Development of a broadly potent neutralizing antibody targeting Nidogen 1 effectively inhibits cancer growth and metastasis in preclinical tumor models

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ABSTRACT

Background and Objectives: Nidogen 1 (NID1) is a highly conserved structural component of the extracellular matrix (ECM), which interacts with different basement membrane (BM) proteins to form a stabilized meshwork. The promoting ability of NID1 in cancer development and metastasis has been demonstrated in multiple cancer types, including ovarian cancer, breast cancer, and hepatocellular carcinoma (HCC). This suggests that NID1 holds great potential as a therapeutic target for cancer treatment. However, currently, there is a lack of commercially available neutralizing antibody for clinical testing and treatment. **Methods:** To address this, we utilized hybridoma technology to develop a monoclonal neutralizing antibody which targets the critical G2 region of NID1. The therapeutic effect of this NID1 neutralizing antibody against a wide range of human cancer cells was evaluated. **Results:** The results showed that NID1 neutralizing antibody effectively attenuated the growth, motility and metastasis of HCC, lung cancer, breast cancer and nasopharyngeal carcinoma cells *in vitro*. The proof-of-concept of targeting NID1 using neutralizing antibody was further demonstrated in various animal models. Mechanistically, our findings indicate that treatment with NID1 neutralizing antibody leads to the deregulation of hypoxia-inducible factor-1 (HIF-1α) pathway in cancer cells. **Conclusions:** Taken together, this study offers promising prospects for a new pan-cancer monoclonal antibody-based strategy by targeting the tumor-associated membrane protein NID1.

Key words: nidogen 1, neutralizing antibody, treatment, cancers, hypoxia-inducible factor-1

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INTRODUCTION

Since the identification of various cancer-specific surface antigens, monoclonal antibody-based therapy has evolved as a major oncological breakthrough. Multiple monoclonal antibodies have been approved by the United States Food and Drug Administration (FDA) due to their positive clinical outcome and minimal immunological reactions. These antibodies exert their anti-tumorigenic effects through various mechanisms. The most common approach involves the direct killing of cancer cells by

blocking critical receptors to induce subsequent apoptosis. Indirect methods include neutralization of enzymatic signaling or delivery of conjugated drugs. The fragment crystallizable (Fc) region of antibodies can also be designed to activate immune-mediated cytotoxicity and phagocytosis. Some antibodies are designed not only to target tumor cells but also to ablate components of the surrounding tumor microenvironment (TME). For instance, bevacizumab, an anti-VEGF antibody, inhibits pro-angiogenic factors and thus limits intratumoral vascular supply.[1] With the discovery of immune checkpoints, such as cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed death receptor-1 (PD-1), antibodies against these checkpoints effectively antagonize self-regulatory immune inhibition. These antibodies have emerged as a breakthrough in cancer therapy and have gained rapid approval from the FDA as first-line treatments. The swift development of targeted antibody-based treatment signifies the importance of identifying and characterizing new specific targets for cancer therapies.

NID1 is an essential structural component of the basement membrane (BM) and extracellular matrix (ECM), which acts as a linker, connecting other components such as collagen, fibronectin, and laminin.[2] These components form a stable structural network that is vital for cell adhesion, survival, and differentiation.[3] Accumulating evidence has revealed the involvement of NID1 in various human cancers (Table 1). The level of NID1 has been found to be higher in breast cancer patients with lung metastasis than in those without metastasis.[4,5] Similarly, upregulation of NID1 has been reported in glioma, kidney renal clear cell carcinoma, and head and neck squamous cell carcinoma.[6] High levels of NID1 are associated with more aggressive clinicopathological parameters of cancer patients.[7] Notably, NID1 has been identified as a factor for stratifying high-risk and low-risk groups in prostatic adenocarcinoma, ovarian cancer, nasopharyngeal carcinoma (NPC), and oral squamous cell carcinoma.[8–11] NID1 has also been shown to predict the chance of relapse in papillary thyroid carcinoma.[12] In addition to its role as a structural protein, NID1 can be released as secretory protein or carried by small extracellular vesicle (sEV). Higher levels of NID1 have been detected in the medium of cancer cell lines and the sera of cancer patients. For instance, saliva from patients with oral cavity squamous cell carcinoma shows higher levels of NID1 compared to that from healthy individuals.[13] Plasma samples from ovarian cancer patients also exhibit increased levels of NID1 compared to those from healthy women.[14] Furthermore, high expression of NID1 has been detected in the secretomes of lung metastatic breast cancer, colorectal tissues, and melanoma cells compared to their normal counterparts.[5,15,16] The level of NID1 in circulating sEVs has been reported to be higher in HCC patients compared to healthy individuals.[17]

The remarkable clinical relevance of NID1 in multiple cancer types underscores its potential as a promising therapeutic target. However, a commercial anti-NID1 neutralizing antibody has yet to be developed. To address this gap, we designed a mouse monoclonal antibody specifically targeting NID1. Subsequently, we evaluated the efficacy of this newly developed antibody in mouse models of four different cancer types, including HCC, lung adenocarcinoma, breast adenocarcinoma, and NPC.

MATERIALS AND METHODS

Human tissues

Clinical tissue specimens were used in this study. A tissue microarray (TMA) comprising paired cases of tumor and adjacent nontumor liver tissues was provided by the Department of Pathology, Sun Yat-sen University Cancer Centre, China. Approval for the use of human tissues was obtained from the Institutional Review Board of Sun Yat-sen University Cancer Centre. All procedures involving human specimens in this study were conducted in accordance with the applicable ethical regulations.

Cell culture

The human embryonal kidney cell lines 293T and 293FT, human lung adenocarcinoma cell line HCC827, and human breast adenocarcinoma cell line MDA-MB-231 were purchased from American Type Culture Collection (ATCC) and cultured according to the ATCC recommendations. The HCC cell lines MHCC97L and MHCCLM3 were obtained from the Cancer Institute, Fudan University, China. The C666-1 cell line is an Epstein-Barr virus-positive NPC cell line.[18] All cell lines were cultured according to the recommendations provided by the respective providers. Cell line authentication was carried out by Short Tandem Repeat (STR) profiling. All cell lines were regularly tested to ensure the absence of mycoplasma contamination.

Establishment of NID1 knockdown stable clones and NID1 overexpression stable clones

All NID1 knockdown stable clones (shNID1) were established using pLKO. 1 shRNA plasmid (Addgene), subcloned with the shRNA sequence constructed by the following oligos, shNID1-F: 5-CCGGCCTCCCAGATAG AATGTCAATCTCGAGATTGACATTCTATCTGGGA GGTTTTTG-3 and shNID1-F: 5'AATTCAAAAACCTCC CAGATAGAATGTCAATCTCGAGATTGACATTCTA TCTGGGAGG-3'. on-target control clones (shCTL) were generated using MISSION™ non-target shRNA control vector (Sigma-Aldrich). NID1 was subcloned and expressed using the CMV-XP-MCS-EF1α-Puro Cloning Lentivector (XPack) (System Biosciences).[17] The MISSION® Lentiviral

Table 1: Expression and functions of nidogen 1 in different types of cancers

Nidogen 1	Cancer types	Expression	Findings	Ref.
Cellular	Melanoma	Patients with higher expression	Correlated with poor prognosis	[5]
	Breast cancer	Elevated in patients with lung metastasis	Correlated with poor prognosis	[4,5]
		High level in highly tumorigenic murine mammary tumor cells Abnormal promoter DNA hypermethylatio	Promote cell proliferation, migration and invasion Risk gene of cancer	[6] [51]
	Ovarian cancer	Upregulated in tumor	Promote EMT, chemoresistance	[19]
	Endometrial cancer	Upregulated in tumor	Promote tumorigenesis, metastasis	[7,52]
	Papillary thyroid carcinoma	Upregulated in tumor and metastatic tissues	Higher likelihood of relapse after treatment	[12]
	Prostatic adenocarcinoma	Immune-related gene-based signature	Risk stratification, tumor immune state assessment	[9,11]
	Nasopharyngeal carcinoma	Upregulated in tumor	Risk gene of cancer	[8]
	Oral squamous cell carcinoma	Upregulated in tumor	Risk gene of cancer	[8]
	Glioma	Upregulated in tumor	Inhibit apoptosis, promote chemoresistance	[6]
	Kidney renal clear cell carcinoma	Upregulated in tumor	ND	[6]
	Head and neck squamous cell carcinoma	Upregulated in tumor	ND	[6]
	Gastric cancer Gastric cancer Oral squamous cell carcinoma Salivary gland adenoid cystic carcinoma	Suppressed by JQ1 inhibitor Upregulated in tumor Upregulated in tumor Upregulated in tumor	Rescued JQ1-inhibited gastric cancer cell growth and motility Risk gene of cancer Promote the proliferation and migration Promote lung metastasis	[45] [53] [26] [54]
Secretory	Breast cancer	Elevated in secretome of metastatic subline	Promote lung metastasis	[5]
	Melanoma	Elevated in secretome of metastatic subline	Promote lung metastasis	[5]
	Oral cavity squamous cell carcinoma	Higher in interstitial fluids of patients	Correlated with the advanced stage and poor prognosis	[13]
	Ovarian cancer	Elevated in plasma of early stage patients	Potential biomarker in blood	[14]
	Colorectal cancer	Upregulated in cancer secretome	Promote growth and migration	[15,16,43]
Extracellular	Melanoma	Elevated in metastatic cell lines	ND	[55]
Vesicles	Hepatocellular carcinoma	Elevated in circulating EVs of patients	Promotes tumor development and metastasis Correlated with tumor stage	[17]

Packaging Mix and Fugene® 6 Transfection Reagent (Promega) were employed to transfect the shNID1 plasmid or NID1 overexpression vector (XP-NID1) into HEK293FT cells. After 24 h, viral supernatant, centrifuged, and filtered through 0.45-μm fillter. Polybrene transduction enhancer together with viral supernatant was then added to seeded cells. Forty-eight h after transduction, cells were selected by puromycin (Thermo Fisher Scientific). The NID1 expression of the resistant clones was examined by western blotting. For NID1 overexpression NID1 clone, the NID1 fragment (nucleotides 21-3123; Accession No. BC045606.1) was released from NID1/Entactin cDNA ORF Clone (Sino Biological) and subcloned into pMH-SFB cloning vector (Addgene) using gateway recombinational cloning (Invitrogen). The plasmid was transfected to HEK293T cells by Lipofectamine™ 2000 Transfection reagent (Thermo Fisher Scientific). Forty-eight h after transduction, cells were selected by puromycin (Thermo Fisher Scientific). Single clone was picked, and NID1 overexpression was examined by western blotting.

Generation and purification of antibody

To generate anti-NID1 antibody, 6 peptide antigens were identified through sequence analysis of NID1. These peptides were synthesized and conjugated to immunogenicity enhancement factors. BALB/c mice were immunized with the conjugated antigen peptides, and the resulting anti-sera were tested for reactivity to

BSA-conjugated antigen peptides using enzyme-linked immunosorbent assay (ELISA). After cell fusion and subcloning of hybridoma clones, antibodies from different clones were evaluated for their inhibitory effects on cancer cell growth and motility. The hybridoma clone that produced the antibody with the strongest inhibitory effect was selected for large-scale production by DiagnoVEX Therapeutics Ltd. The selected anti-NID1 antibody specifically targets a sequence, VHDDSRPALPST, located at residues 619–630 of NID1. The hybridoma cells were cultured until the logarithmic growth phase, and the conditioned medium was collected and centrifuged to remove cell debris. The pH of the culture medium was adjusted by adding 1/10 volume of 1.0 M Tris-HCl pH 8.0. The pH-adjusted medium was then applied to Protein G Sepharose 4 FF columns (GE Healthcare) that were equilibrated with the appropriate buffer, at a flow rate of 2 mL/min. The column was thoroughly washed with the provided binding buffer, and the monoclonal anti-NID1 antibody was subsequently eluted using an elution buffer.

Coimmunoprecipitation and mass spectrometry

The cell pellet was lysed with NETN lysis buffer (150 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L Tris, 0.2% NP-40). One μg of anti-NID1 antibody was added to 500 μg of lysate in a total volume of 500 μL of NETN and incubated overnight at 4°C with gentle rotation. Thirty μL of washed Protein G Sepharose™ 4 Fast Flow beads slurry (GE Healthcare) was added to the immunoprecipitated proteins and rotated for an additional 4 h at 4 °C. The beads were then washed 3 times with 500 μL NETN. Supernatant was completely removed, and the beads were boiled for 5 min with 15 μL 2× SDS loading dye. For mass spectrometry (MS) protein analysis, the proteins were resolved on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel and excised for MS analysis performed by The University of Hong Kong Centre for PanorOmic Sciences Proteomics and Metabolomics core.

Colony formation assay

Tumor cells were seeded at a density of 1×10^3 cells per well in a 6-well plate and incubated in a 37°C incubator for 1 week. Once small colonies had developed, the serum-free medium was replaced with medium containing the anti-NID1 antibody, and the cells were treated for three days. Following treatment, the cells were fixed with methanol for 15 min and stained with crystal violet for 30 min. Four fields were randomly selected, and the number of colonies was counted.

Cell migration and invasion assays

In the migration assay, cells in serum-free medium were seeded in the upper chamber of transwells. For the invasion assay, BD Matrigel Basement Membrane Matrix (BD Bioscience) was used to coat Transwell Permeable Supports (SPL) prior to cell seeding. The cells were seeded at a density of 1.5×10^5 in a mixture of serum-free medium containing the anti-NID1 antibody in the upper chamber of the transwells. Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS was added to the bottom chamber as a chemoattractant for both assays. After 24–48 h, the cells were fixed with methanol for 1 h and stained with crystal violet for an additional 1 h. Four fields were randomly selected from each well, and the number of migrated and invaded cells was counted.

Animal experimentation

Experimental procedures were performed in compliance with the research protocols CULATR 22-066 and 5808-21, which were approved by the Committee of the Use of Live Animals in Teaching and Research (CULATR). All animal studies were conducted strictly according to the Animals (Control of Experiments) Ordinance (Hong Kong, China) and followed guidelines provided by the Centre for Comparative Medical Research (CCMR), Li Ka Shing Faculty of Medicine, the University of Hong Kong. All mice used in the experiments were provided by and housed in a specific pathogen-free area in the CCMR building.

Orthotopic liver implantation model

To obtain tumor seeds for orthotopic liver implantation, luciferase-labeled MHCC97L cells were inoculated into the right flank of male 4-week-old BALB/cAnN-nu mice at a concentration of 1×10^6. After 2 weeks, the mice were killed using a euthanasia agent, and the tumor mass was harvested. The tumor mass was cut into small pieces of about 1 mm^3 in size. For the implantation of tumor seeds, mice were anesthetized, and a laparotomy was performed to expose the liver. To monitor the development of the tumors, the mice received intraperitoneal injection of D-luciferin (GoldBio) were subjected to weekly bioluminescence imaging. The images were captured, and the bioluminescence signal was quantified using IVIS Spectrum imaging system (Perkin Elmer). At the end of experiment, the mice were sacrificed, and their lungs and livers were excised for histological analysis.

Subcutaneous injection model

To access the therapeutic efficacy of the monoclonal anti-NID1 antibody in the growth of tumors derived from cancer cells, BALB/cAnN-nu mice were used. The mice were injected subcutaneously with cells at a concentration of 5×10^6 in 0.1 mL per site. Once the tumors reached approximately 5 mm in diameter, intraperitoneal administration of the anti-NID1 antibody (400 μg/0.15 mL/mouse) was started once every 3 days for a total of 3 weeks. Mice injected with phosphate-buffered

saline (PBS) or mouse IgG antibody were performed as control experiments. Five mice were used in each group. Development of tumor growth was regularly monitored by measuring the length and width of the tumors using a caliper. The tumor volume was calculated according to the formula, 0.5 × length × width^2. All mice were sacrificed at the end of the experiment, and their tumors were harvested for measurement and subsequent immunohistochemical staining.

Lung colonization metastasis assay

In the lung colonization model, a total of 1 × 10^5 mouse p53-/-; Myc hepatoblasts were co-injected with either 5 μg of NID1 antibody, IgG or PBS into 6-week-old male BALB/cAnN-nu mice. The mice were monitored on a weekly basis using bioluminescence imaging performed by the IVIS spectrum imaging system (Perkin Elmer). At the end of experiment, ex vivo bioluminescence imaging of lungs was performed, and the dissected lungs were subjected to histological analysis.

Immunohistochemistry and hematoxylin/eosin staining

Formalin-fixed, paraffin-embedded tissue was cut into 5 μm thick sections and deparaffinized in xylene, followed by rehydration in a series of alcohol gradients (100%, 95%, and 70%) and distilled water. Antigen retrieval was conducted by immersing the sections in preheated EnVision FLEX Target Retrieval Solution, High pH (Agilent). The sections were then microwaved for an additional 15 min and allowed to cool down in the retrieval solution for at least 20 min at room temperature. After blocking the endogenous peroxidase by EnVision FLEX Peroxidase-Blocking Reagent, the sections were subjected to incubation with primary and secondary antibodies. Signal detection was facilitated by adding Dako REAL EnVision Detection System and 3, 3'-Diaminobenzidine (DAB) chromogen for 30 and 2 min, respectively, at room temperature. The specimen sections were also stained with hematoxylin/eosin (H&E) stain. NanoZoomer Digital Pathology System (Hamamatsu) was used to process slides and to create high-quality digital images for analysis. Anti-Entactin/NID antibody (#ab254325, Abcam) and anti-Ki67 (#M7240, Dako) antibodies were used for immunohistochemical staining. The signal was quantified by counting the number of positively stained cells relative to the total number of cells in three randomly selected fields for each sample.

RNA sequencing

Total RNA from antibody-treated cells was extracted using RNA extraction kit according to the manufacturer's protocol (Qiagen). RNA of 900 ng was subjected to RNA sequence performed by The University of Hong Kong Centre for PanorOmic Sciences Genomics Core.

Database mining

The TCGA expression correlation of NID1 in different cancers was generated from (https://www.xiantaozi.com). The survival curve was analyzed from Kaplan-Meier plotter (https://kmplot.com/analysis/).

Statistical analysis

The readings of all assays were calculated as the mean ± standard error of mean (SEM). Student's t-test was performed using Prism (Version 9.5.0, GraphPad) for statistical analysis. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

NID1 upregulation is associated with poor prognosis in HCC

A previous study has reported the elevation of NID1 level in circulating sEV of HCC patients compared to control individuals.[17] However, the cellular expression of NID1 in HCC has not been well documented. To investigate the clinicopathological significance of NID1 in HCC, we utilized TCGA databases and revealed elevated levels of NID1 transcripts in tumor tissues (n = 374) compared to normal tissues (n = 49) (Figure 1A). Kaplan-Meier survival analysis showed that individuals with higher NID1 level exhibited decreased overall survival (Figure 1B). To further confirm the pathological association of NID1 in HCC, we performed immunohistochemistry staining on an HCC TMA comprising 80 paired cases of HCC tissues and adjacent noncancerous tissues. We found that 67.5% (54/80) of tumors exhibited positive staining, whereas only 27.5% (22/80) of non-tumorous tissues showed positive staining (Figure 1C and 1E). NID1 was overexpressed in 47.5% (38/80) of tumor tissues compared to normal tissues, while 32.5% (26/80) and 20% (16/80) of tumor tissues showed no difference or lower expression, respectively, compared to their normal counterpart (Figure 1D and 1F). Altogether, the NID1 staining score was significantly higher in tissue samples than in normal tissues (Figure 1G), which aligns with the TCGA analysis indicating that NID1 expression correlates with the development of HCC.

The newly developed mouse monoclonal antibody specifically detects NID1

The strong correlation between NID1 expression and the disease stage of HCC and other cancer types underscores the potential of NID1 as a promising therapeutic target for cancer treatment. NID1's secretory nature and its localization on the surface of sEVs make it an ideal candidate for recognition and targeting by antibodies. In

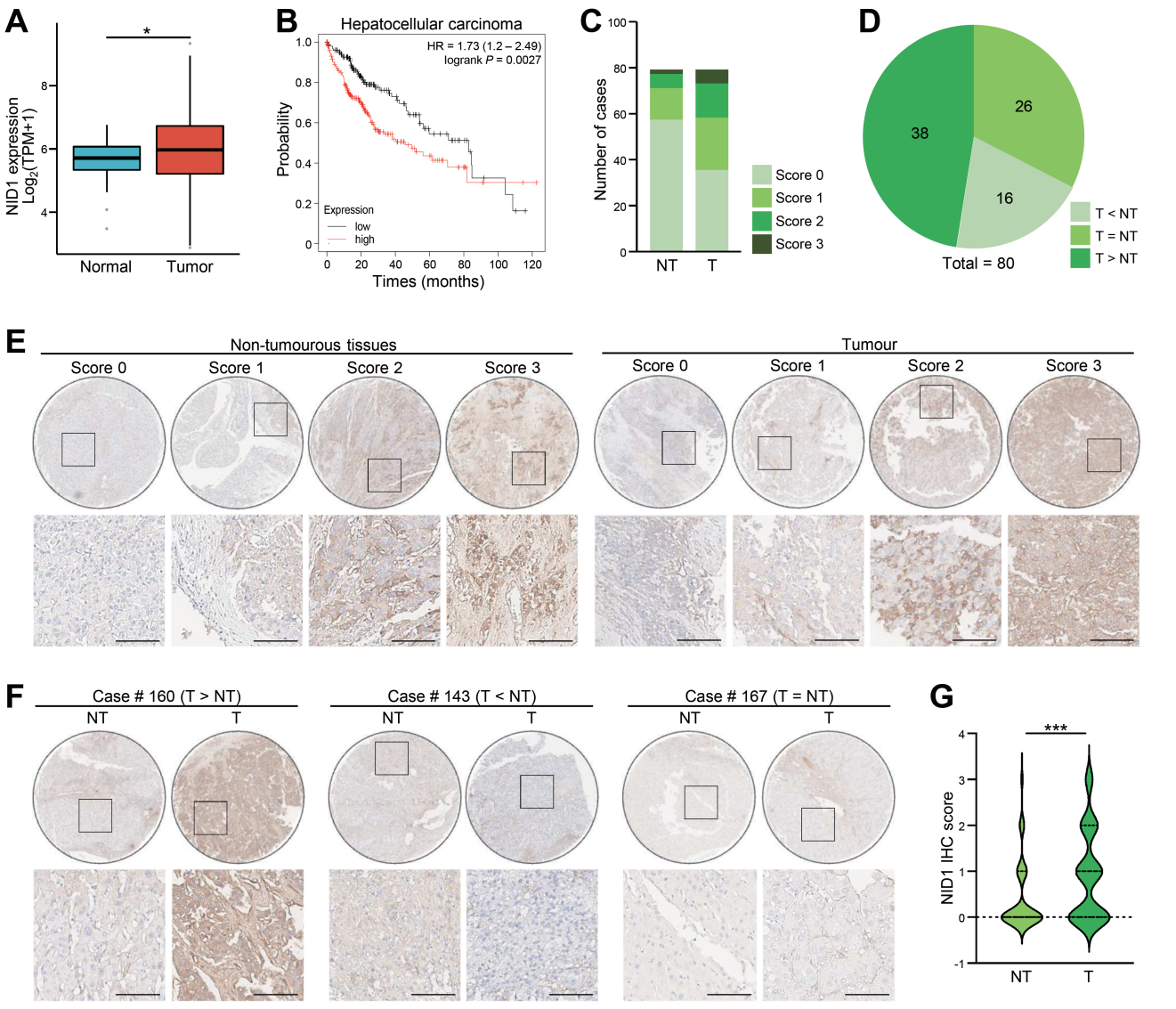

Figure 1: Upregulation of NID1 is associated with poor prognosis in HCC. A, Analysis of NID1 expression in tumor tissue (T, *n* = 374) and non-cancerous tissues (N, *n* = 49) using TCGA datasets of HCC. B, Kaplan-Meier curves comparing progression-free survival (PFS) of patients with high and low NID1 expressions in HCC stratified by median level, based on the Kaplan-Meier Plotter database (http://kmplot.com/analysis). C, Immunohistochemistry of NID1 was performed on TMA comprising paired T and NT tissues (*n* = 80). The NID1 intensity score was analyzed. D, The pie chart illustrates the number of cases with overexpression, underexpression and no change in NID1 (*n* = 80). E, Representative images of NID1 expression with intensity scores (0–3). Scale bar, 100 μm. F, Representative images of cases showing overexpression (T > NT), underexpression (T < NT) and no change (T = NT) in NID1. Scale bar, 100 μm. G, The overall IHC score of NID1 expression was plotted (*n* = 80).

this study, our aim was to develop a monoclonal anti-NID1 neutralizing antibody capable of effectively inhibiting cancer growth and metastasis.

To design suitable epitopes for antibody development, we analyzed the amino acid sequence of NID1 using an algorithm that considered structural features, sequence conservation, and hydrophobicity. Based on these criteria, we selected six 12-residue epitopes with the highest "epitope score" for the immunization of mice (Figure 2A). It is important to note that NID1 shares a structural similarity of 46% with NID2, another protein in the same family (Figure 2B). To ensure specificity and avoid cross-reactivity with NID2, we compared the selected epitope "VHDDSRPALPST" of NID1 with the corresponding region of NID2. The amino acid sequence mismatch indicated a relatively low probability of cross-reactivity with NID2 (Figure 2B). Subsequently, we developed a hybridoma clone producing an anti-NID1 antibody that specifically targets the "VHDDSRPALPST" epitope. The antibody was purified from the conditioned medium of the selected hybridoma cells. To assess its purity, we

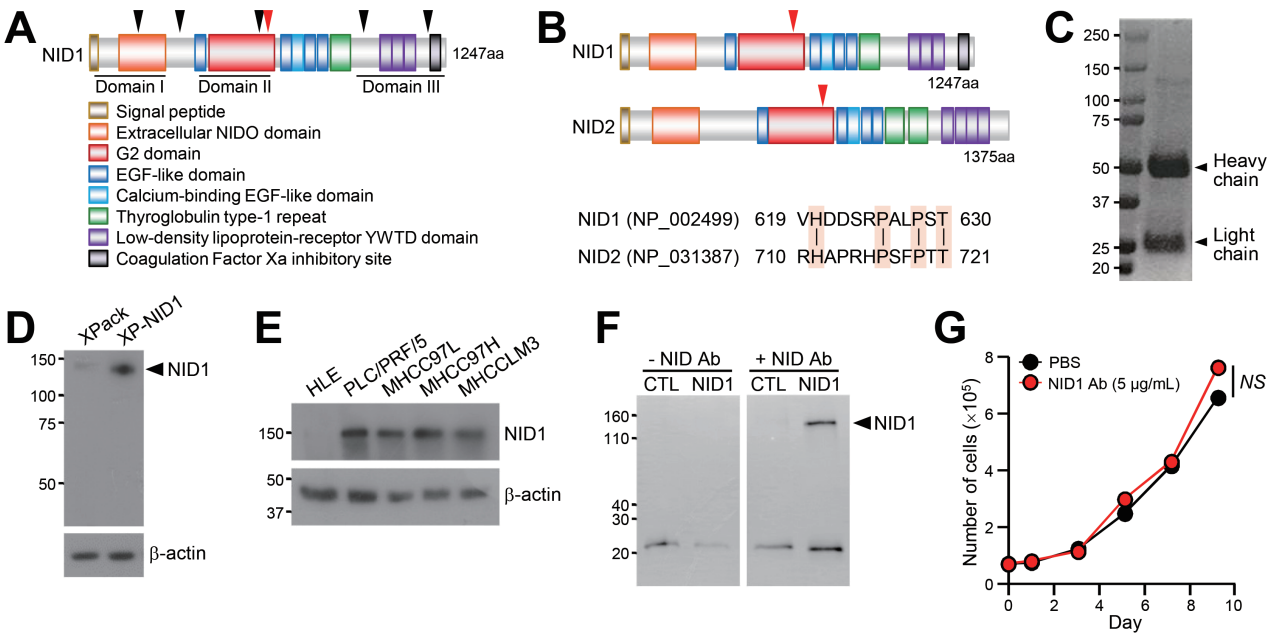

Figure 2: Generation of monoclonal anti-NID1 antibody. A, Schematic diagram illustrates the structural organization of NID1. The location of the 12-residue peptide for anti-NID1 antibody production is indicated by arrowhead. The location of epitope targets by the anti-NID1 antibody generated by the hybridoma clone is indicated by red arrowhead. B, The structural organization of NID1 and NID2. Homologous 12-residue peptide sequences in NID1 and NID2 are indicated by red arrowhead. Alignment of amino acid sequences of the selected epitope sequence between NID1 and NID2. Same residues between NID1 and NID2 are highlighted. C, Coomassie blue-stained polyacrylamide gel showing the purified anti-NID1 antibody obtained from hybridoma cells. D, Western blot analysis of NID1 expression in HLE cells stably transfected with backbone vector (XPack) and plasmid expressing NID1 (XP-NID1) using anti-NID1 antibody. E, Western blot analysis of NID1 expression in HCC cell lines using anti-NID1 antibody. F, Total cell lysates of MHCC97L cells was immunoprecipitated using anti-NID1 antibody. The immunoprecipitated proteins were subjected to immunoblotting using anti-NID1 antibody. G, Normal human hepatocyte MIHA cells were incubated with PBS or 5 μg/mL of anti-NID1 antibody. Number of cells was counted every 2 days. Data are represented as the mean ± SEM. NS, not significant from Student's *t*-test.

resolved the antibody using SDS-PAGE (Figure 2C). The Coomassie blue-stained gel clearly showed the presence of the heavy and light chains of the antibody, confirming its structural integrity.

The anti-NID1 antibody was able to effectively detect exogenously expressed NID1 in the HLE cell line, which exhibits low levels of NID1 expression (Figure 2D). Furthermore, it effectively detected endogenous NID1 in various HCC cell lines (Figure 2E). To validate the specificity of the anti-NID1 antibody, we performed a co-immunoprecipitation assay and confirmed its ability to pull down NID1 from cell lysate extracted from NID1-overexpressing cells (Figure 2F). To confirm the identity of the immunoprecipitated protein, we subjected it to MS protein analysis, which provided further evidence of NID1 (Supplementary Table S1). Considering that the intended use of this antibody is for therapeutic purposes, it is crucial to evaluate its potential side effects on normal cells. Therefore, we treated normal human liver cell line MIHA, which has low NID1 expression, and demonstrated that the addition of the antibody did not affect the proliferation of normal cells (Figure 2G). These findings highlight the high specificity of the homemade monoclonal anti-NID1 antibody and its limited impact on normal cells. As a result, this antibody can confidently be utilized for further *in vitro* and *in vivo* experiments.

Anti-NID1 antibody inhibits HCC growth and tumorigenesis

In our previous research, we demonstrated that metastatic MHCC97L and MHCCLM3 cells exhibit elevated levels of NID1 expression compared to normal hepatocyte and non-metastatic HCC cells.[17] To consolidate the role of NID1 in HCC oncogenesis, control shCTL and shNID1 cells were established in MHCC97L and MHCCLM3 cell lines (Supplementary Figure S1A). Knockdown of NID1 resulted in a marked reduction in colony forming, migratory and invasive capabilities of HCC cells compared to shCTL cells (Supplementary Figure S1B-D). These findings prompted us to explore the neutralizing effect of the anti-NID1 antibody on HCC. Immunoblotting analysis revealed that treatment with the anti-NID1 antibody led to a decrease in NID1 levels in HCC cells compared to untreated cells (Figure 3A). Functional analysis showed that treatment with the anti-NID1 antibody inhibited the colony-forming ability of both metastatic cells (Figure 3B). We further extended the analysis to an *in vivo* setting

using an orthotopic liver implantation model. We observed that treatment with the anti-NID1 antibody significantly hampered tumor growth compared to the PBS and IgG control groups (Figure 3C-D). Importantly, we did not observe any signs of distress or significant changes in body weight among the mice (Figure 3E). Immunohistochemical staining for Ki67, a marker of cell proliferation, showed that tumors treated with the anti-NID1 antibody exhibited the lowest proliferation ability compared to all experimental groups (Figure 3F). These results provide primary evidence highlighting the therapeutic potential of targeting NID1 using a neutralizing antibody in HCC.

Anti-NID1 antibody inhibits HCC motility and metastasis

Taking into account the well-established role of NID1 in promoting metastasis in various cancers, we further examined the inhibitory effect of the anti-NID1 antibody on HCC motility.[19–21] In migration and invasion assays conducted on MHCC97L and MHCCLM3 cells, treatment with the anti-NID1 antibody significantly reduced their migration and invasion abilities compared to cells treated with PBS (Figure 3G and 3H). To access the impact of the anti-NID1 antibody on metastasis, we conducted a lung colonization model by intravenously coinjecting p53-/-; Myc hepatoblasts with either PBS, IgG or anti-NID1 antibody (Figure 3I). The results demonstrated that compared to the PBS group, the colonization ability of hepatoblasts was profoundly diminished in mice injected with the anti-NID1 antibody, while IgG showed no inhibitory effect (Figure 3J-L). Collectively, these findings demonstrate the crucial inhibitory role of the anti-NID1 antibody in the growth, tumorigenesis, motility, and metastasis of HCC. These results strongly suggest the therapeutic potential of the anti-NID1 antibody in the treatment of HCC.

Knockdown of NID1 suppresses colony forming ability and motility of lung cancer, breast cancer and NPC cells

The potent inhibitory activity of the anti-NID1 antibody in HCC motivated us to investigate its effects in other cancer types, including lung cancer, breast cancer, and NPC. In the case of non-small cell lung cancer (NSCLC), which accounts for approximately 80% of all lung cancer cases, a significant proportion of patients are diagnosed at an advanced stage with a low 5-year survival rate.[22,23] Notably, NID1 has been identified as a significant promoter of NSCLC.[24] To examine the role of NID1 in NSCLC cells, shNID1 and shCTL clones were generated in HCC827 cells (Supplementary Figure S2A). Knockdown of NID1 in HCC827 cells resulted in a significant reduction in growth, migration, and invasion abilities compared to the shCTL cells (Supplementary Figure S2B). Furthermore, HCC827 cells with reduced NID1 expression displayed delayed tumor development and formed smaller tumors compared to those derived from the control cells (Supplementary Figure S2C-E).

The functional ability of NID1 in cancer metastasis has also been demonstrated in breast cancer.[5] To confirm the promoting effect of NID1 in breast cancer, shNID1 and shCTL clones were generated in MDA-MB-231 cells (Supplementary Figure S3A). Depletion of NID1 in shNID1 cells significantly impaired the ability of breast cancer cells to grow, migrate, invade and to form tumors compared to shCTL cells (Supplementary Figure S3B-E).

NPC and oral squamous cell carcinoma (OSCC) are clinically relevant diseases that share similar molecular and pathological characteristics.[8,25] Studies have shown that increased levels of NID1 are associated with advanced OSCC stage and poor survival rates and overexpression of NID1 promotes proliferation and migration of OSCC.[13,26] However, due to the limited availability of public gene expression databases for NPC, the clinical relevance of NID1 in NPC remains unknown. Therefore, it was crucial to investigate whether NID1 has a similar promoting effect in NPC. To test this hypothesis, we generated shNID1 and shCTL clones in C666-1 cells (Supplementary Figure S4A). Knockdown of NID1 had a significant impact on the growth, migration, invasion and tumor formation abilities compared to shCTL cells (Supplementary Figure S4B-E).

Anti-NID1 antibody effectively inhibits cell growth, migration and invasion of lung cancer, breast cancer and NPC in vitro

Kaplan–Meier analysis of the TCGA database for lung and breast cancer corroborated the functional role of NID1 in cancer progression. Patients with high expression of NID1 had worse overall survival compared to patients with low NID1 levels (Figure 4A). To further investigate the neutralizing activity of the anti-NID1 antibody, we utilized three cell lines, HCC827, MDA-MB-231 and C666–1 cells. Consistently, treatment with the anti-NID1 antibody resulted in a reduction in cellular NID1 levels in all three cell lines (Figure 4B). To assess the functional impact of the anti-NID1 antibody, colony formation, migration, and invasion assays were conducted on the treated and untreated cells. The *in vitro* results were in line with the findings in HCC, demonstrating that treatment with the anti-NID1 antibody significantly reduced the colony-forming, migration, and invasion abilities of HCC827, MDA-MB-231, and C666–1 cells compared to the respective PBS groups (Figure 4C-E).

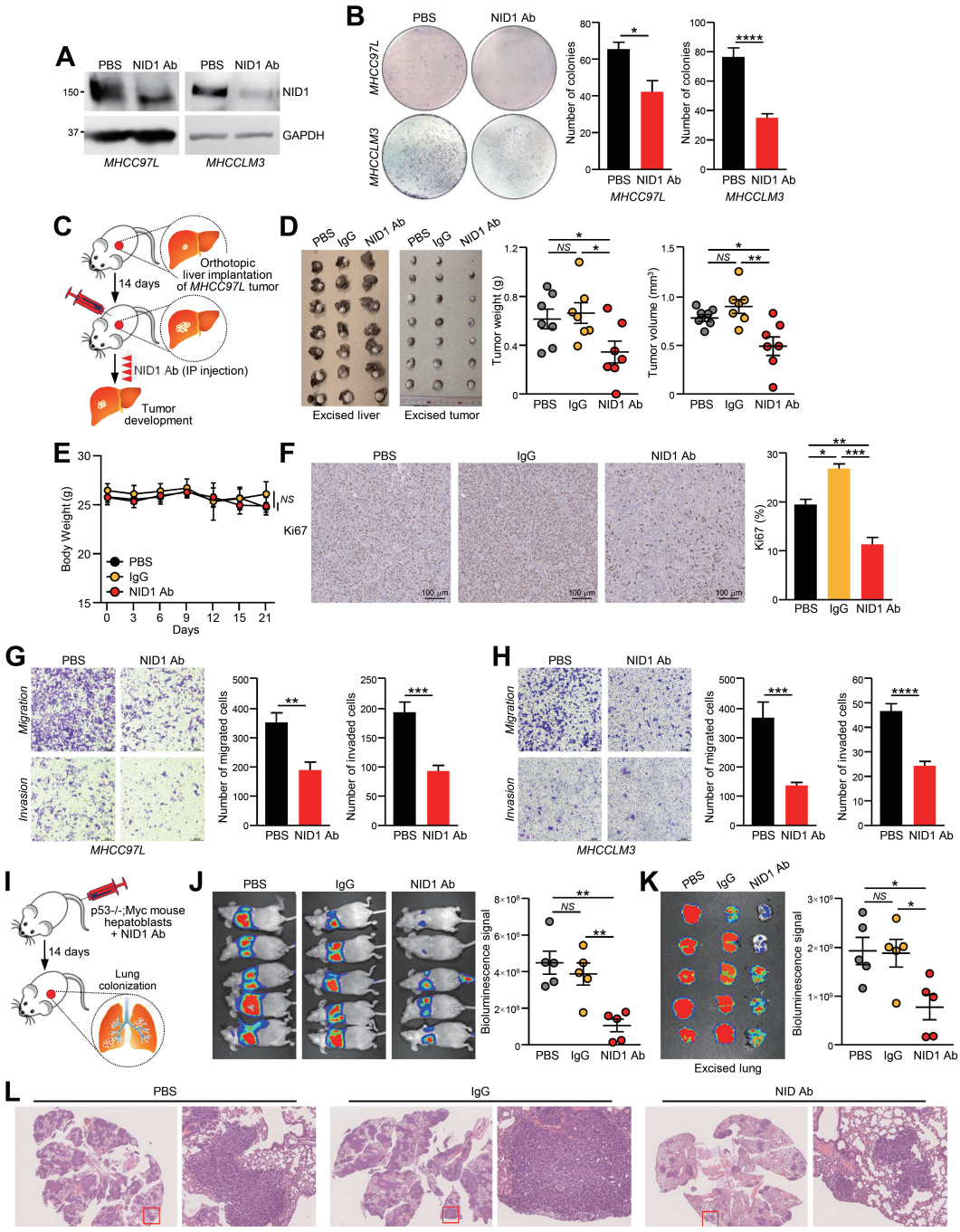

Figure 3: Anti-NID1 antibody inhibits HCC growth and tumorigenesis. A, Immunoblots showing NID1 expression in the total cell lysate (TCL) of MHCC97 and LMHCCLM3 cells treated with anti-NID1 antibody. B, The colony forming ability of MHCC97L and MHCCLM3 cells treated with or without anti-NID1 antibody was assessed by colony formation assay. Representative images of colonies are shown (left). The number of colonies were counted and plotted (right). C, Schematic diagram of orthotopic liver implantation model. orthotopic liver implantation of luciferase-labelled MHCC97L tumor seed ($n = 7$). Two weeks later, PBS, IgG and anti-NID1 antibody (400 µg/mouse) were injected intraperitoneally once a week for 21 days. Development of liver tumor was analyzed 3 weeks after liver implantation. D, At the end of experiment, tumors developed were excised and tumor size and weight were measured. Image showing the liver and tumors (left). Tumor weight (middle) and volume (right) were plotted. E, Body weight was measured regularly for 3 weeks. F, Immunohistochemistry of Ki67 staining of the excised tumors. The number of cells with positive Ki67 staining was counted. Analysis of the migration and invasiveness of MHCC97L (G) and MHCCLM3 (H) cells treated with or without anti-NID1 antibody using migration and invasion assays, respectively. Representative images of migrated and invaded cells are shown (left). Scale bar, 200 μm. Quantification of the number of migrated and invaded cells are plotted (right). I, Lung colonization of murine p53-/-; Myc hepatoblasts (1×10^5) after coinjection with anti-NID1 antibody (10 µg) *via* tail vein ($n = 5$). Mice were subjected to bioluminescence imaging 14 days after injection. J, Bioluminescence imaging of mice at the end of the experiment. Quantification of the luciferase signal is shown. K, Bioluminescence imaging of dissected lung tissues. The intensity of luciferase signal is shown. L, Representative images of H & E staining of lung tissues. Insets show the enlarged area of the metastatic lesions. Scale bar, 150 μm. Data are represented as the mean ± SEM; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. NS, not significant from Student's *t*-test. $P < 0.05$ is considered as statistically significant.

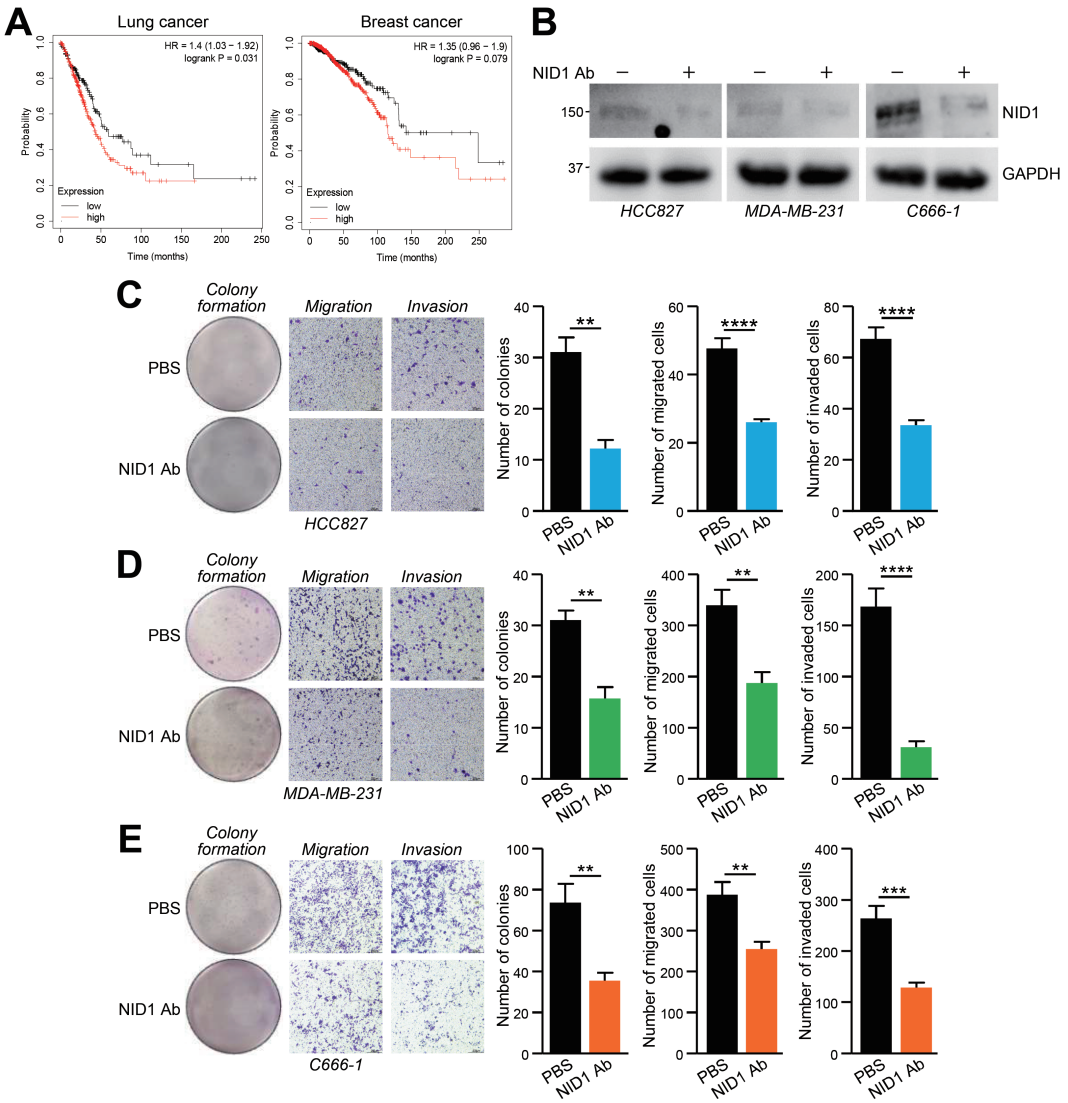

Figure 4: Anti-NID1 antibody effectively inhibits cell growth, migration and invasion of lung cancer, breast cancer and NPC in vitro. A, Kaplan-Meier analysis of overall survival of lung cancer and breast cancer patients with high and low NID1 expression based on the TCGA dataset. B, Immunoblots showing NID1 expression in HCC827, MDA-MB-231, and C666-1 cells treated with anti-NID1 antibody. Examination of the colony forming, migratory and invasive abilities of HCC827 (C), MDA-MB-231 (D), and C666-1 (E) cells treated with or without anti-NID1 antibody. Representative images of colonies, migrated and invaded cells are shown (left). Scale bar, 200 µm. Quantification of the number of colonies, migrated and invaded cells are plotted (right). Data are represented as the mean ± SEM; **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. NS, not significant from Student's *t*-test. $P < 0.05$ is considered as statistically significant.

Anti-NID1 antibody inhibits tumorigenesis of lung cancer, breast cancer and NPC in pre-clinical models

To validate the responsiveness of HCC827, MDA-MB-231, and C666–1 cells to anti-NID1 antibody treatment in an *in vivo* setting, we subcutaneously injected cancer cells into BALB/cAnN-nu mice and administered regular intraperitoneal treatment with the anti-NID1 antibody over a specific time period (Figure 5A, 5D and 5G). Compared to the respective PBS or IgG control groups, tumor development was significantly delayed in the mouse groups injected with the anti-NID1 antibody for all three cancer types (Figure 5B, 5E and 5H). Moreover, the group treated with the anti-NID1 antibody developed smaller tumors compared to the PBS or IgG control groups. Additionally, tumor volume and weight were significantly reduced in the anti-NID1 antibody group (Figure 5C, 5F and 5I). Immunohistochemical staining of the excised tumors from mice treated with the anti-NID1 antibody revealed a significant reduction in Ki67-postive cells (Figure 5J). Importantly, there was no significant change in mouse body weight, indicating that the antibody treatment did not induce major side effects (Figure 5K).

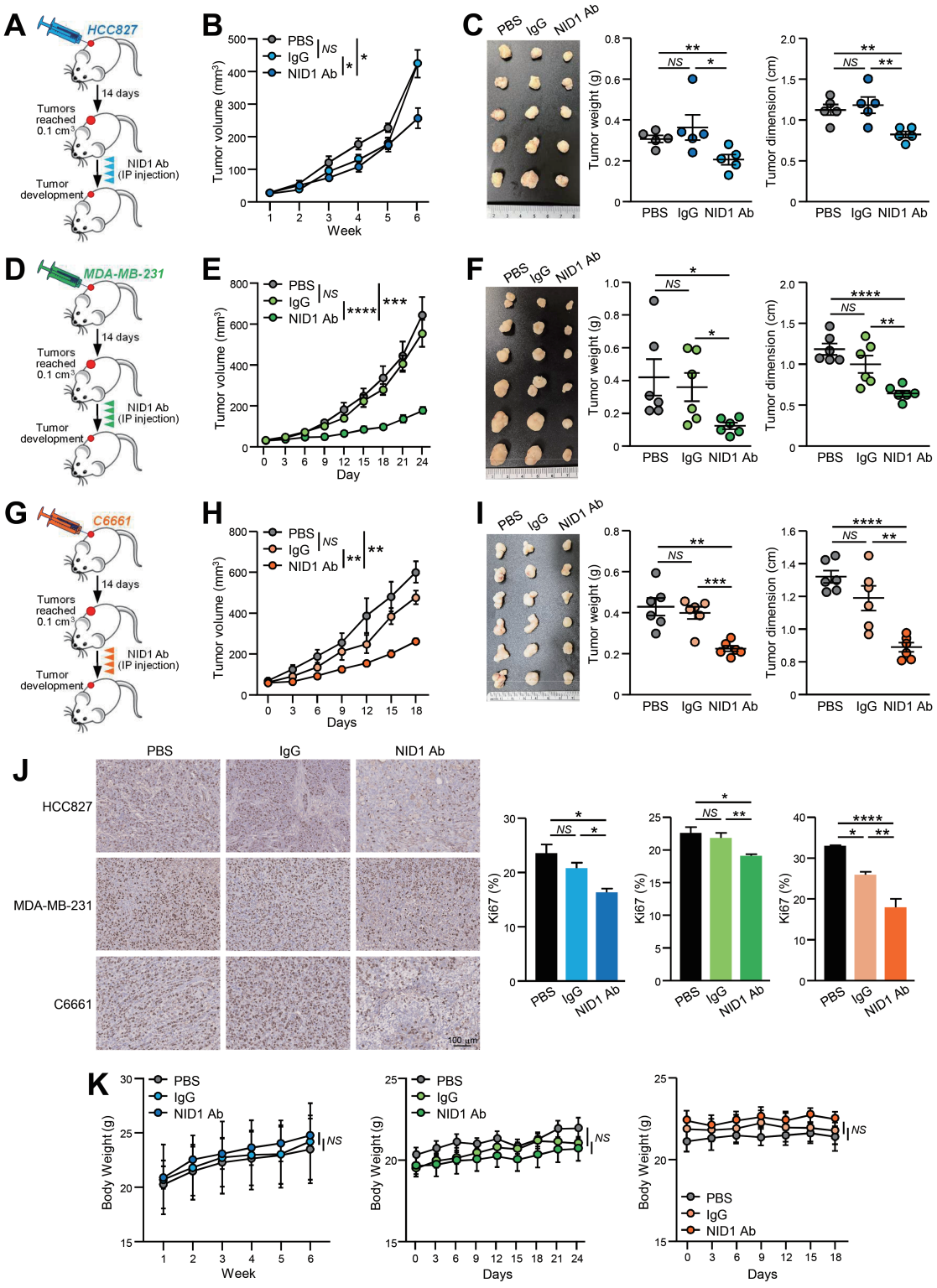

Figure 5: Anti-NID1 antibody inhibits tumorigenesis of lung cancer, breast cancer and NPC in mouse models. HCC827 (A), MDA-MB-231 (D), and C666-1 (G) cells were injected subcutaneously into BALB/cAnN-nu mice. Administration of anti-NID1 antibody was started when the tumor volume reached 0.1 cm3. PBS, control IgG or anti-NID1 antibody (400 μg/mouse) were administered by intraperitoneal injection once every 3 days over a time period. B, E, F, Tumor size was measured regularly and plotted. C. F, I, At the end of experiment, tumors developed were excised and tumor size and weight were measured. Image showing the fixed excised tumors (left). Tumor weight (middle) and dimension (right) were plotted. J, Immunohistochemistry of Ki67 staining of the excised tumors. The number of Ki67 positively stained cells was analyzed. Scale bar, 100 μm. K, Body weight was measured regularly during the course of experiment. Data are represented as the mean ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. NS, not significant from Student's *t*-test. $P < 0.05$ is considered as statistically significant.

Taken together, these findings provide further confirmation of NID1's potential as a therapeutic target for cancer treatment. Targeting NID1 with the anti-NID1 antibody shows promise as an approach for treating various cancer types, including HCC, lung cancer, breast cancer, and NPC.

Anti-NID1 antibody deregulates HIF-1a pathway in cancer cells

To investigate the underlying mechanism of the anti-tumor effect of the anti-NID1 antibody in different cancer cell lines, RNA sequencing was performed on MHCC97L cells treated with and without the anti-NID1 antibody. The analysis revealed significant alterations in gene expression in cells treated with the anti-NID1 antibody, with numerous genes showing upregulation or downregulation (Supplementary Table S2, Supplementary Figure S5A). The analysis revealed that a total of 73 genes exhibited significant deregulation with at least a 2-fold change in expression upon antibody treatment (Supplementary Figure S5B). Further analysis utilizing KEGG pathway analysis was conducted to explore the potential impact of the deregulated genes. The results indicated the alteration of the hypoxia-inducible factor 1-alpha (HIF-1α) pathway by the anti-NID1 antibody treatment (Supplementary Figure S5C). HIF-1α is a key transcription factor that plays a pivotal role in cellular responses under hypoxic conditions, regulating genes involved in angiogenesis, metabolism, and cell survival.[27–29] These processes are essential for tumor growth and metastasis. Therefore, the results suggest that the anti-NID1 antibody may modulate the HIF-1α pathway, affecting genes that promote cell proliferation and motility. This provides a potential mechanistic insight into the anti-tumor effect of the anti-NID1 antibody. Notably, genes related to hypoxia signature were shown to be downregulated (Supplementary Figure S5D). Among these genes, ALDOC, PGK1, LDHA and SLC2A1 are glycolytic-related genes induced by HIF-1α, and are commonly overexpressed in many cancers.[30,31] Studies have explained a strong correlation between hypoxia and glycolysis, suggesting it as a metabolic adaptation for cancer under hypoxic TME.[32] This explains why glycolysis is the second most-deregulated pathway following antibody treatment (Supplementary Figure S5C). Conversely, the expression of LDHB, which encodes an enzyme responsible for pyruvate and lactate interconversion,[33] was shown to be elevated after antibody addition (Supplementary Figure S5D). In fact, it is found that LDHB is commonly upregulated in normoxic growth condition.[34] In conclusion, the treatment of cancer cells with anti-NID1 antibody was demonstrated to extensively deregulate the HIF-1α pathway, suggesting its involvement in modulating genes related to cell proliferation, motility, and metabolic adaptations in response to hypoxic conditions. These findings provide valuable mechanistic insights into the anti-tumor effect of the anti-NID1 antibody.

DISCUSSION

The ECM proteins, as essential components of the TME, play a crucial role in promoting carcinogenesis and metastasis. These dysregulated ECM proteins work in coordination with other oncogenic factors, ranging from cytokines to the vascular network, to establish a conducive TME and a pre-metastatic niche. Gaining a comprehensive understanding of the ECM components is important for the development of effective therapeutic interventions. During the process of cancer metastasis, cancer cells need to protrude the mechanical barrier of the basement membrane for extravasation. BM proteins, such as collagen and integrin, have been demonstrated to assist cancer cell escape by mediating cellular interactions, particularly during the epithelial-to-mesenchymal transition (EMT) remodeling.[35,36] These proteins have emerged a promising target for drug intervention. Notably, studies have demonstrated the synergistic effects of anti-integrin complex monoclonal antibody (mAb) when combined with chemotherapeutic drugs in pancreatic ductal adenocarcinoma [37] and epithelial ovarian cancer.[38] These findings underscore the potential of ECM regulator blockade as an effective therapeutic strategy in various types of cancer.

NID1 is a glycoprotein located in the basement membrane and serves as a critical component of the ECM.[39] It interacts with other basement membrane components, and mediates ECM repair and remodeling after injuries.[40,41] Functionally, NID1 has been found to augment tumor cell proliferation, metastatic potential and angiogenesis.[42] Its role in tumorigenesis and metastasis of cancer has been extensively described in various types of cancer, where it enhances cancer cell migration and invasion.[5] Furthermore, NID1 promotes cancer cell adhesion to the endothelium, disrupts endothelial integrity and enhance vascular tube formation capacity. In colorectal cancer, secretory NID1 released by tumor cells promote tumor progression by inducing EMT in neighboring tumor cells.[16,43] Moreover, sEV-NID1 promotes lung colonization of HCC cells by activating fibroblasts to secrete tumor necrosis factor receptor 1.[17] The overexpression of NID1 in cancer is speculated to be caused by aberrant B-Myb or bromodomain-mediated transcriptional mechanisms.[44] The addition of JQ1 inhibitor can effectively suppress the RUNX2/NID1 signaling pathway.[45] It has been reported that silencing of NID1 results in compromised tumorigenicity. MicroRNA-1298–3p inhibits tumor progression by reducing the expression of NID1 in glioma cells.[46] In this study, we demonstrated the oncogenic role of NID1 in the development of four epithelial-derived

cancers, including HCC, NSCLC, breast cancer, and NPC. Utilizing shRNA, our research revealed that the loss of NID1 resulted in less aggressive tumor behaviors both *in vitro* and *in vivo*, consistent with the previous findings.

Monoclonal antibodies are well-known for their high specificity, prolonged long half-life, and minimal side effects due to their ability to bind to specific molecules on the outer surface of tumor cells.[47,48] Leveraging these advantages, our study aimed to target NID1 by developing a monoclonal antibody using hybridoma technology. We evaluated the therapeutic potential of our homemade antibodies against the four cancer cell lines and observed a reduction in NID1 proteomic expression upon treatment. This suggests that the antibody may function by inhibiting the uptake or entry of NID1 into cancer cells. Additionally, we assessed the anti-tumor effect of the antibody and found that it significantly inhibited the migration, invasion ability and clonogenicity of cancer cells. These observations were further supported by *in vivo* testing, where the anti-NID1 antibody effectively hampered orthotopic and subcutaneous tumor growth. Importantly, treated mice did not experience a significant drop in body weight, indicating that the injection of the anti-NID1 antibody did not pose severe side effects. Building upon our previous findings that delineated the role of EV-NID1 in facilitating HCC extrahepatic metastasis to the lung,[17] we hypothesized that the anti-NID1 antibody could effectively inhibit HCC's metastatic ability. Using a lung colonization model, we demonstrated that antibody treatment could block the colonization ability of hepatoblasts. providing further evidence for the potential therapeutic efficacy of our anti-NID1 antibody in cancer metastasis.

Our findings demonstrated that the anti-NID1 antibody possesses anti-tumorigenic properties against four cancer cell lines. However, the underlying mechanism of action of the anti-NID1 antibody remains to be determined. RNA sequencing of antibody-treated cells revealed HIF-1α pathway was significantly deregulated. Hypoxia, a common characteristic of solid cancers, is accompanied with enhanced HIF-1α expression and transcriptional activity.[49] Aberrant HIF-1α expression results in the upregulation of genes associated with proliferation, metastasis, and drug resistance, contributing to poor prognosis in multiple cancers.[50] Given that antibody treatment reduces cellular NID1 levels, it suggests a potential association between NID1 and the HIF-1α pathway. Further investigations are necessary to elucidate the precise molecular mechanisms through which NID1 and the anti-NID1 antibody influence the HIF-1α pathway and its downstream targets in cancer cells.

This study provides valuable insights into the oncogenic role of NID1 in four epithelial cancers, highlighting its potential as both a biomarker and a therapeutic target. Notably, a monoclonal antibody targeting NID1 has been successfully developed, and its therapeutic efficacy has been demonstrated both *in vitro* and *in vivo*. These findings pave the way for further exploration of NID1 as a promising candidate for diagnostic and therapeutic applications in the context of these cancer types.

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Author Contributions

T. Xue: Data curation, formal analysis, investigation, methodology, writing-original draft. C.L. S. Yeung: Data curation, formal analysis, investigation, methodology, writing-review and editing. X. Mao: Methodology. S.K. Tey: Methodology. K.W. Lo: Resources, Methodology. A.H. N. Tang: Data curation, investigation. J.P. Yun: Resources. J.W. P. Yam: Conceptualization, project administration, supervision, funding acquisition, writing-review and editing.

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Ethical Approval

Approval for the use of human tissues was obtained from the Institutional Review Board of Sun Yat-sen University Cancer Centre. All procedures involving human specimens in this study were conducted in accordance with the applicable ethical regulations.

Informed Consent

The collection of samples was executed with informed consent.

Conflict of Interest

The authors declare no competing interest.

Use of Large Language Models, AI and Machine Learning Tools

None declared.

Data Availability Statement

The data generated in this study are available upon request from the corresponding author.

Author's Disclosure

J.W.P. Yam reports a patent for anti-NID1 antibody pending and shares ownership in DiagnoVEX Therapeutics Ltd. X. Mao reports a patent for anti-NID1 antibody pending. S.K. Tey reports a patent for anti-NID1 antibody pending. No disclosures were reported by the other authors.

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