay was performed using Cell Counting Kit-8 at the indicated time points. Results were presented as Mean $\pm$ SD (n = 3). (C) Netrin-1 promotes cell migration. T24 cells were transfected with mock or with netrin-1 expression plasmid and then subjected to the transwell migration assay. Results were shown as Mean $\pm$ SD (n = 3). (D) Netrin-1 promotes cell invasion. T24 cells in (C) were subjected to cell invasion assay as described in Supplementary Materials. Results were shown as Mean $\pm$ SD (n = 3). (E&F) Cell proliferation assay of

netrin-1-knocked down T24 cells. (E) Netrin-1 knockdown efficiency. T24 cells were transfected with scrambled siRNA or with siRNAs against netrin-1 (netrin-1 si) for 48 hours and then subjected to quantitative RT-PCR and western blot analysis to confirm knockdown efficiency. (F) T24 cells transfected with scramble siRNA or with netrin-1 siRNA (netrin-1 si) were subjected to cell proliferation assay using Cell Counting Kit-8. Results were presented as Mean $\pm$ SD (n = 3). (G) Knockdown of netrin-1 inhibits cell migration. T24 cells in (E) were subjected to the transwell migration assay. Results were shown as Mean $\pm$ SD (n = 3). (H) Knockdown of netrin-1 inhibits cell invasion. T24 cells in (E) were subjected to cell invasion assay. Results were shown as Mean $\pm$ SD (n = 3). (I) Colony formation assay. T24 and EJ cells were transfected with scrambled RNA or with siRNAs targeting netrin-1 for 48 hours, and then subjected to colony formation assay as described in Supplementary Materials. Colonies with a diameter $\geq$ 100 μ m were counted. Results were presented as Mean $\pm$ SD (n=3). The plates were fixed and stained with Giemsa's solution. (J) Sphere formation assay. T24 cells stably transfected with an empty vector (ctr) or with netrin-1 expression plasmid were seeded into 6-well ultra-low attachment plates with 1 $\times$ 10⁴ cells per well in 2 ml of sphere formation medium and cultured for 10 days. Representative images of the tumor spheres were indicated (200 $\times$ magnification, left). The number of tumor spheres with a diameter $\geq$ 100 μ m were scored (right). (K & L) Western blot analysis. T24 cells were transfected with netrin-1 expression plasmid (K) or with netrin-1 siRNAs (L) along with the negative controls for 48 hours, and subjected to western blot analysis using the indicated antibodies. *, P < 0.05; **, P < 0.01.

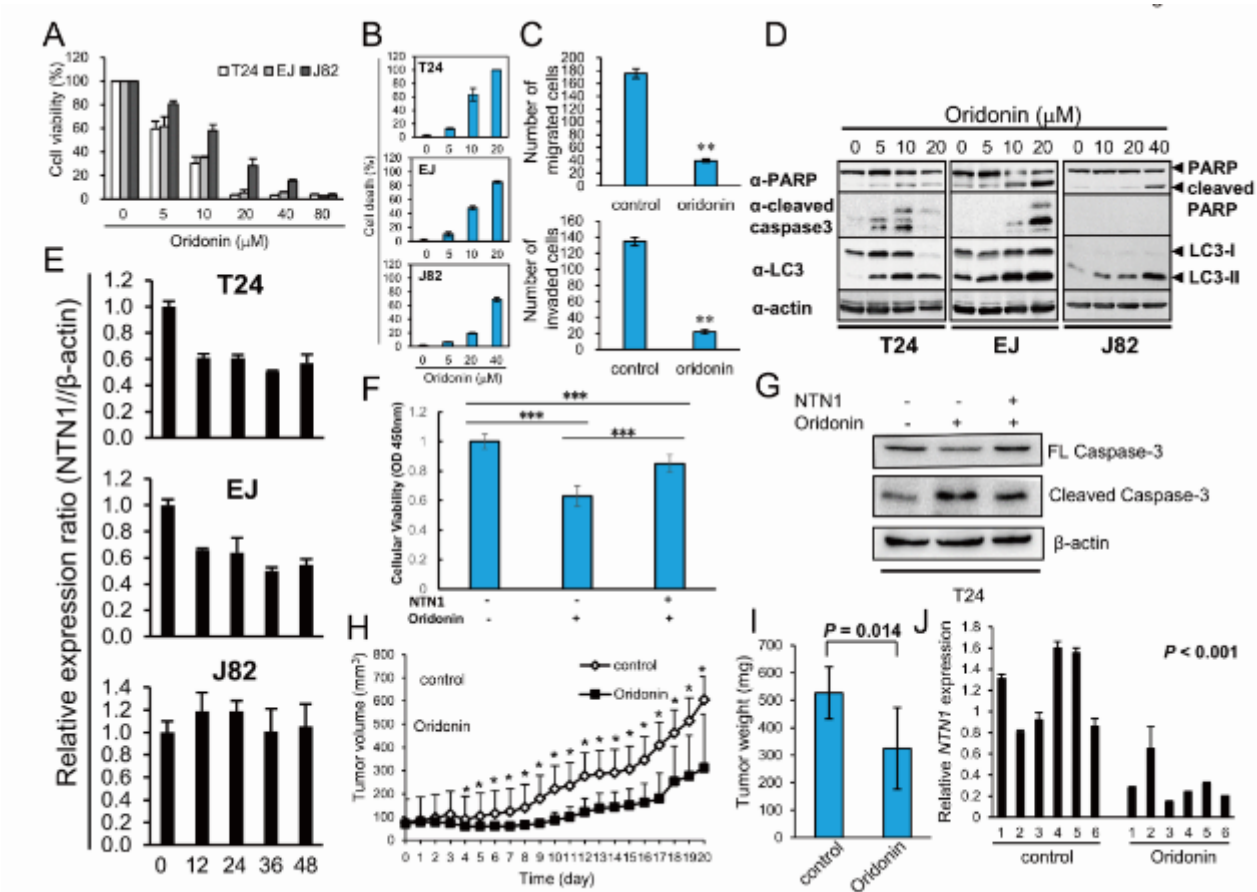


Figure 8

Oridonin inhibits cell proliferation and invasion and induces cell death of invasive TCC cells. (A). Oridonin inhibits cell proliferation of TCC. T24, EJ and J82 cells were treated with an increasing amount of oridonin for 24 hours and cell growth was measured using Cell Counting Kit-8. Results were presented as Mean $\pm$ SD (n = 3). (B). Oridonin induces cell death of TCC. T24, EJ and J82 cells were treated with oridonin at the indicated concentrations for 24 hours and cell death was evaluated by trypan blue exclusion assay. Results were presented as Mean $\pm$ SD (n = 3). (C). Oridonin reduces the migration and the invasion abilities of T24 cells. T24 cells were treated with oridonin (10 μ M) and subjected to the migration and the invasion assays as described in Supplementary Materials. Results were shown as Mean $\pm$ SD (n = 3). (D). Western blot analysis of oridonin-treated TCC cells. T24, EJ and J82 cells were treated with oridonin at the indicated concentrations for 24 hours and subjected to western blot analyses using the indicated antibodies. (E). Quantitative RT-PCR. T24, EJ and J82 cells were exposed to oridonin for the indicated periods. netrin-1 expression was measured by quantitative RT-PCR. (F&G). T24 cells stably expressing netrin-1 display the resistance to oridonin-induced cell death. T24 cells with or without netrin-1 were treated with oridonin (10 μ M) for 24 hours and cell death was checked by Cell Counting Kit-8 (F) and western blot analysis (G). (H). Images of TCC xenografts and tumor growth curve of the vehicle- or oridonin-treated mice. EJ cells were subcutaneously inoculated into the right flank of nude mice. Mice were randomly separated into two groups (n = 6 per group) and administrated with vehicle or oridonin (15 mg/kg/day) by intraperitoneal injection daily for 20 days. Tumor growth was monitored and tumor volume was estimated. (I). Comparison of tumor weight between the vehicle- and oridonin-treated mice. Xenograft tumors were excised on day 21 and weighted. Results were presented as Mean $\pm$ SD. (J). Expression levels of netrin-1 in xenograft tumors. Total RNA was isolated from the xenograft tumors and the expression level of netrin-1 was examined by quantitative RT-PCR. *, P < 0.05; **, P < 0.01.

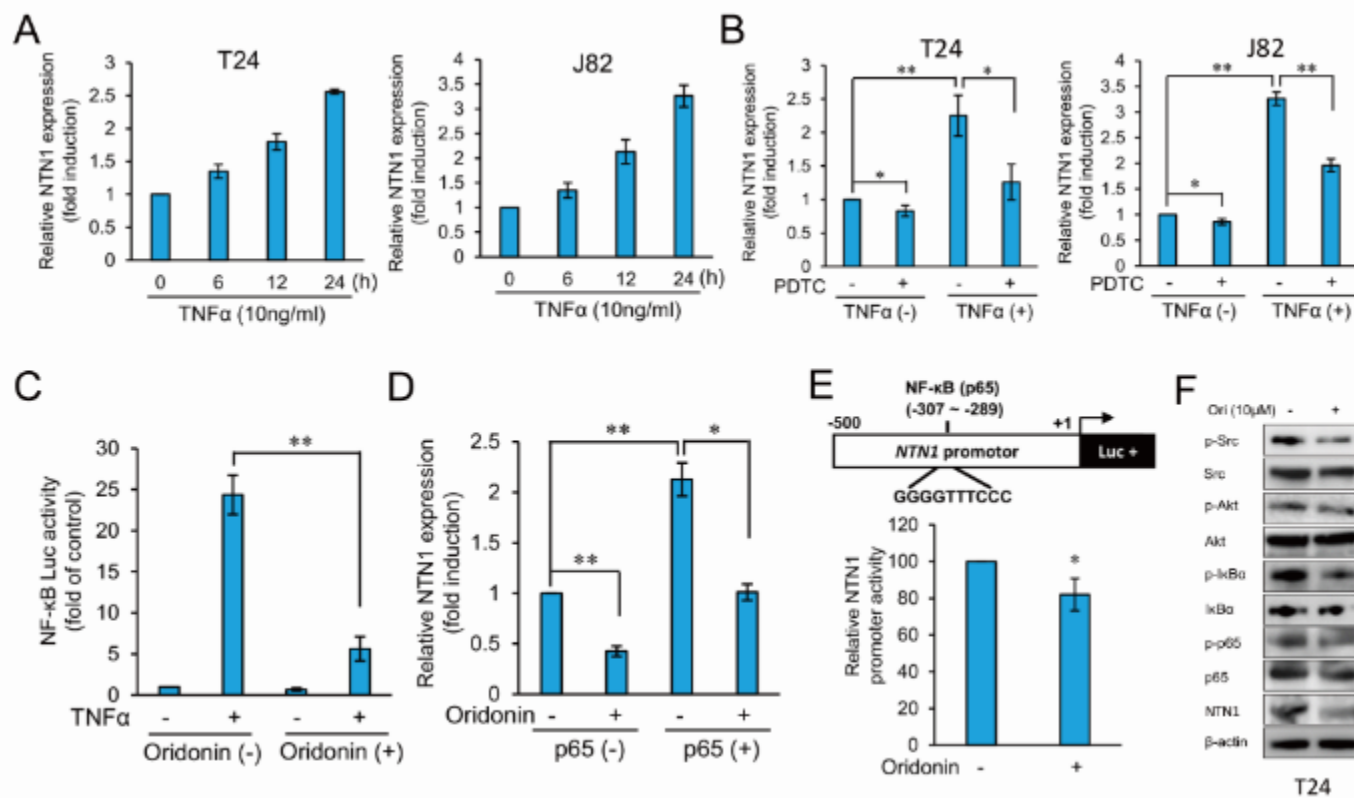


Figure 10

Oridonin suppresses NF-κB-mediated transcriptional up-regulation of netrin-1. (A). Netrin-1 expression in TNFα-treated T24 and J82 cells. Cells were serum starved for 6 hours and then treated with TNFα (10 ng/ml) for the indicated time periods. netrin-1 expression level was measured by quantitative RT-PCR. (B). TNFα-mediated activation of NF-κB up-regulates netrin-1. T24 and J82 cells were treated with or without TNFα (10 ng/ml) in the absence or presence of pyrrolidine dithiocarbamate (PDTTC, 100 μM), a potent NF-κB inhibitor, for 6 hours. Expression of netrin-1 was detected by quantitative RT-PCR. (C). Oridonin inhibits the transcriptional activity of NF-κB. T24 cells were transfected with pGL3-NF-κB vector containing three tandem NF-κB-binding sites attached to luciferase reporter gene. Cells were pretreated with or without oridonin (10 μM) for 1 hour and then treated with or without TNFα (10 ng/mL) for additional 6 hours. Luciferase activity was measured using the dual luciferase reporter assay system. Relative luciferase activity was presented as fold change in luciferase activity relative to the untreated cells. Results were presented as Mean $\pm$ SD (n = 3). (D). Oridonin inhibits p65-mediated induction of netrin-1. T24 cells were transfected with mock or with p65 expression vector for 24 hours and then treated with vehicle or with oridonin (10 μM) for additional 6 hours. netrin-1 expression was detected by quantitative RT-PCR. (E). Oridonin suppresses netrin-1 promoter activity. Luciferase reporter vector carrying a 500-bp fragment of human netrin-1 promoter that encompasses the putative NF-κB-binding motif was shown (top). T24 cells were transfected with the above construct for 36 hours and treated with or without oridonin (10 μM) for additional 6 hours. Relative luciferase activity was calculated as fold change over that of the untreated cells. Results were expressed as Mean $\pm$ SD (n = 3). (F). Western blot analysis of oridonin-treated T24

cells. T24 cells were treated with or without oridonin (10 μ M) for 24 hours and subjected to western blot analysis with the indicated antibodies.

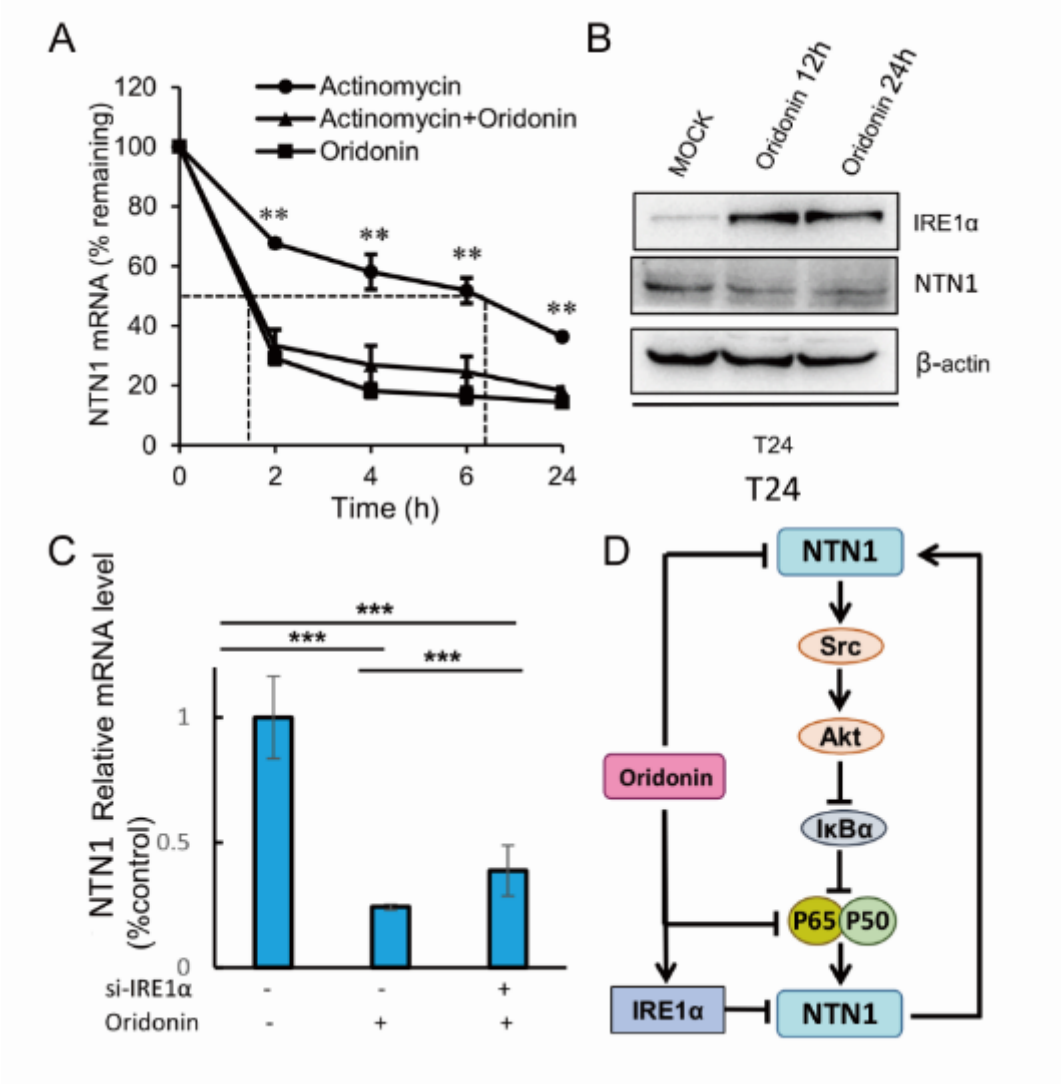


Figure 12

Oridonin reduces the stability of netrin-1 mRNA.(A). T24 cells were treated with oridonin (10 μ M), actinomycin D (10 mg/ml) or both for the indicated time periods. Netrin-1 mRNA was quantified by quantitative RT-PCR. (B). Oridonin efficiently increases the expression level of IRE1 α , and thereby reduces netrin-1 expression level. T24 cells were exposed to oridonin (10 μ M) for the indicated time periods and then subjected to western blot analysis with the indicated antibodies. (C). Knockdown of IRE1 α partially attenuated the inhibitory effect of oridonin on netrin-1 mRNA expression. T24 cells transfected with scramble siRNA or with IRE1 α siRNA (IRE1 α si) were treated with oridonin (10 μ M) for 24 hours and subjected to quantitative RT-PCR. (D). Schematic representation of the molecular mechanisms underlying netrin-1-mediated proliferative signaling and the antitumor effect of oridonin targeting netrin-1 in TCC. *, P < 0.05; **, P < 0.01.

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