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## Comprehensive pan-cancer analysis reveals NTN1 as an immune infiltrate risk factor and its potential prognostic value in SKCM

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Netrin-1 (NTN1) is a laminin-related secreted protein involved in axon guidance and cell migration. Previous research has established a significant connection between NTN1 and nervous system development. In recent years, mounting evidence indicates that NTN1 also plays a crucial role in tumorigenesis and tumor progression. For instance, inhibiting Netrin-1 has been shown to suppress tumor growth and epithelial-mesenchymal transition (EMT) characteristics in endometrial cancer. To further elucidate the influence of genes on tumors, we utilized a variety of machine learning techniques and found that NTN1 is strongly linked to multiple cancer types, suggesting it as a potential therapeutic target. This study aimed to elucidate the role of NTN1 in pan-cancer using multi-omics data and explore its potential as a prognostic biomarker in SKCM. Analysis of the TCGA, GTEx, and UALCAN databases revealed significant differences in NTN1 expression at both the mRNA and protein levels. Prognostic value was evaluated through univariate Cox regression and Kaplan-Meier methods. Mutation and methylation analyses were conducted using the cBioPortal and SMART databases. We identified genes interacting with and correlated to NTN1 through STRING and GEPIA2, respectively. Subsequently, we performed GO and KEGG enrichment analyses. The results suggested that NTN1 might be involved in crucial biological processes and pathways related to cancer development and progression, including cell adhesion, axon guidance, immune response, and various signaling pathways. We then explored the correlation between NTN1 and immune infiltration as well as immunotherapy using the ESTIMATE package, TIMER2.0, TISIDB, TIDE, TIMSO, and TCIA. The relationship between NTN1 and tumor heterogeneity, stemness, DNA methyltransferases, and MMR genes was also examined. Lastly, we constructed a nomogram based on NTN1 in SKCM and investigated its association with drug sensitivity. NTN1 expression was significantly associated with tumor immune infiltration, molecular subtypes, and clinicopathological features in various cancers. Genetic analysis revealed that Deep deletions were the most common type of NTN1 alteration. Additionally, a positive correlation was observed between NTN1 CNAs and its expression levels. In most cancers, NTN1 showed positive correlations with immune and stromal scores, as well as with specific immune cell populations. Its predictive value for immunotherapy response was comparable to that of tumor mutational burden. Furthermore, NTN1 exhibited positive correlations with tumor heterogeneity, stemness, DNA methyltransferase genes, and MMR genes. In SKCM, NTN1 was identified as an independent risk factor and demonstrated potential associations with multiple drugs. NTN1 exhibits substantial clinical utility as a prognostic marker and indicator of immune response across various tumor types. This comprehensive analysis provides insights into its potential implications in pan-cancer research.

Keywords NTN1, Pan-cancer, Biomarker, Tumor microenvironment, Nomogram

#### Abbreviations

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ACC	Adrenocortical carcinoma
AUC	Area under the curve
BLCA	Bladder urothelial carcinoma
BP	Biological process
BRCA	Breast invasive carcinoma
CAFs	Cancer-associated fibroblasts
CC	Cellular component
CESC	Cervical squamous cell carcinoma and endocervical adenocarcinoma
CHOL	Cholangiocarcinoma
CNA	Copy number alteration
CNV	Copy number variants
COADREAD	Colon adenocarcinoma Colon adenocarcinoma/restum adenocarcinoma
DEI	Disease free interval
	Jumphoid noonloom diffuon large P cell lumphome
DLBC	Disease specific survival
ESC A	Esophageal carcinoma
GBM	Glioblastoma multiforme
GBMLGG	Glioma
GO	Gene ontology
GSEA	Gene set enrichment analysis
HCC	Hepatocellular carcinoma
HNSC	Head and neck squamous cell carcinoma
HRD	Homologous recombination deficiency
IC50	Half-maximal inhibitory concentration
ICB	Immune checkpoint blockade
IPS	Immune phenotype scores
KEGG	Kyoto encyclopedia of genes and genomes
KICH	Kidney chromophobe
KIPAN	Pan-kidney cohort (KICH + KIRC + KIRP)
KIRC	Kidney renal clear cell carcinoma
KM	Kaplan–Meier
LAML	Acute myeloid leukemia
LGG	Brain lower grade glioma
LIHC	Liver hepatocellular carcinoma
	Loss of heterozygosity
LUAD	Lung adienocarcinoma
LUSC МАТН	Mutational and clonal intratumoral batarogeneity
MDSC	Mutational and cional initiatumoral neterogeneity
MESO	Mesothelioma
ME	Molecular function
MMR	Mismatch renair
MSI	Microsatellite instability
NEO	Neoantigen load
NSCLC	Non-small cell lung cancer
OS	Overall survival
OV	Ovarian serous cystadenocarcinoma
PAAD	Pancreatic adenocarcinoma
PCPG	Pheochromocytoma and paraganglioma
PFI	Progression-free interval
PPI	Protein-protein interaction
PRAD	Prostate adenocarcinoma
READ	Rectum adenocarcinoma
ROC	Receiver operating characteristic
SARC	Sarcoma
SKUM	Skin cutaneous melanoma
	Single nucleonde variants
STAD	Stomach and econhageal carcinoma
TGCT	Testicular germ cell tumors
THCA	Thyroid carcinoma
THYM	Thymoma
TILS	Tumor-infiltrating lymphocytes
TMB	Tumor mutational burden
TME	Tumor microenvironment
TNBC	Triple-negative breast cancer
UCEC	Uterine corpus endometrial carcinoma
UCS	Uterine carcinosarcoma

#### UVM Uveal melanoma

NTN1, also known as Netrin-1, located on human chromosome 17p13.1, encodes a 604-amino acid protein<sup>1</sup>. Belonging to the Netrin family, NTN1 is crucial for nervous system development, particularly in axon guidance and cell migration<sup>2</sup>. NTN1 is a critical protein in nervous system development and a significant player in cancer biology. Its abnormal regulation can lead to various malignancies, making it a potential target for cancer diagnosis, prognosis, and therapy<sup>3</sup>.

NTN1 plays a crucial role in various tumors by influencing multiple aspects of cancer progression<sup>4–10</sup>. In breast cancer, high NTN1 expression is linked to increased proliferation, migration, and invasion of cancer cells. This promotion of survival and growth is mediated through interactions with its receptors, activating signaling pathways such as PI3K/Akt and MAPK/ERK<sup>4</sup>. In colorectal cancer, high NTN1 levels activate the ERK and PI3K/Akt pathways, enhancing the immunosuppressive activity of MDSCs and facilitating tumor progression by effectively inhibiting T cell antitumor responses<sup>5</sup>. Similarly, in pancreatic cancer, NTN1 overexpression leads to NF-KB activation and subsequent upregulation of SENP1. Inhibition of NF-KB results in decreased SENP1 expression, indicating that NTN1 promotes pancreatic cancer cell proliferation and survival through NF-κB-mediated SENP1 upregulation<sup>6</sup>. In non-small cell lung carcinoma, high NTN1 expression correlates with tumor progression and poor prognosis. NTN1 induces EMT in NSCLC cells by downregulating E-cadherin and upregulating N-cadherin and vimentin, significantly enhancing invasion, migration, and vasculogenic mimicry capabilities<sup>7</sup>. NTN1 overexpression activates the ERK and PI3K pathways via receptor interactions, promoting tumor cell proliferation and survival in gastric cancer<sup>8</sup>. Glioma growth is facilitated by NTN1 through the NTN1-UNC5A-NF-KB axis, leading to increased cell proliferation, migration, and resistance to apoptosis<sup>9</sup>. In prostate cancer, the high expression of NTN1 and its dependence receptor UNC5B significantly enhances cancer cell proliferation, migration, and invasion, emphasizing the critical role of NTN1-UNC5B interactions in tumor progression<sup>10</sup>.

Previous studies have paid limited attention to the connection between NTN1 and tumors. Moreover, the role of NTN1 in tumor immunity and its regulation of the tumor microenvironment (TME) remains unclear. This research aimed to thoroughly examine the potential importance of NTN1 across various tumor types by integrating multi-omics data from pan-cancer studies. We conducted a detailed investigation into NTN1, exploring its expression variations, prognostic relevance, genetic mutations, methylation status, pathway enrichment, immune landscape, immunotherapy potential, tumor heterogeneity, tumor stemness, and more. Additionally, we developed a nomogram based on NTN1 expression levels specifically for SKCM and examined the correlation between NTN1 and drug sensitivity or resistance. By adopting a pan-cancer approach, we obtained extensive insights into the role of NTN1. Our results strongly indicate that NTN1 may be a valuable biomarker for tumor diagnosis, prognosis, and a promising therapeutic target. This comprehensive analysis enhances our understanding of the potential clinical significance of NTN1 in tumor management.

#### Materials and methods

#### Data collection and analysis of expression levels

We leveraged multiple databases and resources to examine the expression data of NTN1 in both normal and tumor tissues. Normal tissue expression data was obtained from the GTEx database (https://www.gtexportal.o rg/)<sup>11</sup>. Gene expression levels in tumor cell lines were obtained from the CCLE database (https://portals.broadi nstitute.org/ccle/)<sup>12</sup>. Expression data for 33 types of tumors and normal tissues were obtained from the Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/). Because of the limited number of normal tissues available in TCGA, we downloaded a harmonized pan-cancer dataset, known as the PANCAN cohort, from UCSC for further analysis (https://xenabrowser.net/)<sup>13</sup>. Samples for differential analysis were sourced from Solid Tissue Normal, Primary Solid Tumor, Primary Tumor, Normal Tissue, Primary Blood Derived Cancer-Bone Marrow, and Primary Blood Derived Cancer-Peripheral Blood. Tumor types with fewer than three tumor or normal samples were excluded from the analysis. To investigate the protein levels of NTN1, we utilized the CPTAC module of the UALCAN database (https://ualcan.path.uab.edu/index.html)<sup>14</sup>. Additionally, we acquired immunohistochemically stained tissue section images of NTN1 in various tumors from the HPA database (https://www.proteinatlas.org/)<sup>15</sup>, using the antibody HPA056419. We collected information on antibody staining, intensity, and quantity for subsequent analysis.

#### Diagnostic and prognostic value and clinical correlation analyses of NTN1

To evaluate the diagnostic value of NTN1 in pan-cancer, we constructed receiver operating characteristic (ROC) curves using combined cohorts from the TCGA and GTEx datasets, with the standard of area under receiver operating curve (AUC) > 0.7. By integrating the expression data of NTN1 with relevant prognostic information, including overall survival (OS), disease-specific survival (DSS), disease-free interval (DFI), and progression-free interval (PFI), we conducted Cox proportional hazards regression analysis using the coxph function from the "survival" package. To determine the optimal cut-off value for NTN1 in specific tumor types, we employed the "maxstat" package, setting the minimum sample number in each group to be more than 25% and the maximum sample number to be less than 75%. Based on this cut-off value, patients were divided into two groups, and Kaplan–Meier (KM) curves were generated for prognostic analysis. We also utilized ROC curves to evaluate the performance of NTN1 in predicting survival probabilities at 1-, 3-, and 5-year intervals. Furthermore, we explored the correlation between NTN1 expression and clinicopathological parameters, such as gender, grade, stage, T stage, lymph node metastasis, and lymphatic invasion. In the SKCM cohort, we conducted prognostic stratification analyses based on clinical parameters such as age, gender, lymph node status (N), distant metastasis (M), and stage to investigate the value of NTN1 in SKCM. Univariate and multivariate Cox regression analyses were performed to assess the independent prognostic value of NTN1 in SKCM. Additionally, we developed a

nomogram incorporating NTN1 expression levels and clinical parameters to predict 1-, 3-, and 5-year survival probabilities. The predictive accuracy of the nomogram was evaluated using calibration curves.

#### Mutation and methylation status of NTN1

Mutations in the NTN1 gene were examined utilizing the cBioPortal databas e (https://www.cbioportal.org/)<sup>16</sup>. We analyzed the frequency and variety of genomic alterations in NTN1 across different tumor types and compared the mutation counts among these tumors. Furthermore, we explored the relationship between NTN1 expression and copy-number alterations, determining the correlation using Spearman or Pearson tests. Furthermore, we utilized the "Mutations" module in the cBioPortal database to identify and display the mutation sites and types in the NTN1 gene. To visualize the structure of the NTN1 protein, we obtained a 3D view of the protein (PDB ID: 40VE) from the Protein Data Bank (http://www.rcsb.org/)<sup>17</sup>. The ten genes exhibiting the highest mutation rates in the NTN1 mutated group, as opposed to the NTN1 wildtype group, were identified and presented. We employed the SMART database (http://www.bioinfo-zs.com/smartapp/) to investigate the connection between NTN1 and its methylation levels<sup>18</sup>. With methylation data, we assessed the variations in methylation levels at individual sites between normal and tumor tissues. Furthermore, we explored the link between NTN1 expression and 10 distinct methylation sites. CNV and methylation analysis of NTN1 was analyzed by GSCALite (http://b ioinfo.life.hust.edu.cn/web/GSCALite/), an open algorithm for integrating genomic and immunogenomic data of 16 cancer types from TCGA, and normal tissue data from GTEx<sup>19</sup>

#### Functional enrichment and PPI network analysis of NTN1 in pan-cancers

STRING (https://cn.string-db.org/) online tool was used to conduct the protein–protein interaction (PPI) network analysis of NTN1<sup>20</sup>. Additionally, we utilized the "Gene\_Corr" module of the TIMER2.0 database (http: //timer.cistrome.org/) to explore the relationship between NTN1 and the aforementioned 10 genes across various tumors<sup>21</sup>. The correlation and p-values were adjusted based on tumor purity, and the results were visualized using heatmaps. We used the "clusterProfiler" package to gene ontology (GO) (http://www.geneontology.org) analysis (biological process, cellular component, and molecular function), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis (https://cn.string-db.org/) and Reactome pathways (https://rea ctome.org), focusing on genes that interact with or are associated with NTN1<sup>22</sup>. Additionally, we conducted GO, KEGG, and gene set enrichment analysis (GSEA) related to NTN1 to identify the functional modules implicated in SKCM.

### Examination of the immune environment, tumor heterogeneity, stemness, MMRs, and DNMTs

To comprehensively understand the relationship between the immune landscape and NTN1 across various tumors, we analyzed immune-related scores, immune cell infiltration, immunomodulators, and tumor immunophenotypes. Using the "ESTIMATE" package, we assessed the immune, stromal, and estimate scores for different tumor patients. Additionally, to determine the correlation between NTN1 and specific immune cell populations, we employed the TIMER2.0 webserver, which incorporates multiple algorithms such as TIMER, CIBERSORT, quanTIseq, xCell, MCP-counter, and EPIC to estimate immune cell infiltration. Moreover, we employed the TISIDB webserver (http://cis.hku.hk/TISIDB/) to investigate how NTN1 expression correlates with diverse immune factors, including tumor-infiltrating lymphocytes (TILs), immunoinhibitors, immunostimulators, MHC molecules, chemokines, and receptors<sup>23</sup>. Through investigating these relationships, we obtained a deeper understanding of how NTN1 expression interacts with the immune system. Furthermore, we computed the tumor mutational burden (TMB) and measures of mutational and clonal intratumoral heterogeneity (MATH) for all patients using the "TMB" and "inferHeterogeneity" functions within the "maftools" package. We also extracted additional parameters related to tumor heterogeneity, such as microsatellite instability (MSI), neoantigen load (NEO), tumor purity, ploidy, homologous recombination deficiency (HRD), and loss of heterozygosity (LOH), from prior investigations<sup>24,25</sup>. Additionally, we obtained stemness-related scores from a previous study. These scores included RNAss and EREG.EXPss, which are based on gene expression, as well as DNAss, DMPss, ENHss, and EREG-METHss, which are derived from DNA methylation signatures<sup>26</sup>. These scores offered valuable insights into the stemness traits of tumor samples. Finally, we used the TIMER2.0 database to examine the correlation between NTN1 expression and five mismatch repair (MMR) genes as well as four DNA methyltransferases, adjusting the correlation analyses for tumor purity.

#### Role of NTN1 in immunotherapy cohorts

The TIDE database (http://tide.dfci.harvard.edu/) serves as an online tool that assesses the likelihood of tumor immune evasion by analyzing gene expression data<sup>27,28</sup>. In our research, we employed the "Biomarker Evaluation" module to compare the biomarker value of NTN1 with other established biomarkers, examining their predictive power for response outcomes in various immune checkpoint blockade (ICB) therapy cohorts. Additionally, we used the TIDE database's "Regulator Prioritization" module to investigate the relationship between NTN1 and T cell dysfunction, as well as its impact on immunotherapy across different cohorts. This analysis offered valuable insights into the potential role of NTN1 in immune regulation and its significance for immunotherapy outcomes. The TIMSO database (http://tismo.cistrome.org/) features an extensive compilation of 1518 samples derived from mouse models, encompassing 68 cell lines and 19 tumor types, particularly emphasizing immune checkpoint blockade (ICB) studies<sup>29</sup>. We analyzed the expression levels of NTN1 both before and after ICB treatment, comparing responders with non-responders across various tumor models. Additionally, we investigated the alterations in NTN1 expression before and after cytokine treatment in different cell lines. Furthermore, we used the TCIA database (http://tis.di/) to examine the correlation between NTN1

expression and immune phenotype scores (IPS), focusing on genotype-immunophenotype relationships and the prediction of responses to ICB therapy<sup>30</sup>.

#### Potential therapeutic agents targeting NTN1 in SKCM

Drug response information was sourced from three databases: GDSC (https://www.cancerrxgene.org/), CTRP (https://portals.broadinstitute.org/ctrp/), and PRISM (https://depmap.org/portal/prism/)<sup>31-33</sup>. To examine the connection between NTN1 expression and the half-maximal inhibitory concentration (IC50) of potential drugs, we conducted an analysis across eight SKCM cohorts. We identified the top 10 drugs that exhibited either positive or negative correlations with NTN1 and presented the findings using heatmaps.

#### **Statistical analysis**

We normalized gene expression data using log2 transformation. Comparisons between normal and cancer tissues were evaluated using t-tests. For survival analysis, Kaplan–Meier curves, Cox proportional hazards models, and log-rank tests were applied. Spearman's or Pearson's tests assessed correlations, with statistical significance set at p < 0.05. All statistical analyses were performed using R software (Version 4.2.1).

#### Results

#### Expression levels of NTN1 in pan-cancer

Initially, we conducted an analysis of NTN1 mRNA and protein levels in various tumor types to gain preliminary insights into its role in carcinogenesis.

The CCLE database reveals significant differences in NTN1 expression across various cancer cell lines. In some cell lines, NTN1 expression levels are relatively high, particularly in SARC, LUSC, KIRC, and LUAD. Conversely, NTN1 expression levels are relatively low in certain cell lines, such as BLC, SKCM, ALL, LCL, THCA, PRAD, CESC, and CLL, where the median expression levels are relatively lower. In cell lines such as LAML, MESO, ESCA, UCEC, PAAD, STAD, HNSC, and LGG, NTN1 expression levels are intermediate (Fig. 1a).

The upregulated NTN1 mRNA expression was observed consistently in tumor tissues versus paired normal tissues in the KICH, SKCM and LUSC, using the data directly from the TCGA dataset. While, downregulated mRNA expression of NTN1 was also observed in BLCA, BRCA, COAD, KIRC, KIRP, LUAD, PRAD, STAD and THCA. (Fig. 1b).

Comparative analysis of normal and tumor tissues in the TCGA database demonstrated that NTN1 was significantly downregulated in most tumors, except for GBM, GBMLGG, LGG, LUSC, SKCM and KICH, where it was upregulated (Fig. 1c).

To investigate the expression of NTN1 in human cancers better, we integrated normal tissue data from the TCGA and GTEx databases with tumor tissue data from the TCGA database, the results indicate that it is significantly upregulated in GBM, GBMLGG, LGG, LUSC, OV, PAAD, ALL, LAML, and KICH, while it is significantly downregulated in UCEC, BRCA, CESC, LUAD, ESCA, STES, KIRP, KIPAN, COAD, COADREAD, PRAD, STAD, HNSC, KIRC, WT, SKCM, BLCA, THCA, READ, TGCT, UCS, and CHOL (Fig. 1d).

We obtained immunohistochemical figures from the HPA database, displaying pan-cancer protein expression patterns to further investigate NTN1 protein levels (Fig. S1a). The analysis indicated that NTN1 protein was moderately to expressed in over 60% of samples across different tumor types (Fig. S1b).

Moreover, the CPTAC data base compared protein levels between normal and tumor tissues, revealing higher NTN1 protein expression in GBM, while lower expression was observed in BRCA, COAD, OV, KIRC, HNSC (Fig. 1e).

#### Pan-cancer analysis of the diagnostic and prognostic value of NTN1

To estimate the diagnostic value of NTN1, we conducted the receiver operating characteristic (ROC) analysis in pan-cancer based on the dataset from TCGA and GTEx. NTN1 had a certain accuracy (AUC>0.700) in predicting at least these 13 cancers: READ (AUC : 0.956), COAD (AUC : 0.947), THCA (AUC : 0.892), BLCA (AUC : 0.884), ESCA (AUC : 0.859), KIRP (AUC : 0.842), KIRC (AUC : 0.805), PRAD (AUC : 0.767), UCS (AUC : 0.763), SKCM (AUC : 0.756), TGCT (AUC : 0.737), STAD (AUC : 0.727), CESC (AUC : 0.719), highlighting excellent diagnostic potential (Fig. 2).

We retrieved survival data from the TCGA database and performed univariate Cox regression and KM analyses. OS analysis revealed that NTN1 was identified as a risk factor in multiple tumor types, including STAD, THCA, SKCM, UVM and BLCA, while it acted as a protective factor for CESC, KIPAN, HNSC and KIRC patients (Fig. 3a). KM plots displayed consistent results for OS, and NTN1 was found to be a risk factor for OV, KIRP, STES and MESO, while being a protective factor for ACC and THYM (Fig. S2a). Besides, NTN1 was negatively associated with DSS in STES, KIRP, STAD, COAD, MESO, SKCM and UVM, while was positive in HNSC, KIRC and THYM (Fig. 3b). KM analyses confirmed these findings, furthermore, NTN1 serves as a risk factor in BLCA, COADREAD, and OV, while acting as a protective factor in ACC, LUSC, and SARC, which correlates with prolonged survival periods (Fig. S2b). Furthermore, we evaluated the relationship between NTN1 and DFI. low NTN1 expression was associated with worse survival outcomes in SKCM and CHOL (Fig. 3c). Kaplan–Meier analyses validated these findings. In CHOL, KIRP, PAAD, PRAD, and TGCT, high expression of NTN1 was significantly correlated with longer disease-free intervals (DFI). Conversely, in LUAD, high expression of NTN1 was associated with shorter DFIs (Fig. S3a).

Similarly, in terms of PFI, NTN1 was a significant risk factor for LGG, STES, TGCT, UVM, and BLCA, while it was a significant protective factor in KIPAN, HNSC, KIRC, and THYM (Fig. 3d). Additionally, NTN1 was found to be a detrimental factor in TGCT and THYM, while it serves as a risk factor in BLCA, COAD, COADREAD, HNSC, KIRC, KIRC, KIRP, LGG, PRAD, SARC, SKCM, STES, UCEC, UCS, and UVM (Fig. S3b). Finally, we assessed the ability of NTN1 to predict the 1-, 3-, and 5-year survival rates of tumor patients using ROC analysis. The



**Fig. 1.** Expression levels of NTN1 in pan-cancer. (a) NTN1 expression in tumors using the CCLE database. (b) NTN1 mRNA expression across The Cancer Genome Atlas (TCGA) tumor tissues compared with paired normal tissues showed tumor-specific expression (blue point: normal, yellow point: tumor). (c) Comparison of NTN1 mRNA expression between tumor tissues in TCGA and normal tissues in TCGA and GTEx databases (red organ, tumor; blue organ, normal). (d) Difference of NTN1 mRNA expression between tumor tissues and normal tissues in TCGA (red organ, tumor; blue organ, normal). (e) NTN1 protein levels between normal and tumor tissues using the UALCAN database (red organ, tumor; purple organ, normal). \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001; \*\*\**P*<0.001; ns or -, not significant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

results indicated that in KIRP, LGG, MESO, OV, SKCM, STAD, THCA and BLCA, the AUC values were greater than 0.5, suggesting that NTN1 has some value in predicting patient survival (Fig. S4).

#### NTN1 expression across immune, molecular subtypes, and clinical features

Identifying tumor subtypes is essential for assessing prognosis and guiding personalized precision therapy. In this study, we examined the relationships between NTN1 expression and immune and molecular subtypes in



#### Fig. 2. (a) Diagnostic value of NTN1.

various tumors. The analysis demonstrates that NTN1 expression varies significantly across different subtypes of BRCA, ESCA, HNSC, KIRP, LGG, OV, PCPG, SKCM, STAD, and UCEC, highlighting its potential role in the molecular and clinical heterogeneity of these cancers, for example, in BRCA, the Basal subtype exhibits the highest NTN1 expression and the HM-Indel subtype has the highest NTN1 expression in ESCA (Fig. S5a). Furthermore, NTN1 expression has significant differential distribution across immune subtypes in BLCA, CESC, COAD, GBM, HNSC, KIRC, LGG, LUAD, LUSC, PCPG, PRAD, READ, SARC, SKCM, STAD, TGCT, UCEC, and UCS, with specific subtypes in each cancer type exhibiting notably higher or lower expression levels (Fig. S5b). NTN1 expression is highest in the C2 subtype for BLCA, CESC, PCPG, and STAD, and in the C4 subtype for COAD, GBM, HNSC, LUAD, LUSC, READ, TGCT, UCEC, and UCS, suggesting that these high-expression subtypes are associated with poorer prognosis.

Furthermore, we examined the association between NTN1 expression and clinicopathological parameters. We observed elevated NTN1 expression in females with BLCA and LUAD, while in males with HNSC, LUAD, LUSC, and MESO (Fig. S6a). Moreover, NTN1 expression significantly varies across different histological grades in five cancer types, with higher expression in G2 compared to G1 in ESCC, G2 and G3 compared to G1 in HNSC, G3 compared to G1 in OSCC, and lower expression in G3 compared to G1 and G2 in UCEC (Fig. S6b). In three cancer types, NTN1 expression varies significantly across tumor stages, being higher in T2 than T1 in BLCA, in T2 and T3 compared to T1 in SKCM, and in T2, T3, and T4 compared to T1 in STAD (Fig. S6c). In COAD, COADREAD and KIRP patients, NTN1 expression was higher in cases with lymph node metastasis (Fig. S6d). We found that NTN1 expression is significantly higher in M1 compared to M0 in TGCT (Fig. S6e). Regarding tumor stage, NTN1 expression demonstrated an increasing trend with advanced stages in BLCA, COAD, and STAD, while the opposite trend was observed in UCEC (Fig. S6f.). Meanwhile, we found that NTN1 expression is significantly invasion compared to those without in COAD and COADREAD, and in cases with less than 50% tumor invasion compared to those with 50% or more in UCEC (Fig. S6g).

#### Mutation and methylation status of NTN1

We examined the mutation status of NTN1 using the cBioPortal database, leveraging the TCGA pan-cancer datasets. Analysis revealed that UCS and UCEC have the highest alteration rates, with significant contributions from structural variants, mutations, amplifications, and deep deletions, while other cancers such as COAD, SKCM, and SKCM show moderate alteration frequencies primarily due to mutations and structural variants (Fig. 4a). The NTN1 protein's 3D structure was displayed (Fig. 4b). Then, the types and sites of the NTN1 SNVs alterations are further presented in Fig. 4c. The deep deletion of NTN1 was the main type of genetic alteration, marked in blue. These mutations are predominantly located in the Laminin\_N domain and the





Laminin domains. A specific notable mutation, P459T, is found in the NTR domain (Fig. 4c). Subsequently, we investigated the correlation between mutation count, copy number alteration (CNA), and gene expression of NTN1 across pan-cancer datasets. UCEC exhibits the highest mutation count for NTN1, primarily featuring missense mutations, structural variants, amplifications, and both shallow and deep deletions, while ACC had the lowest (Fig. 4d). There was a significant positive correlation between CNA and NTN1 expression across multiple tumor types (Fig. 4e). Additionally, NTN1 mRNA expression levels were significantly higher in cases with gene amplifications and gains, and lower in cases with deep and shallow deletions (Fig. 4f), indicating a strong correlation between copy-number alterations and NTN1 expression. Besides, compared to the unaltered group, the NTN1-altered group had higher mutation frequencies in TP53, PIK3R6, PIK3R5, DNAH9, MYH10, DNAH2, MYH2, MFSD6L, SPDYE4 and CCDC42 (Fig. 4g). Moreover, we examined the single nucleotide



**Fig. 4.** NTN1 Mutation analysis in various tumors. (**a**) Genetic alteration frequency of NTN1 in pan cancer (green, Mutation; purple, structural variant; red, amplification; blue, deep deletion; gray, multiple alterations). (**b**) The 3D view of the NTN1 protein. (**c**) Mutation sites of NTN1. (**d**) Mutation counts of NTN1. (**e**) The correlation of NTN1 expression and copy-number values. (**f**) NTN1 expression difference among different copy-number alterations. (**g**) The top ten genes with significant difference in mutation rate between NTN1 altered and unaltered groups (red, altered groups; bule, unaltered groups). (**h**) The waterfall plot showed the mutation profile of NTN1 and its related genes across different cancer types. The tumor mutation burden is marked on the top of the plot. The names of cancer types are located on the right side of the bottom, with different mutation types on the left side. The names of genes are marked on the left. The stacked column chart on the right represents the frequency of SNVs mutation types, with different colors indicating various SNVs mutation types. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

polymorphisms (SNVs) mutations of these genes across pan-cancer, including the missense mutation, frame shift del, nonsense mutation, splice site, in frame del, frame shift ins, in frame ins, translation start site and multi hit. The information of nucleotide variants was combined with 33 cancer types, including ACC, BLCA, BRCA, CESC, CHOL, COAD, DLBC, ESCA, GBM, HNSC, KICH, KIRC, KIRP, LAML, LGG, SKCM, LUAD, LUSC, MESO, OV, PAAD, PCPG, PRAD, READ, SARC, SKCM, STAD, TGCT, THCA, THYM, UCEC, UCS, UVM. The results revealed that the top 5 mutated genes were TP53 (76%), DNAH9 (16%), DNAH2 (11%), MYH2 (10%), and MYH10 (5%). Among these, the most frequent type of mutation was missense mutation (dark blue symbol). Additionally, the cumulative SNV frequency exceeded half in the following cancer types: GBM, HNSC, KIRP, and LUSC (Fig. 4h).

We obtained data from the SMART database and conducted a comparative analysis between normal and tumor tissues to assess the methylation status of NTN1 in pan-cancer. In ACC, BLCA, CHOL, KIRP, SKCM, LUAD, LUSC, PAAD, and THCA, the methylation level of the NTN1 was significantly higher in tumor tissues, while in KICH, LAML, OV, SKCM, and TGCT, it was lower (Fig. S7a). Additionally, an analysis was conducted on 10 methylation sites, uncovering variations across different tumor types. Notably, cg00284375, cg12840740, cg17133388, cg06505000, cg22956489, and cg01385253 displayed differences in more than 10 tumor types (Fig. S7b). In addition, we explored the location of these probes on the genome and their distance from the transcription start site of NTN1(Supplementary Table 1), and investigated whether CpG islands are present at these probe locations by using the MethPrimer 2.0 website (http://www.urogene.org/methprimer/) (Fig. S7c). Furthermore, we explored the relationship between NTN1 expression and methylation. The results revealed significant correlations in 24 cancer types, with positive correlations observed in COAD, DLBC, READ, and STAD, and negative correlations in ACC, BLCA, BRCA, CESC, CHOL, ESCA, HNSC, KICH, KIRP, SKCM, LUAD, LUSC, PAAD, PCPG, PRAD, SARC, SKCM, TGCT, THCA, and UCEC. In the heatmap, STAD shows significant associations with all methylation sites, exhibiting positive correlations with most, except cg14615768 and cg17072465 (Fig. S7d).

To further investigate the relationship between NTN1 and DNA methylation in pan-cancer, we used the UALCAN database to compare the promoter methylation levels of the NTN1 gene in normal and tumor tissues. The results showed that the promoter methylation level of NTN1 is higher in normal tissues for KIRC, KIRP, and UCEC, whereas it is higher in tumor tissues for BRCA, COAD, SKCM, LUAD, LUSC, PRAD, and READ (Fig. S8a). Herein, a significant increase in the CNV level of NTN1 was observed in BLCA, PRAD, HNSC, SARC, SKCM, SKCM, LUSC, STAD, PCPG, CESC, OV, COAD, ESCA, BRCA and TGCT (Fig. S8b). We also investigated the methylation level of NTN1 in pan-cancer. In PRAD, STAD, THYM, TGCT, ESCA, COAD, THCA, READ, and BRCA, methylation is negatively correlated with NTN1 expression, while in LUSC, LGG, SKCM, SKCM, LAML, PCPG, and HNSC, the opposite is observed (Fig. S8c).

#### NTN1-related networks and pathways

To elucidate the potential networks and pathways associated with NTN1, a total of the top 10 NTN1 targeted binding proteins was collected through the String online tool (Fig. 5a). Subsequently, we analyzed the correlation between the aforementioned proteins and NTN1 using the GEPIA2 database. The scatter plot analysis revealed the 5 genes (DSCAM, DRAK1, DOCK, RGMB, NEO1) that displayed a highly positive correlation with NTN1 (Fig. 5b). The heatmap demonstrated the correlation between NTN1 and these genes across all tumor types. In TGCT, these genes significantly correlate with NTN1, all positively except for DCC (Fig. 5c). Besides, we identified some co-expressed genes in the cBioPortal database for GO, KEGG, and Reactome pathway enrichment analysis. In the GO enrichment analysis, NTN1 is likely crucial for nervous system development (axonogenesis, axon development), signal transduction (neuron projection guidance, synapse organization), and cell-cell adhesion (regulation of cell-cell adhesion, adherens junction). It is also involved in immune response and cytokine receptor activity, such as in the immune response-activating signaling pathway, cytokine receptor activity, regulation of cell-cell adhesion, adherens junction, axonogenesis, and axon development (Fig. 5d). In the KEGG enrichment analysis, NTN1 is involved in signaling pathways related to cell growth, differentiation, proliferation, apoptosis, immune response, and nervous system development. These pathways include the calcium signaling pathway, Rap1 signaling pathway, MAPK signaling pathway, Hippo signaling pathway, Ras signaling pathway, and PI3K-Akt signaling pathway. Additionally, NTN1 is associated with various cancerrelated pathways, such as those in breast cancer, gastric cancer, pancreatic cancer, and hepatocellular carcinoma, indicating its potential role in cancer initiation and progression (Fig. 5e). Additionally, according to the results of the Reactome pathway analyses, NTN1 may play an important role in regulating protein synthesis, cellular metabolism, and stress response (Fig. 5f).

#### Immuno-related analysis of NTN1 in pan-cancer

The "ESTIMATE" package was employed to evaluate immune, stromal, and estimate scores across various tumors. We examined the association between these scores and NTN1 expression in pan-cancer. The results showed a significant positive correlation between these scores and NTN1 expression in LGG, COAD, PRAD, STAD, HNSC, BLCA, READ, OV, and UVM. Conversely, a significant negative correlation was observed in SARC, KIPAN, and KIRC (Fig. 6a). To further illustrate this relationship, we selected the three tumors with the highest correlation in stromal or immune scores and presented them in scatter plots. Notably, NTN1 displayed a strong positive association with stromal score in COADREAD (R=0.53), STAD (R=0.52), and BLCA (R=0.51). Similarly, it exhibited a positive correlation with immune score in COAD (R=0.36), READ (R=0.40), and BLCA (R=0.36) (Fig. 6b, c). Moreover, the TIMER2.0 database was utilized to explore the connection between NTN1 expression and immune cell infiltration. This heatmap shows that the majority of immune cells are significantly positively correlated with NTN1 in most tumors (Fig. 6d). Notably, in STAD, 20 types of immune cells have a



**Fig. 5.** (a) PPI network of NTN1 and its interacting protein performed through the String platform. (b) The top ten genes with the highest correlation with NTN1 in pan-cancer. (c) The heatmap of the relationship between NTN1 and the above ten genes in various tumors (red, positive correlation; bule, negative correlation). (d) GO analysis of NTN1. (e) KEGG analysis of NTN1. (f) The Reactome pathway analyses of NTN1. \*< 0.05, \*\*P<0.01, \*\*\*P<0.001, and ns, not significant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

significant correlation with NTN1. Among them, except for MDSC, common lymphoid progenitor, resting mast cell, and resting NK cell, all show a significant positive correlation (Fig. 6e).

Subsequently, we investigated the relationship between NTN1 and immune checkpoints, including immuinhibitors, immunostimulators, chemokines, and receptors. The results show that in many cancers, most immunostimulators are significantly positively correlated with NTN1, such as in STAD, OV, and BRCA. Conversely, in some cancers like KIRC and HNSC, certain immunostimulators are significantly negatively correlated with NTN1 (Fig. S9a). Similarly, NTN1 shows a significant positive correlation with most chemokines across various cancer types, particularly in STAD, BRCA, OV, and COAD (Fig. S9b). NTN1 is significantly positively correlated with most receptors across various cancer types, especially in STAD, PRAD, and UVM. In contrast, in cancers like KIRC and TGCT, certain receptors show a significant negative correlation



**Fig. 6.** NTN1 significantly related to immune infiltration in various cancers based on multiple algorithms. (**a**) The relationship between NTN1 and stromal, immune, and ESTIMATE scores (red, positive correlation; bule, negative correlation). (**b**) The top three tumors with the highest correlation between NTN1 and stromal score. (**c**) The top three tumors with the highest correlation between NTN1 and immune score. (**d**) The heatmap of the relationship between NTN1 and immune cells. (red, positive correlation; bule, negative correlation) (**e**) The correlations between NTN1 and immune cells in STAD. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with NTN1 (Fig. S9c). Similarly, NTN1 shows a significant positive correlation with immunoinhibitors across various cancer types (Fig. S9d).

#### Immunotherapy evaluation of NTN1

We conducted a comparative analysis of NTN1 and other published biomarkers, focusing on their predictive power for treatment response and OS. The results indicate that the majority of biomarkers exhibit high AUC values across many studies, except for TMB, T.Clonality and B.Clonality (Fig. 7a). Furthermore, we explored the association between NTN1 and immunotherapy. In the TCGA Endometrial and Patel 2017 1, NTN1 was found to be highly expressed according to the T dysfunction value in the core dataset and normalized Z score calling from selection log2FC in the CRISPR screen dataset. Conversely, in the GSE12417\_GPL570, METABRIC and ICB\_Liu2019\_PD1 Ipi\_Prog, NTN1 exhibited low expression according to T dysfunction value in the core dataset and normalized Z score calling from Cox-PH regression in the immunotherapy dataset (Fig. 7b). Moreover, we investigated the relationship between NTN1 and immunotherapy response. NTN1 demonstrated predictive capabilities for the effects of immunotherapy in 6 cohorts utilizing an in vivo tumor model (Fig. 7c), as well as for the response to cytokine treatments in 6 cohorts using in vitro cell lines (Fig. 7d). We analyzed the relationship between NTN1 and IPS scores, including ips\_CTLA4\_pos\_PD1\_pos, ips\_CTLA4\_pos\_PD1\_ neg, ips\_CTLA4\_neg\_PD1\_pos, and ips\_CTLA4\_neg\_PD1\_ neg. The results indicated a positive correlation between NTN1 and these four scores in PRAD, UCEC and KICH (Fig. 7e).

#### Association of NTN1 with tumor heterogeneity, stemness, MMRs, and DNMTs

To further elucidate the role of NTN1 in tumorigenesis, we meticulously examined its associations with tumor heterogeneity, stemness, MMRs, and DNMTs. Tumor heterogeneity, a hallmark of malignant tumors, involves diverse aspects such as growth rate, invasive potential, drug response, and prognosis, which are shaped by genomic alterations. In the majority of tumors, NTN1 shows a significant correlation with these tumor heterogeneity-related indicators, particularly with tumor purity (Fig. S10a). NTN1 demonstrated positive associations with RNA-based stemness scores (RNAss and EREG.EXPss) in the majority of tumors, while it was positively correlated with DNA-based stemness scores (DNAss, DMPss, ENHss, and EREG-METHss) in CHOL, KIPAN and KIRP (Fig. S10b). Notably, in KIPAN, NTN1 shows a significant positive correlation with both RNA-based and DNA-based stemness scores. This suggests that NTN1 may play a crucial role in the tumorigenicity and progression. Finally, we investigated the correlation between NTN1 and DNA mismatch repair genes (MLH1, MSH2, MSH6, PMS2, and EPCAM), as well as DNA methyltransferases (DNMT1, DNMT3L, DNMT3A, and DNMT3B). The heatmap analysis revealed significant positive correlations between NTN1 and these genes in most tumors, particularly in KIRC, HNSC, and other tumor types (Fig. S10c).

#### Investigating the role of NTN1 in SKCM

To evaluate the prognostic significance of NTN1 across various clinicopathological subgroups, we performed a stratified analysis. Patients were stratified based on age, gender, tumor stage, M stage and N stage. These results indicate that high expression of NTN1 is generally associated with poor prognosis, particularly in younger patients (age <= 60), male patients, patients with pathological M0 stage, and those with pathological N0&N1 stage. Additionally, patients with Stage III&IV pathological stages also show significantly worse prognosis with high NTN1 expression (Fig. 8a). Additionally, we conducted univariate and multivariate Cox regression analyses to evaluate the independent prognostic significance of NTN1 alongside clinical parameters. The findings revealed that NTN1 serves as an independent risk factor for SKCM (Fig. 8b). To enable personalized survival predictions, we constructed a nomogram that integrates NTN1 expression along with other clinical parameters to estimate 1-, 3-, and 5-year survival probabilities in SKCM patients (Fig. 8c). The calibration curves demonstrated good agreement between the predicted and observed OS, validating the reliability of the nomogram (Fig. 8d).

We further explored the possible pathways by which NTN1 might be implicated in SKCM through overrepresentation analysis and GSEA. The over representation analysis revealed that NTN1 was primarily associated with developmental process or anatomical structure development, as well as RNA or protein binding (Fig. 9a). Additionally, the KEGG pathway analysis identified several signaling pathways, including calcium signaling pathway and CAMs, malignant tumors, metabolic diseases, and metabolic pathways, in which NTN1 may play a role in SKCM (Fig. 9b). Furthermore, GSEA analysis based on GO, KEGG, and HALLMARK pathways indicated NTN1 was positively correlated with categories such as "Keratinization," "Keratinocyte differentiation" and "Extracellular matrix assembly", while it showed negative associations with various cell cycle-related pathways (Fig. 9c). The KEGG analysis revealed positive associations of NTN1 with pathways related to tumorigenesis, tumor progression, certain metabolic pathways in SKCM (Fig. 9d). Consistently, the GSEA hallmark analysis further highlighted the strong correlation between NTN1 and pathways such as Wnt beta catenin signaling, UV response down, Apical surface in SKCM (Fig. 9e).

To investigate the relationship between NTN1 expression and drug resistance in SKCM, we performed correlation analyses using NTN1 expression and three drug-gene databases (GDSC, CTRP, and PRISM) across sixteen independent SKCM cohorts. The results indicate a consistent positive correlation between NTN1 expression and resistance to multiple drugs across different datasets, such as PLX-4720, Dabrafenib, Obatoclax Mesylate, Selumetinib, and AZD1332. indicating that high NTN1 expression may indicate drug resistance in SKCM (Fig. 9f).

#### Discussion

NTN1, a member of the laminin-associated secreted protein family, plays a pivotal role in the regulation of neuronal guidance during nervous system development<sup>34,35</sup>. Beyond its function in neuronal navigation, NTN1 is implicated in various cellular processes, including adhesion, motility, proliferation, and differentiation,



**Fig.** 7. Immunotherapy response evaluation for NTN1. (a) Biomarker relevance of NTN1 compared to standardized biomarkers on tumor immune evasion in ICB therapy cohorts. (b) The analyses of NTN1 associations with T cell dysfunction, and outcomes using ICB treatment. (c) Immunotherapy response of NTN1 in vivo tumor model in murine ICB therapy cohorts. (d) Immunotherapy response of NTN1 in vitro cell lines. (e) The association between NTN1 and IPS scores using the TCIA database. \*P<0.05; \*\*P<0.01; \*\*\*P<0.001.



**Fig. 8**. The relationship between NTN1 and clinical features in SKCM. (a) Prognostic stratification analysis based on age, gender, tumor grade, M stage, N stage and tumor stage. (blue line: low expression of NTN1, red line: high expression of NTN1) (b) Univariate and multivariate Cox regression identified NTN1 as the independent risk factor. (c) Construction of a nomogram to predict 1-, 3-, and 5-year survival probability. (d) The calibration curves of the nomogram at 1-, 3-, and 5-year (blue line: 1-year survival status, red line: 3-year survival status, green line: 5-year survival status, grey line: ideal line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

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which are critical for epithelial tissue development<sup>36–39</sup>. Despite these known functions, the role of NTN1 in malignancies remains inadequately explored. This study represents the first comprehensive bioinformatics analysis of NTN1 across multiple cancer types. Previous research has documented elevated NTN1 expression in colorectal and gastric cancers<sup>8,40</sup>, yet data on other malignancies are sparse. Our findings indicate that NTN1 is markedly upregulated in several cancers, including KICH, LIHC, and LUSC, while it is downregulated in other such as BLCA, BRCA, and COAD. Prognostic analyses reveal that high NTN1 expression correlates with poor outcomes in cancers such as STAD, THCA, SKCM, and BLCA, underscoring its potential as a negative prognostic biomarker. Conversely, in cancers such as CESC and KIRC, NTN1 exhibits protective properties, suggesting that its prognostic value may be context-dependent and influenced by tumor-specific factors. Notably, other members of the Netrin family, including NTN4<sup>41,42</sup> and NTN5<sup>43</sup>, have also been associated with adverse prognoses when upregulated in tumors.

In this study, we systematically evaluated the biological networks and signaling pathways of NTN1 (Netrin-1) through GO, KEGG, and Reactome analyses. These analyses revealed significant enrichment of NTN1 in various crucial biological processes and signaling pathways, suggesting its potential key roles in nervous system development, tumorigenesis, and immune regulation. GO analysis demonstrated that NTN1 is enriched in several neuro-related biological processes (BP), including axonogenesis, axon guidance, neuron projection guidance, and synaptic signaling. In terms of cellular components (CC), NTN1 is significantly enriched in synapse-associated structures such as postsynaptic specialization, postsynaptic density, and dendritic spines. Molecular function (MF) analysis indicated that NTN1 is significantly involved in various molecular binding activities, including protein binding, G-protein coupled receptor activity, and metal ion transmembrane transporter activity. KEGG pathway analysis showed that NTN1 participates in multiple critical signaling pathways, including the calcium signaling pathway, Ras signaling pathway, PI3K-Akt signaling pathway, and MAPK signaling pathway. These pathways play pivotal roles in cell proliferation, differentiation, survival, and stress responses. Reactome analysis further uncovered significant enrichment of NTN1 in biological networks such as rRNA processing, eukaryotic translation elongation and initiation, and cellular responses to starvation. These results suggest that NTN1 may play a vital role in protein synthesis and metabolic stress conditions. Additionally, the enrichment of NTN1 in immune-related pathways, such as eukaryotic translation termination and selenocysteine synthesis, indicates its critical role in cellular responses to environmental changes.





Research has shown that Netrin-1 is expressed in various regions of the central nervous system, including during the development of the spinal cord and brain<sup>44</sup>. Additionally, Netrin-1 is involved in synapse formation and the maintenance of synaptic stability, playing a crucial role in neuronal signaling and synaptic plasticity<sup>45</sup>. NTN1 operates through multiple signaling pathways in cellular signal transduction. By binding to its receptor DCC, NTN1 activates the PI3K-Akt signaling pathway, promoting cell survival and growth<sup>46</sup>. Moreover, Netrin-1 influences cell proliferation and differentiation by activating the MAPK signaling pathway<sup>47</sup>. The Src family kinases play a critical role in cell motility and adhesion<sup>48</sup>. NTN1, through its interaction with DCC or

other receptors, activates the Src family kinase signaling pathway, thereby playing a pivotal role in neuronal growth cone guidance. The cAMP/PKA pathway regulates various cellular processes, including cell proliferation and migration<sup>49</sup>. NTN1 can modulate cAMP levels to activate the PKA signaling pathway, thus regulating tumor cell migration and invasion<sup>50</sup>. These mechanisms further elucidate the potential roles of NTN1 in tumor biology and provide a theoretical foundation for its consideration as a therapeutic target.

Our mutation and methylation analyses further elucidate the regulatory mechanisms underlying NTN1 expression in cancer. In 32 tumor types, NTN1 mutations were identified in 25, exhibiting various alteration types, including mutations, structural variants, amplifications, deep deletions, and multiple alterations. Notably, deep deletions predominated among NTN1 variations and were observed in 13 tumor types, including UCS, USEC, LIHC, STAD, SARC, COADREAD, SKCM, ACC, DLBCL, CESC, LUSC, ESAD, and LUAD. Deep deletions of oncogenes play a critical role in tumorigenesis through multiple mechanisms, such as the loss of tumor suppressor genes, genomic instability, disruption of cellular signaling pathways, deregulation of the cell cycle, enhancement of anti-apoptotic mechanisms, and increased resistance to therapy. These mechanisms collectively drive cancer initiation and progression<sup>51,52</sup>. Promoter region methylation can regulate gene expression, with hypermethylation typically suppressing and hypomethylation promoting gene expression<sup>53</sup>. In our analysis, we observed a negative correlation between NTN1 expression and specific methylation sites in multiple cancers. These findings underscore the importance of investigating these specific methylation sites in future studies. Additionally, the significant correlation between copy number variations and NTN1 expression levels further supports the notion that genomic instability leads to its dysregulation. The differential methylation patterns observed between normal and tumor tissues, along with the correlation between methylation levels and NTN1 expression, highlight the complex epigenetic landscape influencing NTN1 regulation.

DNA methylation regulates gene expression by silencing genes in promoter regions, and is involved in genomic imprinting, allele-specific expression, and maintaining genome stability<sup>54</sup>. In mammals, it is essential for X-chromosome inactivation<sup>55</sup>. In cancer, aberrant methylation patterns disrupt gene expression, thereby driving tumorigenesis<sup>56</sup>. Even small-scale methylation changes in promoter regions can significantly affect gene transcription by blocking transcription factor binding or altering chromatin structure. Minor methylation changes in promoter regions may directly block the binding of transcription factors (SP1, AP-1) to DNA, disrupting the foundational steps required for transcriptional activation<sup>57</sup>. Additionally, methylation can recruit methyl-CpG-binding proteins (MBD2, MeCP2), which further attract histone deacetylases (HDACs) and other chromatin-modifying complexes. This process shifts chromatin from an open to a compact structure, restricting the accessibility of transcriptional machinery and resulting in gene silencing<sup>58</sup>. Studies also indicate that minor methylation changes not only affect DNA itself but also induce widespread alterations in histone modification patterns, such as the removal of active chromatin marks (H3K9ac and H3K4me3) and the addition of repressive marks (H3K27me3), further reinforcing the repressive chromatin state<sup>59</sup>. In cancer, even subtle methylation changes in promoter regions often mark the beginning of critical tumor suppressor gene silencing. For instance, low-level methylation of the BRCA1 promoter is significantly associated with an increased risk of breast cancer<sup>60</sup>. Similarly, in neurological and autoimmune diseases, minor promoter methylation changes also play crucial roles. For example, aberrant methylation of immune-related gene promoters in systemic lupus erythematosus (SLE) patients has been implicated in dysregulated gene expression and abnormal immune responses<sup>61</sup>. Promoter hypomethylation, even at a few CpG sites, can lead to gene silencing, particularly in cancer-related genes. In colorectal cancer, minor methylation changes in promoter regions can disrupt the transcription of key genes, driving disease progression<sup>62</sup>. Additionally, subtle methylation changes in promoter regions can induce alterations in histone modifications, further impacting gene expression significantly<sup>63</sup>. Thus, minor methylation changes in promoter regions are not merely localized events but act as amplifiers in gene regulation by blocking transcription factor binding, recruiting repressive complexes, and remodeling chromatin structure. Even small-scale methylation changes can significantly influence gene expression by altering critical nodes within the epigenetic network, ultimately driving disease progression.

Research on NTN1 methylation is limited, but previous studies have suggested that methylation-induced silencing of NTN1 is commonly observed in various cancer types, particularly in colon cancer and breast cancer. DNA methylation in the NTN1 promoter region can lead to transcriptional repression, thereby affecting NTN1 expression and promoting tumor aggressiveness, metastasis, and chemotherapy resistance<sup>64,65</sup>. Our research indicates that there are differences in the methylation levels of NTN1 between tumor samples and normal tissues in some cancers, although the beta values are relatively low, which reflect a low degree of methylation. We further analyzed the correlation between NTN1 promoter methylation and its mRNA expression. The results show that even at lower methylation levels, NTN1 transcriptional activity is still influenced, suggesting that this process may involve complex regulatory mechanisms. Further in-depth investigation is needed to explore these mechanisms.

Immune analyses reveal that NTN1 expression is positively correlated with stromal, immune, and ESTIMATE scores, as well as with immune infiltrating cells across various tumor types in multiple databases. CD8 + T cells play a crucial role in tumor immune responses, exerting direct cytotoxic effects by recognizing and binding to peptide-MHC I complexes on tumor cells during the early immune response<sup>66</sup>. Additionally, CD8 + T cells regulate the function of other immune cells by secreting cytokines such as interferon-gamma (IFN- $\gamma$ ), thereby enhancing the overall anti-tumor immune response<sup>67</sup>. CD4 + T cells are pivotal in modulating tumor immune responses, shaping the tumor microenvironment, and being utilized in immunotherapy<sup>68–70</sup>. However, CD4 + T cells have dual roles in cancer. They include regulatory T cells (Tregs), which maintain immune tolerance and prevent autoimmunity. In the tumor microenvironment, Tregs are often recruited and expanded, suppressing antitumor immune responses and promoting tumor growth<sup>71</sup>. Some studies report that increased Treg infiltration correlates with improved prognosis<sup>72,73</sup>. Natural killer (NK) T cells are critical in anti-tumor immunity, attacking tumor cells through direct cytotoxicity early in disease progression<sup>74,75</sup>. They also interact with other immune



**Fig. 10**. The schematic figure illustrates the potential mechanisms of NTN1 within the tumor microenvironment in COADREAD (**a**), COAD and NSCLC (**b**), as well as BRCA and OV (**c**). Created with Biorender.com.

cells, secreting cytokines, chemokines, and growth factors<sup>76</sup>. Higher NK T cell infiltration is associated with better prognosis in patients with solid malignancies<sup>77</sup>. Macrophages have dual roles in cancer. Early in tumor development, they can directly kill or inhibit tumor cell growth through phagocytosis and secretion of cytokines like tumor necrosis factor-alpha (TNF- $\alpha$ ), nitric oxide (NO), and reactive oxygen species (ROS)<sup>78</sup>. As tumors progress, macrophages are often reprogrammed into tumor-associated macrophages (TAMs), exhibiting protumor characteristics. TAMs mainly differentiate into M2 macrophages, secreting various growth factors such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and transforming growth factor-beta (TGF- $\beta$ ), promoting tumor angiogenesis, cell proliferation, and invasion<sup>79–81</sup>. Cancer-associated fibroblasts (CAFs) are the main stromal cells in the tumor microenvironment (TME), promoting immunosuppression and creating a supportive environment for tumors<sup>82</sup>. Endothelial cells are a major source of CAFs<sup>83</sup>, contributing to tumor metastasis<sup>84</sup>, and their proliferation provides protection for tumor cells<sup>85</sup>. NTN1 is also negatively correlated with various immune-regulatory factors, chemokines, and receptors. Immune checkpoints play a critical role in regulating immune responses. High expression of immune checkpoints in patients may indicate activated immune cells, enhanced T cell activity, and improved tumor antigen presentation, leading to more effective anti-tumor immune responses.

Previous studies have found that genes play a crucial role in the tumor microenvironment<sup>86</sup>. Through a series of immunological analyses based on the TCGA database, we identified a significant correlation between NTN1 and immune infiltration across various cancer types. In COADREAD, cancer cells overexpress NTN1, leading to the secretion of high levels of Netrin-1. This binds to A2BR on MDSCs, activating the cAMP/ PKA/CREB pathway, which subsequently drives the production and release of Arg1, ROS, IL-10, and TGF- $\beta$ , thereby suppressing T cell function and promoting immunosuppression<sup>5</sup> (Fig. 10a). Interestingly, a recent study demonstrated that in COAD and NSCLC, direct interactions between CAFs and cancer cells significantly upregulate the expression of NTN1 and its receptor UNC5B. The increased secretion of Netrin-1 by CAFs binds to UNC5B receptors on both CAFs and cancer cells, inducing IL6 secretion. IL6 then binds to its receptor on tumor cells, activating the JAK2/STAT3 signaling pathway, enhancing cancer stem cell properties and tumor plasticity<sup>87</sup> (Fig. 10b). Similarly, Netrin-1 is highly expressed in BRCA and OV. On one hand, Netrin-1 binds to

UNC5B and DCC on cancer cells, activating pathways such as PI3K/AKT and MAPK/ERK, promoting cancer cell survival and the accumulation of immunosuppressive cells like MDSCs. On the other hand, Netrin-1 binds to UNC5B and DCC on MDSCs, inducing the release of inhibitory molecules such as ARG1, ROS, NO, iNOS, TGF- $\beta$ , IL-10, IDO, and PGE2. These inhibitory molecules suppress T cell activity, weakening the immune system's anti-tumor response<sup>88</sup> (Fig. 10c). In summary, building upon previous research, we further explored the specific mechanisms by which NTN1 influences the tumor microenvironment, highlighting NTN1 as a potential therapeutic target in cancers.

Earlier studies have shown that NTN1 is closely associated with mast cells in SKCM<sup>89</sup>, which aligns with our findings. Mast cells secrete chemokines such as TNF-a and CCL5 to recruit immunosuppressive cells (MDSCs and Tregs) to the tumor site, thereby inhibiting anti-tumor immune responses and aiding SKCM in evading immune surveillance. Additionally, TGF-β produced by SKCM promotes the expression of the TIM-3 receptor on mast cells, suppressing the secretion of IFN- $\gamma$  by T cells, which weakens the immune attack on the tumor and facilitates melanoma progression. Chemokines such as CXCL12 released by mast cells promote the EMT of melanoma, increasing their invasiveness and metastatic potential. Furthermore, proteases secreted by mast cells, such as tryptase and chymase, degrade the extracellular matrix, assisting tumor cells in breaching the basement membrane and facilitating the spread of melanoma cells to surrounding tissues<sup>90</sup>. Our findings also indicate a strong positive correlation between NTN1 and macrophages in SKCM. Cancer cells attract macrophages by secreting chemokines such as CCL2 and CSF-1 in SKCM. In turn, these macrophages secrete factors like MMP-9 and SPARC, which degrade the extracellular matrix, creating conditions conducive to melanoma invasion and metastasis. Additionally, macrophages release pro-angiogenic factors such as VEGF, which promote neovascularization, supplying nutrients and oxygen to tumor cells. Simultaneously, macrophages secrete IL-10 and TGF- $\beta$ , and highly express PD-L1, which binds to PD-1 on T cells, inhibiting T cell function and further reinforcing the immunosuppressive nature of the tumor microenvironment<sup>91</sup>.

Our study results indicate that NTN1 expression is negatively correlated with tumor mutational burden (TMB), microsatellite instability (MSI), loss of heterozygosity (LOH), and homologous recombination deficiency (HRD) in most tumors. TMB and MSI have emerged as effective biomarkers for predicting responses to tumor immunotherapy<sup>92,93</sup>. TMB is closely associated with the efficacy of PD-1/PD-L1 inhibitors and can serve as a predictor of immunotherapy response<sup>92</sup>. Notably, our research suggests that the predictive value of NTN1 in immunotherapy response can rival that of TMB. MSI is a clinically relevant tumor marker resulting from defects in the DNA mismatch repair mechanism in tumors. HRD status is a critical indicator for treatment selection and prognosis across various tumor types, closely linked to the sensitivity to platinum-based chemotherapeutics and PARP inhibitors<sup>94</sup>. Additionally, we observed a positive correlation between NTN1 expression and tumor stemness. Studies have shown that tumor stemness is positively correlated with tumor heterogeneity but negatively correlated with anti-tumor immune response<sup>95</sup>. Stemness burden is a valuable prognostic predictor for solid tumors<sup>96</sup>, with high stemness burden being an independent predictor of recurrence<sup>97</sup>. Therefore, NTN1 may influence the tumor immune microenvironment and treatment response by regulating cancer stem cells.

Our study has several limitations. The pan-cancer research data is derived from online open databases, which may have limitations regarding data sources, sample size constraints, potential biases, and certain systematic deviations. Additionally, the analysis did not account for other factors that might influence tumorigenesis and tumor progression. Future research should incorporate clinical and epidemiological data to validate the prognostic and diagnostic value of NTN1 in cancer, aiming to comprehensively enhance our understanding of the disease. Moreover, the mechanisms by which NTN1 influences tumorigenesis and tumor progression require further investigation through rigorous experimental studies.

#### Conclusion

In conclusion, this study provides a comprehensive pan-cancer analysis that confirms the potential role of NTN1 as a prognostic biomarker and predictor of immune response in cancer. NTN1 is associated with the tumor microenvironment, immune regulation, immune cell infiltration, prognosis, and drug sensitivity across various tumor types. These findings hold significant translational potential for cancer diagnosis and therapy. Therefore, NTN1 may emerge as a novel biomarker for cancer diagnosis and prognosis, as well as a new therapeutic target for future cancer treatment.

#### Data availability

RNAseq data in TPM format for TCGA and GTEx for pan-cancer and PAAD were provided by UCSC XENA (https://xenabrowser.net/datapages/), which are publicly available.

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#### Author contributions

FL, YC, RH, and ZY participated in the conception, design, acquisition of data, analysis, and interpretation. All authors participated in drafting the article and gave final approval.

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#### Declarations

#### **Competing interests**

The authors declare no competing interests.

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