

**Abstract**

**Purpose:** Epigenetic alterations play important roles in metastasis and drug resistance through gene regulation. However, the functional features and molecular mechanisms of epigenetic changes remain largely unclear in nasopharyngeal carcinoma (NPC) metastasis.

**Experimental Design:** Gene regulatory network analysis was used to identify metastatic-specific dysregulated genes between normal and NPC tissues and the expression was validated in published Gene-Expression Omnibus data set. The regulatory and functional role of RAB37 downregulation was examined in NPC and was validated in *vivo* and *in vitro*, and downstream target of RAB37 was explored. The clinical value of RAB37 methylation was evaluated in NPC metastasis and chemosensitivity.

**Results:** We identified RAB37 as a specific hypermethylated gene that is most commonly downregulated in NPC. Moreover, RAB37 downregulation was attributed to hypermethylation of its promoter and was significantly associated with metastasis- and docetaxel chemoresistance-related features in NPC. Ectopic RAB37 overexpression suppressed NPC cell metastasis and enhanced chemosensitivity to docetaxel. Mechanistically, RAB37 colocalized with TIMP2, regulated TIMP2 secretion, inhibited downstream MMP2 activity, and consequently altered NPC cell metastasis. Furthermore, RAB37 hypermethylation was correlated with poor clinical outcomes in patients with NPC. We developed a prognostic model based on RAB37 methylation and N stage that effectively predicted an increased risk of distant metastasis and a favorable response to docetaxel-containing induction chemotherapy (IC) in NPC patients.

**Conclusions:** This study shows that RAB37 hypermethylation is involved in NPC metastasis and chemoresistance, and that our prognostic model can identify patients who are at a high risk of distant metastasis and might benefit from for docetaxel IC.

**Introduction**

Nasopharyngeal carcinoma (NPC), one of the common head and neck cancers in Southeast Asia, especially southern China, is characterized by high invasion and early metastasis (1). Due to the obscure symptoms and aggressiveness of NPC, more than 70% of patients with NPC are diagnosed with locoregionally advanced disease at the initial visit and often have poor prognoses (2, 3). Patients with NPC are diagnosed with locoregionally advanced disease (LA-NPC) and result in substantial improvement in locoregional control (4, 5). However, the increased likelihood of systemic metastasis remains the major cause of treatment failure and cancer-related mortality for these patients.

Accumulating studies report that induction chemotherapy (IC) might improve metastasis control in patients at a high risk of distant metastasis (6–8). Moreover, our phase III trial confirmed that the addition of docetaxel, cisplatin, and fluorouracil (TPF) IC to concurrent chemoradiotherapy (CCRT) can significantly improve the 3-year distant metastasis-free survival (DMFS) for LA-NPC patients (6). However, this treatment strategy only results in 7% improvement in metastasis control. This survival difference suggests the presence of unknown heterogeneity among NPC patients and highlights the urgent need for an effective strategy to identify patients at a high risk of metastasis who will benefit from IC. Currently, the anatomic tumor–node–metastasis (TNM) staging system is the major indicator to assess prognosis and guide therapeutic decision, but this system is not very effective (4, 9). Therefore, exploration of the molecular mechanisms and identification of new biomarkers reflecting tumor heterogeneity can help to provide innovative strategies to stratify precise risk and guide individual treatment for metastasis in patients with NPC.

Epigenetic alterations have been shown to profoundly influence the maintenance and evolution of cancer (10). DNA methylation is a ubiquitous and dynamic epigenetic alteration critical for cellular development, function, and gene-expression regulation (11). A substantial number of studies have reported widely aberrant DNA methylation patterns in various tumor types (12). Altered DNA methylation patterns may be relevant for the
Translational Relevance

Metastasis is the major reason of treatment failure in nasopharyngeal carcinoma (NPC). Our phase III clinical trial confirmed that docetaxel, cisplatin, and fluorouracil (TPF) induction chemotherapy (IC) significantly improved the distant metastasis control in NPC. However, this pattern provides only results in 7% improvement in metastasis control, which promotes the urgent need to develop a better patient stratification to efficiently identify patients at high risk of metastasis for IC. Here, we identified RAB37 as a specific hypermethylated gene that is consistently downregulated, contributing to metastasis and docetaxel-based chemoresistance in NPC. In patients with NPC, RAB37 hypermethylation was correlated with poor clinical outcomes. We developed a prognostic model based on RAB37 methylation and N stage that effectively predicted an increased risk of distant metastasis and a favorable response to docetaxel-contained IC in patients with NPC, thereby facilitating the development of novel predictive and therapeutic strategies against NPC metastasis.

dysregulation of specific genes or signaling pathways, leading to tumor initiation, progression, and treatment resistance (13). Thus, there is a strong rationale for evaluating the interaction and enrichment of dysmethylated genes involved in identified features and signaling pathways. Although several abnormally methylated genes have been identified in NPC (14–16), their interaction and contribution to metastasis and chemoresistance remain elusive.

Here, using gene regulatory network analysis, we identify RAB37, a member of RAS superfamily, as a specific hypermethylated gene that is consistently downregulated in NPC. RAB37 hypermethylation contributes to its decreased expression and is relevant to metastasis- and chemoresistance-related properties in NPC. Restored RAB37 expression inhibits metastasis through the tissue inhibitor of the metalloproteinase 2 (TIMP2)/matrix metalloproteinase 2 (MMP2) axis and enhances sensitivity to docetaxel in NPC cells. Moreover, RAB37 hypermethylation is correlated with unfavorable survival and, when combined with advanced N stage, can effectively identify patients who are at a high risk for distant dissemination and are likely to benefit from docetaxel-based IC in NPC. Therefore, this study highlights the oncogenic role of RAB37 hypermethylation and provides innovative strategies to identify patients at a high risk for metastasis who will benefit from docetaxel IC, thereby providing novel perspectives for the precise treatment of NPC.

Materials and Methods

Methylation microarray analysis

Our previous Infinium Human Methylation 450 K BeadChip microarray data were reanalyzed to identify gene interaction networks between NPC samples and normal nasopharyngeal epithelial tissues (GSE52068). We used GenomeStudio Methylation Module V.1.9.0 to extract image intensities and the algorithm in "methylumi" and "lumi" packages in R Bioconductor for normalization and background correction. The DNA methylation levels for per-probe loci were determined using $\beta$-values of normalized signal intensities. Using the "IAMA" Bioconductor package, we filtered significantly different methylation loci with the criteria of single-nucleotide polymorphism probes, $P \geq 0.01$ in 12 of 48 samples and probes located in the X and Y chromosomes. Finally, 473,788 CpG sites were subjected to further analysis. The nonparametric Wilcoxon rank-sum test with false discovery rate (FDR) adjustment was used to identify significantly different DNA methylation loci.

"GRENITS" package from R Bioconductor was used to cluster differentially dysmethylated loci into causal interaction networks from methylation microarray data in groups of NPC and normal tissues with fully Bayesian spline autoregression (17). Differential loci with interaction network probability $>0.99$ between groups were filtered for further function-associated network analysis using Cytoscape software. The final differential loci involved in functional network were clustered and visualized by heat map analyses ($P < 0.05$ and $\Delta \beta \geq 0.2$).

Clinical specimens

For RAB37 promoter methylation level analysis, we obtained 8 freshly frozen NPC specimens and 8 normal nasopharyngeal epithelial specimens from Sun Yat-sen University Cancer Center (Guangzhou, China). For methylation level and survival analysis, we collected a total of 110 paraffin-embedded NPC specimens from Sun Yat-sen University Cancer Center between March 2004 and April 2007. None of the patients had received radiotherapy or chemotherapy before biopsy, and all patients were diagnosed with nonmetastatic NPC. The 7th edition of the AJCC Cancer Staging Manual was used to reclassify TNM stages (18). The median follow-up period was 102.95 months (range, 6.9–133.0 months). The pathologic types were all WHO III. All patients received radiotherapy, and patients with stage III–IV NPC received IC plus concurrent platinum-based chemotherapy or concurrent chemotherapy alone. The detailed clinicopathologic characteristics are shown in Supplementary Table S3. This study has been approved by the Institutional Ethical Review Boards of the Sun Yat-sen University Cancer Center and conducted in accordance with the Declaration of Helsinki. Written informed consents obtained from all patients.

Propensity score matching analysis was used to adjust for patient selection bias and create two clinicopathologic matched cohorts with or without docetaxel IC. The propensity score was calculated with host and tumor factors, including age, sex, T stage, N stage, WHO pathologic type, Epstein–Barr virus antigen immunoglobulins (VCA-IgA and EA-IgA) and radiotherapy methods using the caliper algorithm with 0.05.

DNA extraction and bisulfite pyrosequencing analysis

Genomic DNA was extracted from all tissues and cells using an AllPrep RNA/DNA Mini Kit (Qiagen), QIAamp DNA FFPE Tissue Kit (Qiagen), or EZ1 DNA tissue Kit (Qiagen), respectively. Bisulfite modification of DNA was conducted using an EpTect Bisulfite Kit (Qiagen). The bisulfite pyrosequencing primers were designed with PyroMark Assay Design Software 2.0 (Qiagen) and listed as follow: Forward: 5′-TTAGGGTAAAGAGATTA- TAGGGG-3′, Reverse: 5′-AATCTACCATTTAACCACAACCC- TCC-3′, and sequencing: 5′-GGTTAGGGTGTGAT-3′. The PyroMark Q96 ID System and software (Qiagen) were applied to quantify the methylation level. The percentage of individual CpG
methylations were calculated by average methylation level. For methylation and survival analysis, cutoff methylation value with 5.8% was determined by receiver-operating characteristic (ROC) curve analysis (19).

**Cell culture**

NP69, a human immortalized nasopharyngeal epithelial cell line, was maintained in Keratinocyte/serum-free medium (Invitrogen) supplemented with bovine pituitary extract (BD Biosciences). Human NPC cell lines (CNE2, CNE1, SUNE1, HONE1, HNE1, and C666-1) were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gibco). NP69 and all NPC cell lines, which had been authenticated, were bountifully provided by Dr. Mu-sheng Zeng (Sun Yat-sen University Cancer Center). 293FT cells obtained from ATCC were generously provided by Dr. Mu-sheng Zeng (Sun Yat-sen University Cancer Center). Gene expression profiles of 31 NPC specimens (GSE12452) were used to conduct GSEA to identify gene signatures between groups with high and low RAB37 expression. We used the Molecular Signatures Database (MSigDB) C2 collection of chemical and genetic perturbations ($n = 3,409$ gene sets) and the GSEA algorithm (http://software.broadinstitute.org/gsea/msigdb; ref. 25) to rank genes with expression levels correlated with RAB37 expression levels across NPC samples. GSEA results are shown using normalized enrichment scores, accounting for the size and degree to which a gene set is overrepresented at the top or bottom of the ranked list of genes ($P < 0.05$ and FDR $\leq 0.25$).

An enrichment heat map and a STRING network (https://string-db.org/) were used for visualization of the GSEA results with the top 100 ranked genes (50 with positive score and 50 with negative score) in RAB37-low vs. RAB37-high groups.

**RNA extraction and real-time PCR**

Total RNA from cultured cells and clinical samples were isolated with TRizol reagent (Invitrogen). Total RNA was reverse transcribed using random primers and M-MLV reverse transcriptase (Promega). SYBR Green-based (Invitrogen) real-time PCR analysis was carried out in a CFX96 Touch sequence detection system (Bio-Rad). Real-time PCR primers for RAB37 and TIMP2 were as follows: Forward: 5'-TGGCTGCTAGGCGAACAGG-3', Reverse: 5'-CCCCAAGGCGTACTCCA-3', Forward: 5'-AGATG-CTTGATAGGCTTGAAC-3', Reverse: 5'-ACGCCCTTTGAC-3'. GAPDH was regarded as an endogenous control for all the genes. The $2^{-\Delta\Delta CT}$ equation was utilized to calculate the relative gene expression (20).

**Western blotting assay**

Total protein was obtained using RIPA buffer (Beyotime Biotechnology) containing EDTA-free Protease Inhibitor Cocktail (Roche). Protein extracts were separated via 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Merck Millipore). The membranes were blocked in 5% nonfat milk and incubated with primary antibodies targeting RAB37 (1:400; Proteintech, 13051-1-AP), TIMP2 (1:600; Proteintech, 13051-1-AP), $\alpha$-tubulin (1:500; Sigma-Aldrich, T5266), and GAPDH (1: 5,000; Abcam, ab70699) overnight at 4°C. Secondary antibody incubation was performed using horseradish peroxidase-conjugated antibodies (anti-mouse or anti-rabbit; 1:2,000; Proteintech) at room temperature. Finally, the target protein bands were detected using an enhanced chemiluminescence system (Thermo Fisher Scientific).

**Gene-expression profiling and gene-expression profiling interactive analysis (GEPIA)**

Gene-expression profiles of NPC were derived from Gene-Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under the series accession numbers GSE12452, GSE53819, and GSE64634. GEPIA (21) was conducted to examine the expression level of RAB37 in different cancer types and corresponding normal tissues (http://gepia.cancer-pku.cn/index.html) based on The Cancer Genome Atlas (TCGA; ref. 22) and GTEx data (23, 24).

**Oligonucleotides, plasmids, and stable cell lines**

Effective siRNA oligonucleotides targeting RAB37 and TIMP2 were obtained from GenePharma. The sequences were as follows: siRAB37-1, 5'-CCATGCTATTACAGAGA-3'; siRAB37-2, 5'-GCCATCCCATGCTTATTA-3'; siTIMP2-1, 5'-GAGATCAAGCAGATAAAAGA-3'; and siTIMP2-2, 5'-CGAAGAGAGGATATCCTCA-3'. The pSin-EF2-puro-vector and TIMP2 plasmids were purchased from Vigeone Biosciences. The sequence of the human RAB37 gene was synthesized and cloned into the lentiviral plasmid pSin-EF2-puro-VCMD (Addgene). The primers used for amplification were as follows: RAB37-Forward, 5'-CGGATCATGAGGCAGGACCGCAGCGGCAC-3', RAB37-Reverse, 5'-GGAGCGCAGGATGAGATCCTCA-3'. Lentivirus packaging expression plasmids were cotransfected into 293FT cells to generate stably transfected cell lines. The lentiviral particles were subsequently harvested and infected into NPC cells 48 hours later. Stable clones were then selected using 0.5 μg/mL puromycin (Sigma-Aldrich), and real-time RT-PCR or Western blotting assays were used to validate the infection efficiency.

**Wound-healing, Transwell migration, and invasion assays**

For wound-healing assay, transfected cells were seeded in 6-well plates and starved for 24 hours. Confluent monolayers were scratched using a 10-μL tip, and images were captured at 0 hour and 36 hours. For migration and invasion assays, 5 × 10³ or 1 × 10⁴ transfected cells were resuspended in serum-free medium and plated in the upper transwell chamber (Corning) with 8-μm pore size membrane with or without Matrigel (BD Biosciences). Then, medium supplemented with 10% FBS was placed in the lower chamber. After 12 or 24 hours of incubation, the cells that migrated or invaded through the upper membrane were fixed, stained with hematoxylin, and counted using an inverted microscope.

**Docetaxel treatment**

For chemotherapy, 5 × 10⁴ SUNE1 and HONE1 cell lines stably expressing RAB37 or vector were seeded in 96-well plates. The next day, the cells were treated with or without docetaxel for 48 hours at the dose of 0, 2.5, 5, 10, 20, and 40 μg/mL. Cell viability was measured, and the concentration of 50% inhibition of cell growth (IC₅₀) was calculated using SPSS 22.0 software (IBM).
Mass spectrometry and coimmunoprecipitation (co-IP) assay

For immunoprecipitation (IP) assays, SUN1E or HONE1 cells were lysed with IP lysis buffer. Primary anti-HA (2 μg; Sigma-Aldrich, H6908) or anti-lgG (negative control, 3 μg; Proteintech, 30000-0-AP) antibodies were incubated with the lysis over-night at 4°C. Protein A/G Sepharose beads (Thermo Scientific) were used to recover the immune complexes, which were then washed and collected after being isolated. Mass spectrometry was performed by Huijun Biotechnology. For co-IP assay, Western blotting was performed to determine protein levels. Cells were trypsinized and rinsed in PBS and then lysed with RIPA lysis buffer. The protein extracts were subsequently incubated using anti-RAB37 and anti-TIMP2 antibodies, followed by precipitation with protein A agarose (Invitrogen) and immobilized on protein G Sepharose beads. The precipitated proteins were separated and detected by Western blotting using rabbit antibodies. Finally, the blots were visualized using a chemiluminescence system.

Immunofluorescence staining

For immunofluorescence staining assays, cells were grown on coverslips (Thermo Fisher Scientific). After 24 hours, cells were fixed with 2% paraformaldehyde, permeabilized in 0.5% Triton X-100 in PBS, and incubated with primary anti-E-cadherin (1:100; BD Biosciences, 610181), anti-Vimentin (1:100; Proteintech, 10366-1-AP), anti-RAB37 (1: 200; Proteintech, 13051-1-AP), and anti-TIMP2 (1: 200; Proteintech, 17353-1-AP) antibodies overnight at 4°C. The coverslips were incubated with Alexa Fluor 488 or 594 goat IgG secondary antibody (1:1,000; Life Technologies; A-11008 or A-11001) and counterstained with 4',6-diamidino-2-phenylindole (DAPI). Images were captured using a confocal laser-scanning microscope (Olympus FV1000).

Gelatin zymography and ELISA assay

The level of MMP2 in the medium was tested using gelatin zymography assays. Briefly, conditioned medium (CM) was collected and analyzed using 10% SDS–PAGE containing 0.1% gelatin. After electrophoresis, the gel was washed with 2.5% Triton X-100 at room temperature for 1 hour to remove SDS and renature MMP2 and then incubated at 37°C overnight in the developing buffer. The gel was subsequently stained with 0.25% Coomassie Blue R-250 for 2 hours and destained in 50% methanol and 10% glacial acetic acid to reveal zones with β-casein activity. Clear bands indicate proteolytic activity. ELISA was performed using the TIMP2 ELISA Kit according to the manufacturer’s instructions (Jianglaibio).

In vivo lung metastasis model

Female BALB/c nude mice (4–6 weeks old, 15–18 g) were purchased from Beijing Vital River Laboratory Co., Ltd. For the lung metastasis model, SUN1E cells that stably overexpressed the vector or RAB37 were resuspended in PBS, and 2 × 105 cells were injected into the tail veins of mice (n = 8 per group). Eight weeks later, the mice were sacrificed, and lung metastatic colonies were quantified. For the lung metastasis model following docetaxel treatment, HONE1 cells with or without RAB37 overexpression (2 × 105 cells in 100-μL PBS) were injected into the tail veins of mice. Two weeks later, 100-μL saline or docetaxel at a dose of 2 mg/g were delivered via intraperitoneal injection, once per week for 3 weeks. Three weeks later, the mice were sacrificed for further analysis. All animal experiments were performed according to protocols provided by the Institutional Animal Care and Use Committee of Sun Yat-sen University Cancer Center (approval number: L102012017002J). The experimental methods complied with the Declaration of Helsinki.

IHC staining

IHC was performed to detect the expression levels of RAB37 and TIMP2 on paraffin-embedded sections prepared from in vivo experiments. Briefly, the sections were deparaffinized, rehydrated, subjected to endogenous peroxidase activity blocking, antigen retrieval, nonspecific binding blocking, and incubated with primary antibodies (RAB37, 1:200, Proteintech, 13051-1-AP; TIMP2, 1:200, Proteintech, 17353-1-AP) at 4°C overnight. All sections were scored and validated by the two experienced pathologists.

Statistical analysis

All data are presented as the mean ± SD from at least three independent experiments. Two-tailed Student t tests were used for comparisons between groups. Comparisons among categorical variables were performed with χ2 and Fisher exact tests. The Kaplan–Meier method and log-rank test were, respectively, used to construct survival curves and compare the differences. Independent prognostic factors were determined by multivariate analysis using a Cox proportional hazards regression model. P values < 0.05 were considered statistically significant. Statistical analyses were performed using SPSS 22.0 software (IBM).

Data availability

The microarray data sets used in this paper have been deposited at Gene-Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under the series accession numbers GSE52068, GSE12452, GSE53819, and GSE64634. Gene-expression profiling interactive analysis in different cancer types and corresponding normal tissues was deposited at GEPIA based on TCGA and GTEx data (http://www.ncbi.nlm.nih.gov/geo/). The authenticity of this article has been validated by uploading the key raw data onto the Research Data Deposit public platform (http://www.researchdata.org.cn), with the approval RDD number as RDDDB2018100395.

Results

RAB37 promoter is hypermethylated in NPC

The regulatory network constructed by genomic or epigenetic aberrations has been revealed to reflect the core molecular mechanisms underlying tumorigenesis and progression (26–28). Previously, our methylation microarray study (GSE52068) identified 473,788 differential methylated gene CpG locus between normal human nasopharyngeal (n = 24) and NPC tumor (n = 24) samples (13). However, the regulatory interaction among these dysmethylated loci remains unclear. Here, using a semiparametric Bayesian model, we found differentially coregulatory methylation loci in networks between NPC and normal tissues with the criteria of interaction link probability > 0.99 (Supplementary Table S1). To further evaluate function-associated networks, 19 methylation loci were identified to be involved in regulatory network interactions (Supplementary Fig. S1). Interestingly, these methylation loci could distinguish NPC tissues from normal tissues and clustered together in hierarchical cluster analysis (P < 0.05 and ABC ≥ 0.2, Fig. 1A). To identify genes for
Figure 1.
RAB37 promoter is hypermethylated in NPC tissues. A, Heat map clustering of dysmethylated genes involved in protein-protein interaction networks between NPC (n = 24) and normal nasopharyngeal tissues (n = 24). B, Schematic location of CpG islands (blue area) within the RAB37 promoter region. The transcriptional start site is indicated with an arrow. Regions analyzed by bisulfite pyrosequencing are listed (bottom). C–E, Bisulfite pyrosequencing analysis and quantitative methylation levels of freshly frozen normal nasopharyngeal epithelial tissues (n = 8) and NPC tissues (n = 8) (C, D) and immortal NP69 and NPC cell lines (E). Cell line methylation results are presented as the mean (n = 3) ± SD. Student t-test, **, P < 0.01.
which the altered methylation status was causally linked to expression dysregulation, we examined the mRNA expression of methylated genes in public data sets from the GEO. Notably, 10 genes were differentially expressed in NPC in at least one data set, but only RAB37 was significantly downregulated in all three data sets (GSE12452, GSE53819, and GSE64634; Supplementary Table S2).

To further validate the dysregulated methylation status of RAB37, we performed bisulfite pyrosequencing analysis in other normal (n = 8) and NPC tissues (n = 8). Figure 1B shows the CpG islands and bisulfite pyrosequencing regions in the RAB37 promoter. Compared with those in normal tissues, the methylation levels of RAB37 were significantly upregulated in NPC tissues (Fig. 1C and D, P < 0.01). Consistently, the RAB37 methylation level was particularly higher in NPC cell lines than in immortalized normal human nasopharyngeal epithelial cells (Fig. 1E, P < 0.05). Collectively, these data suggest that RAB37 is hypermethylated in NPC.

RAB37 promoter hypermethylation contributes to its downregulation in NPC

To investigate the causal link between RAB37 promoter methylation and RAB37 downregulation, we first examined RAB37 expression by quantitative RT-PCR and Western blotting assays in tissues and cell lines. The results showed that compared with that in normal epithelial samples, RAB37 was notably downregulated in freshly frozen NPC samples (Fig. 2A, P < 0.01). Similarly, the RAB37 expression level was significantly lower in NPC cell lines than in immortalized normal human nasopharyngeal epithelial cells (Fig. 2B, P < 0.01). To further verify these findings, we performed external validation using public GEO database and demonstrated that RAB37 was also notably downregulated in patients with NPC in three data sets (GSE12452, GSE53819, and GSE64634; Figure 2C, Supplementary Fig. S2, and Supplementary Table S2, P < 0.05). Moreover, RAB37 expression levels were broadly downregulated across different cancer types, including urothelial bladder carcinoma, lymphoma, glioma and lung carcinoma, based on TCGA (22) and GTEx data (23, 24) by using GEPIA tool (Fig. 2D, P < 0.05). Taken together, these results indicate that RAB37 is downregulated in NPC and potentially in multiple other tumor types, including lymphoma, glioma, bladder, and lung carcinoma.

Next, we investigated whether RAB37 promoter hypermethylation leads to RAB37 expression reduction. To this end, both NP69 and NPC cells were treated with or without 5-aza-2’-deoxycytidine (DAC, an inhibitor of DNA methyltransferase) for 72 hours. Following DAC treatment, RAB37 promoter methylation levels were found to be significantly lower in NPC cells than in NP69 cells (Fig. 2E; Supplementary Fig. S3, P < 0.05). Conversely, the mRNA and protein levels of RAB37 were obviously increased in NPC cells following DAC treatment (Fig. 2F, P < 0.01). Thus, these findings illustrate that RAB37 downregulation results from its promoter hypermethylation in NPC.

RAB37 downregulation is associated with metastasis and chemoresistance in NPC

To understand the potential impact of RAB37 downregulation on the NPC phenotype, we performed GSEA and compared gene profiles of NPC samples with high and low RAB37 levels in the GSE12452 data set to identify biological differences between the two subgroups (Fig. 3A). GSEA revealed that compared with RAB37-high NPCs, RAB37-low NPCs were significantly enriched in gene sets related to metastasis (Bidus_Metastasis_Up, Alonso_Metastasis_Up, Rickman_Metastasis_Up, Jaeger_Metastasis_Up, and Zucchi_Metastasis_Up) and chemoresistance features (Honma_Docetaxel_resistance; Fig. 3B). To obtain further insights into the biological pathways involved in NPC pathogenesis, STRING analysis showed that the top-ranked gene signatures from GSEA were clustered in network and enriched in cell metastasis and chemoresistance pathways in patients with low RAB37 expression (Supplementary Fig. S4). These data suggest that RAB37 may be an important modulator of NPC metastasis and chemoresistance.

RAB37 suppresses metastasis and docetaxel resistance in NPC

To functionally validate the above findings, we transfected RAB37-encoding plasmids into SU8NE1 and HONE1 cells to explore cell motility and invasion ability (Supplementary Fig. S5). Wound-healing assays showed that NPC cells with RAB37 overexpression migrated less than the control cells (Fig. 3C, P < 0.01). In transwell assays, ectopic RAB37 expression significantly inhibited the migratory and invasive abilities of NPC cells (Fig. 3D and E, P < 0.01). In addition, we investigated the effects of RAB37 on cell viability and colonization. The results showed that RAB37 overexpression had no effects on NPC cell proliferation in vitro (Supplementary Fig. S6, P > 0.05). Moreover, DAC treatment significantly suppressed NPC cell proliferation, migration, and invasion, whereas only the inhibitory effects of migration and invasion were significantly sensitized by RAB37 overexpression (Supplementary Fig. S7, P < 0.05). Altogether, these data suggest that RAB37 inhibits NPC cell migration and invasion in vitro.

Furthermore, we investigated whether RAB37 is involved in resistance to docetaxel, which we had previously demonstrated in a randomized controlled phase III clinical trial to be an effective IC agent in improving DMFS. Indeed, RAB37 overexpression impaired the survival of NPC cells (Fig. 3G and H, P < 0.01). The IC50 for docetaxel strikingly decreased to 13.1 μg/mL and 1.9 μg/mL in RAB37-overexpressing SU8NE1 and HONE1 cells, respectively, compared with that in vector-transfected cells (25.3 and 10.5 μg/mL, Fig. 3G and H). Therefore, RAB37 suppresses NPC cell migration and invasion and enhances sensitivity to docetaxel chemotherapy in vitro.

RAB37 regulates TIMP2 secretion and inhibits downstream MMP2

To explore the mechanism underlying the RAB37-mediated suppressive effects in NPC, we sought to identify molecular pathways associated with RAB37 dysregulation. GSEA showed that gene sets related to epithelial-to-mesenchymal transition (EMT, Sarrio_Epithelial_Mesenchymal_Transition_Up) and GO Microtubule Cytoskeleton Organization and Protein Secretion features were correlated with RAB37 downregulation (Fig. 4A–C). Strikingly, the morphology of NPC cells overexpressing RAB37 changed from a spindle shape or elongated mesenchymal form to an epithelial-like form (Fig. 4D), suggesting that RAB37 functions to inhibit EMT. In line with these findings, immunofluorescence staining showed that ectopic RAB37 expression markedly increased the protein levels of epithelial markers (E-cadherin) but decreased the protein levels of mesenchymal markers.
Figure 2.

RAB37 hypermethylation is associated with its downregulation in NPC. A and B, Real-time PCR analysis and representative Western blot of RAB37 expression in freshly frozen normal nasopharyngeal epithelial tissues (n = 16) and NPC tissues (n = 16) (A) and immortal NP69 and NPC cell lines (B). Cell line methylation results are presented as the mean (n = 3) ± SD. Student t test, **, P < 0.01.

C. Expression of RAB37 in an independent GEO set of 31 NPC and 10 normal tissues (GSE12452). Student t test, **, P < 0.01.

D. RAB37 expression level in different cancer based on TCGA and GTEx databases. Student t test; *, P < 0.05; **, P < 0.01. BLCA, urothelial bladder carcinoma; DLBC, diffuse large B-cell lymphoma; GBM, glioblastoma; LGG, lower grade glioma; and LUSC, lung squamous cell carcinoma.

E and F. Methylation levels (E) and RAB37 expression (F) in NP69 and NPC cell lines with or without 5-aza-2'-deoxycytidine (DAC) treatment. Mean (n = 3) ± SD. Student t test; *, P < 0.05; **, P < 0.01.
Together, these results indicate that RAB37 suppresses EMT in NPC cells.

To determine whether RAB37 regulates protein secretion, we next performed IP plus mass spectrometry in SUNE1 and HONE1 cells with stable RAB37 overexpression. The results showed that TIMP2 had the highest interaction score with RAB37 (Fig. 4F; Supplementary Data 1). TIMP2 is a member of the TIMP family, a secreted protein that inhibits MMPs and extracellular matrix turnover, thereby decreasing cell motility, migration, and invasion (29). However, the regulatory interaction between RAB37 and TIMP2 has not been reported. Co-IP assay results verified that RAB37 could physically interact with TIMP2 in NPC cells (Fig. 4G). To address whether RAB37 regulates TIMP2 secretion, we determined the expression levels of TIMP2 in CM from control and ectopic RAB37-expressing cells. Concordant increase in TIMP2 expression was observed in the CM of NPC cells overexpressing RAB37 (Fig. 4H). Immunofluorescence staining revealed that endogenous RAB37 localized with TIMP2 in NPC cells (Fig. 4I). Moreover, DAC treatment significantly upregulated the secretion of TIMP2 in NPC cells (Supplementary Fig. S8, P < 0.05). Importantly, RAB37 overexpression notably decreased the level of active MMP2 in CM (Fig. 4H). Thus, RAB37 promotes TIMP2 secretion and inhibits downstream MMP2.
Figure 4. 

RAB37 regulates EMT and TIMP2 secretion. A–C, EMT- (A), microtubule cytoskeleton- (B) and protein secretion-related (C) signaling pathways enriched in response to low RAB37 expression. D, Representative images of NPC cells with or without RAB37 overexpression. E, Immunofluorescence images of E-cadherin and Vimentin in NPC cells with or without RAB37 overexpression. F, Staining of RAB37-associated proteins. Two RAB37-specific bands (red box) were excised and analyzed by mass spectrometry in NPC cells overexpressing RAB37. G, Western blot analysis of RAB37 and TIMP2 from co-IP assays using anti-HA antibodies. H, Western blot analysis of RAB37 and TIMP2 in CM, and proteolytic activity of MMP2 in CM. I, Immunofluorescence images of RAB37 and TIMP2 in NPC cells with or without RAB37 overexpression. Data are representative of three independent experiments. Scale bar, 50 μm.
RAB37 suppresses NPC cell metastasis by regulating TIMP2 secretion

We next investigated whether TIMP2 is required for RAB37-mediated inhibitory effects on NPC cells. TIMP2-targeting siRNAs were transfected into SUNE1 and HONE1 cells with stable RAB37 overexpression. The results showed that the knockdown of TIMP2 expression significantly abrogated the RAB37-mediated inhibition of migration and invasion (Fig. 5A and B, P < 0.05). Notably, TIMP2 downregulation resulted in decreased secretion of TIMP2 in CM and thus reversed the RAB37-mediated attenuation in the level of MMP2 in CM (Fig. 5C, Supplementary Fig. S9). Together, these data demonstrate that RAB37 inhibits NPC cell migration and invasion by regulating TIMP2 secretion in vitro.

To further evaluate the effect of RAB37 on NPC metastasis in vitro, we used lung metastasis colonization models. The mice in RAB37 overexpression group displayed significantly fewer lung metastatic nodules than did the mice in the control group (Fig. 5D, P < 0.01). Hematoxylin and eosin staining confirmed that mice with RAB37 overexpression exhibited remarkably fewer and smaller metastatic tumors in the lungs (Fig. 5E, P < 0.01). Moreover, we constructed xenograft lung metastasis colonization model to determine the effects of RAB37 on docetaxel chemosensitivity in NPC cell metastasis. Docetaxel treatment signifi-
cantly reduced lung metastatic tumors in mice, which was further sensitized by RAB37 overexpression (Supplementary Fig. S10, P < 0.05). Of note, concordantly high expression of RAB37 and TIMP2 was confirmed in oligometastatic lung nodules in the RAB37-overexpressing group in vitro, suggesting that RAB37 and TIMP2 expression was pathologically relevant (Fig. 5F). Hence, RAB37 suppresses NPC cell metastasis by regulating TIMP2 secretion both in vitro and in vivo.

RAB37 hypermethylation is associated with adverse prognosis for distant metastasis in patients with NPC

To assess the clinical relevance of our findings, we evaluated the relation between RAB37 methylation status and clinicopathologic parameters in 110 patients with NPC. Bisulfite pyrosequencing analysis revealed that the RAB37 promoter was differentially methylated in NPC specimens. With a cutoff value of 5.8%, patients were divided into high RAB37 methylation (methylation level ≥5.8) or low RAB37 methylation groups (methylation level <5.8%). As shown in Supplementary Table S3, no significant correlation was found between RAB37 methylation status and patient age, sex, viral capsid antigen immunoglobulin A (VCA-IgA), early antigen immunoglobulin A (EA-IgA), or clinical stage. However, RAB37 methylation status was significantly correlated with death (P = 0.010) and distant metastasis (P = 0.009; Supplementary Table S3). Kaplan–Meier survival analysis showed that patients with high RAB37 methylation levels had significantly shorter overall survival (OS; 5-year OS; 76.7% vs. 100%, P = 0.010) and DMFS rates (5-year DMFS; 75.5% vs. 95.8%, P = 0.010; Fig. 6A and B) than patients with low RAB37 methylation levels.

Furthermore, multivariate analysis revealed that the RAB37 methylation status [OS, hazard ratio (HR), 4.265; 95% confidence interval (CI), 1.291–14.10; P = 0.017; DMFS, HR, 3.316; 95% CI, 1.119–61.77; P = 0.038] and N stage [OS, HR, 2.409; 95% CI, 1.036–5.601; P = 0.041; DMFS, HR, 3.365; 95% CI, 1.303–8.695; P = 0.012] were independent prognostic indicators for OS and DMFS (Supplementary Table S4). These findings suggest that RAB37 hypermethylation is clinically associated with unfavorable outcomes for metastasis and may serve as an independent prognostic predictor for patients with NPC.

Prognostic model combining RAB37 methylation and N stage for predicting NPC metastasis and docetaxel IC benefit

To provide a clinically applicable method to stratify patients at various risk of distant metastasis, we built a predictive model based on RAB37 methylation status and N stage. According to this model, patients were classified into the following three separate groups: 23 (20.9%) patients in the low-risk group (low RAB37 methylation and early N stage), 72 (65.5%) patients in the intermediate-risk group (high RAB37 methylation or advanced N stage), and 15 (13.6%) patients in the high-risk group (high RAB37 methylation and advanced N stage). Importantly, patients in the three groups were at significantly different risk of distant metastasis, and the combination of high RAB37 methylation and advanced N stage identified patients with notably high risk of distant metastasis (Fig. 6C; Supplementary Fig. S11, P = 0.002). The data indicate that the combination of the RAB37 methylation status and N stage is more precise than clinical stage alone for predicting distant metastasis in NPC.

Furthermore, we evaluated the clinical value of the RAB37 methylation status in the prediction of sensitivity to docetaxel-containing IC. In combined intermediate- and high-risk groups, patients who received docetaxel IC experienced significantly better control of distant metastasis than patients who did not receive docetaxel IC (5-year DMFS; 81.9% vs. 50%, P = 0.047, Fig. 6D). In contrast, patients in the low-risk group did not benefit from docetaxel-containing IC (Supplementary Fig. S12). These findings suggest that our prognostic model based on the RAB37 methylation status and N stage is effective in stratifying patients who are at a high risk of distant metastasis and likely to benefit from docetaxel-containing IC.

Discussion

The present study highlights the oncogenic role of RAB37 hypermethylation in NPC with broad biological and clinical relevance. We demonstrated that NPCs with methylation-mediated RAB37 downregulation are enriched in metastasis- and chemo resistance-related pathways. Moreover, reversal of RAB37 overexpression can suppress NPC cell metastasis by regulating TIMP2 secretion and can increase docetaxel chemosensitivity, offering opportunities for novel therapeutic intervention. Furthermore, we provided evidence that our prognostic model combining RAB37 hypermethylation status with advanced N stage effectively identified patient at a high risk for distant metastasis who are likely to benefit from docetaxel-based IC, representing a promising strategy for the treatment of NPC metastasis.

Metastasis is the major reason of therapeutic failure and death in patients with cancer (30, 31). At present, the prognosis and treatment decisions for patients with metastatic NPC mainly depend on clinical TNM stage (2). Although patients with the same stage of LA-NPC receive similar therapy, they eventually display dramatic differences in clinical outcomes, with approximate 30% distant dissemination rate (5). Hence, heterogeneity exists in patients with NPC, and the traditional TNM stage is not sufficient to stratify patients at individual risk of metastasis. Substantial evidence confirms that epigenetic alterations, particularly DNA methylation, play profound roles in cancer development, progression, metastasis, and drug resistance (32–34).
Figure 5.
RAB37 suppresses NPC cell metastasis through regulating TIMP2. SUNE1 and HONE1 cells overexpressing RAB37 were transfected with either siTIMP2 or negative control (NC). A and B, Transwell migration (A) or invasion (B). Representative images (left) and quantification (right) of transwell assays. Mean (n = 3) ± SD. Student t test, *P < 0.05; **P < 0.01. C, Western blot analysis of RAB37 and TIMP2 in CM, and proteolytic activity of MMP2 in CM. *P < 0.01. D and E, SUNE1 cells (2 × 10^5 cells in 100-μL PBS) were injected into the tail veins of mice. Representative images and quantification of macroscopic (D) and microscopic metastatic nodules (E) in the lungs of mice. Mean (n = 8) ± SD. Student t test, **P < 0.01. F, IHC images of RAB37 and TIMP2 in lung metastatic nodules with or without RAB37 overexpression. Scale bar, 100 μm.
In this study, a group of dysmethylated genes were identified to closely interact with each other and to preferably distinguish NPC tissues from normal tissues. Based on the regulatory role of DNA methylation in gene expression, significant RAB37 downregulation in NPC was validated across three public GEO data sets and shown to result from promoter hypermethylation, consistent with the findings in lung cancer and esophageal squamous cell carcinoma (ESCC; refs. 35–37). Moreover, TCGA and GTEx data analysis confirmed the downregulated RAB37 expression in a wide range of cancer types, including lymphoma, glioma, urothelial bladder carcinoma, and lung squamous cell carcinoma. These results indicate that RAB37 might function as a pan-cancer suppressor. However, the roles and mechanisms of RAB37 remain undefined in NPC. GSEA demonstrated that metastasis-related pathways were enriched in response to RAB37 downregulation. Functionally, RAB37 suppressed NPC metastasis in vitro and in vivo, consistent with the inhibitory effects of RAB37 in other cancer types (35–37).

Therefore, RAB37 hypermethylation contributes to its downregulation and leads to metastasis in NPC.

RAB37 is a member of the RAB GTPase family of proteins that are critical regulators for membrane vesicle trafficking (36–38). Disregulation of the RAB pathway is associated with a wide range of human diseases, including cancer (39–42). However, there is limited knowledge regarding the roles and mechanisms of RAB37 in NPC. Our study demonstrated that RAB37 not only plays highly important roles in metastasis and EMT but also inhibits docetaxel-based chemoresistance in NPC, which provide new insights into the biological and pathologic roles of RAB37.

Bioinformatics analysis revealed that RAB37 alterations are highly relevant to microtubule cytoskeleton organization and protein secretion pathways in NPC, which have been verified to be important drivers of tumor progression, metastasis, and chemotherapy resistance (43–48). Based on mass spectrometry data, we confirmed that TIMP2 has the highest score and is the only member of the secreted TIMP protein family that physically

Figure 6. RAB37 hypermethylation is associated with poor survival and docetaxel-based IC sensitivity in patients with NPC. A and B, Kaplan–Meier analysis of overall survival (OS, A) and distant metastasis-free survival (DMFS, B) according to the RAB37 methylation status in 110 patients with NPC. C, Kaplan–Meier analysis, based on the prognostic model, of DMFS in low-risk (low RAB37 methylation and early N stage), intermediate-risk (high RAB37 methylation or advanced N stage), and high-risk (high RAB37 methylation and advanced N stage) patients with NPC. D, DMFS in intermediate- and high-risk patients treated with IC with and without docetaxel. $P$ values were determined using the log-rank test.
interacts with RAB37 in NPC. Furthermore, overexpressed RAB37 exerts its suppressive effects through the regulation of TIMP2 secretion and downstream MMP2 inhibition in NPC cells, which is in line with previous findings in other cancer types (33, 34), thereby uncovering the role of a novel RAB37/TIMP2/MMP2 axis in tumor biology.

Accumulating evidence on epigenetic aberrations in cancer has emphasized their crucial roles in diagnosis, prognosis, and the prediction of therapy response (10, 31). Here, we found that patients with RAB37 hypermethylation exhibited poor survival, which is similar to the progression-free survival tendency observed in lung cancer and ESCC patients (32–34). Both univariate and multivariate analyses showed that the RAB37 methylation status and N stage were independent prognostic factors for OS and DMFS. The prognostic model combining RAB37 methylation with N stage could accurately stratify patients at risk of metastasis. In addition, we showed that docetaxel-based IC is beneficial for the control of distant metastasis in patients classified into the intermediate- to high-risk groups by our model but not in patients in the low-risk group. IC has advantages of acceptable toxicity and early elimination of micrometastases. Our prospective phase III study confirmed that the addition of TPF IC to CCRT can effectively reduce distant metastasis and prolong survival. However, the criteria for enrollment mainly depend on TNM stage [4], and the precise candidates exactly benefiting from TPF IC remain undefined. Therefore, we conclude that patients in the intermediate- to high-risk groups might be cured by docetaxel-based IC, whereas low-risk patients may be candidates for less aggressive therapeutic strategies that prevent tumor metastasis. Our study may thus improve prediction strategies for evaluating individualized risk of distant metastasis for IC treatment.

In conclusion, we identified a key oncogenic role of RAB37 hypermethylation in NPC metastasis via the RAB37/TIMP2/ MMP2 axis. We also highlighted the effective value of an RAB37 methylation-based prognostic model and confirmed docetaxel-based IC as an effective treatment for patients with NPC at a high risk of metastasis (with RAB37 hypermethylation or/and advanced N stage), thereby facilitating the development of novel predictive and therapeutic strategies against NPC metastasis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Y. Li, X. Du, N. Liu
Development of methodology: X. Yang, X. Tang, P. Zhang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Li, X. Yang, X. Du, Y. Lei, Q. He, X. Hong
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Li, X. Yang, X. Du, Y. Lei, X. Hong, X. Tang, X. Wen, Y. Sun, J. Zhang, Y. Wang, J. Ma, N. Liu
Writing, review, and/or revision of the manuscript: Y. Li, X. Du, Y. Sun, J. Ma, N. Liu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Li, X. Du, N. Liu
Study supervision: N. Liu

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Yingqin Li, Xiaojing Yang, Xiaojing Du, et al.


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