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Research Article
LAMB1 Promotes Nasopharyngeal Carcinoma Cell Growth and Motility

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Abstract

Background and Objective: Nasopharyngeal Carcinoma (NPC) is a rare tumour that has a high mortality rate despite the availability of diagnostic and treatment methods. This study aims to screen Differentially Expressed Genes (DEGs) from the NPC microarray dataset for therapeutic targets using a bioinformatics approach. **Materials and Methods:** The GSE64634 microarray dataset was downloaded to analyze DEGs, which were further subjected to gene ontology analysis, kyoto encyclopedia of genes and genomes pathway enrichment analysis and protein-protein interaction network construction. LAMB1, a gene potentially associated with migration and Epithelial Mesenchymal Transition (EMT), was screened out and its possible involvement in biological processes and pathways was investigated by enrichment analysis. We also performed *in vitro* experiments to assess the effects of LAMB1 on NPC cell proliferation, migration, invasion and EMT. **Results:** A total of 1283 DEGs, including 316 up-regulated and 967 down-regulated genes, were analyzed from the GSE64634 dataset containing 12 NPC tissue samples and 4 normal nasopharyngeal tissues. Three genes, including FGF2, LAMB1 and THBS4, were predicted to be associated with positive regulation of cell migration and epithelial cell proliferation. Among these, LAMB1 had the highest fold change and was the only gene located in one of the top 3 hub modules. **Conclusion:** Oncogenic effects of LAMB1 were predicted by enrichment analysis and validated *in vitro* experiments. Through bioinformatics and *in vitro* validation, this study screened out LAMB1, a gene associated with NPC metastasis, which could be used as a prognostic and diagnostic biomarker for NPC.

Key words: Nasopharyngeal carcinoma, differentially expressed genes, bioinformatic analysis, LAMB1, proliferation, migration, epithelial-mesenchymal transition

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Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Nasopharyngeal Carcinoma (NPC) is one of the most prevalent tumours in China, mostly seen in people aged 30-60 years, accounting for more than half of the new cases each year worldwide[1]. The development of NPC is closely related to genetic factors, Epstein-Barr Virus (EBV) infection and environmental factors[2,3]. Under main treatments, such as intensity-modulated radiation therapy and chemotherapy, the 5 years survival rate for early-stage NPC has improved significantly but a significant proportion of patients still have unsatisfactory outcomes due to local recurrence and metastasis[4,5]. Therefore, it is urgent to further identify more diagnostic and therapeutic biomarkers for NPC.

With the rapid development of gene microarray technology, capable of detecting thousands of genes simultaneously at the transcriptome level[6], bioinformatics analysis is playing an important role in screening candidate biomarkers for various diseases, especially cancer[7], providing new clues for the identification of reliable functionally Differentially Expressed Genes (DEGs) and non-coding RNAs. As an uncommon tumour, there is no NPC data in The Cancer Genome Atlas (TCGA) database, so most of the biomining of NPC is currently carried out using the datasets from Gene Expression Omnibus (GEO) database. For example, Zhimin Ye *et al*.[8] screened 5 potential biomarkers, consisting of DNALI1, RSPH4A, RSPH9, DNAI2 and ALDH3A1, for NPC by integrating GSE64634 and GSE12452 microarray data. Ji-Zhou Zhang *et al*.[9] identified 13 hub genes related to carcinogenesis or progression in NPC through analyzing three microarray datasets from the GEO database. Combining Oncomine, GEO and Gene Expression Profiling Interactive Analysis (GEPIA) database, Weiqian Guo *et al*.[10] identified SPP1 as a possible diagnostic and therapeutic target for NPC. Although many bioinformatic analysis have been performed on NPC, it remains doubtful whether the genes obtained can influence the progression and prognosis of NPC as many studies have not been followed up with experimental validation and therefore these findings have limited clinical applications.

Laminin subunit beta 1 (LAMB1), also known as laminin β1, is ubiquitously expressed in most tissues[11]. Laminin is an Extracellular Matrix (ECM) glycoprotein assembled from 3 disulfide-linked polypeptides, including α, β and γ chains[12,13]. Reportedly, the β subunit (LAMB) plays a role in initiating cell assembly by binding to the γ subunit (LAMC), which contributes to tumour cell invasion and metastasis[14]. Current researches suggest that LAMB1 plays an important role in a variety of tumours, such as hepatocellular carcinoma[15], colorectal cancer[16] and glioblastoma multiforme[17]. However, the relationship between LAMB1 expression and NPC metastasis has never been reported.

In this study, we screened DEGs between normal nasopharyngeal tissues and NPC tissues from the GSE64634[18] dataset based on the GEO database. Subsequently, through Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis and Protein-Protein Interaction (PPI) networks analysis, we finally screened out the upregulated gene LAMB1 and validated its effects on proliferation, migration, invasion and Epithelial Mesenchymal Transition (EMT) in NPC. LAMB1 might be a novel therapeutic target of NPC.

MATERIALS AND METHODS

Study area: The study was carried out at the Department of Otolaryngology, The Affiliated Hospital of Putian University, China from March-October, 2021.

Microarray data information: Based on the GPL570 platform, a microarray dataset GSE64634 containing 4 normal nasopharyngeal tissues and 12 NPC tissues was retrieved from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). This is a public microarray dataset sequenced by the Zhaoyang Zeng Lab at Central South University and uploaded to the GEO database. As a bioinformatics analysis, this study did not require ethical approval.

Data processing and DEG screening: Principal Component Analysis (PCA) was performed on specimens from the above dataset using R 3.6.3 software and drawn by ggplot2 3.3.3 package to observe the distribution between groups. DEGs from the above dataset were analyzed using R 3.6.3 software with limma package[19] according to the screening criteria of $|\log_2(FC)|>1$ and $p<0.05$. Then the volcano plot of all genes and heat map of the top 50 dysregulated DEGs were drawn by ggplot2 3.3.3 package and ComplexHeatmap 2.2.0 package[20] of R 3.6.3 software, respectively.

Enrichment analysis of DEGs: The above DEGs were extracted for GO and KEGG enrichment analysis in R 3.6.3 software with Cluster Profiler 3.14.3[21] and ggplot2 3.3.3 package and the main enriched Biologic Processes (BPs), Cellular Components (CCs) and Molecular Functions (MFs) and pathways were screened at a threshold of adjusted $p<0.05$.

PPI network analysis of DEGs: PPI network of DEGs was analyzed in the search tool for the retrieval of interacting

genes (STRING, http://www.string db.org/) database[22] and was visualized in Cytoscape 3.8.0 software[23] with the Molecular Complex Detection (MCODE) plug-in. Hub modules were screened with some cutoff parameters: 0.2 node score, 2 degrees, 100 max. the depth and 2 k-core.

Screening for up-regulated DEGs associated with positive regulation of proliferation and migration: Positive regulation of proliferation and migration-related genes were obtained through the NCBI website and intersected with up-regulated genes in GSE64634 as shown by the Venn diagram based on online tool Draw Venn Diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/). The expression of the overlapped gene, including FGF2, LAMB1 and THBS4, was counted by t-test between NPC tissues and normal nasopharyngeal tissues and visualized by R 3.6.3 software with ggplot2 3.3.3 package. Then, according to the expression level of LAMB1, NPC samples from GSE64634 were divided into 2 different groups. Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/)[24] was used to define the biological processes and pathways enriched in the DEGs between the 2 groups. Terms with $p<0.05$ were identified.

Cell lines: All cells were purchased from the Chinese Academy of Science (Shanghai, China). In keratinocyte/serum-free medium (Invitrogen), normal nasopharyngeal cells NP69 were cultured and in RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum (HyClone), NPC cell lines (6-10B, 5-8F) were cultured. All cells were cultured at 37 with 5% CO_2.

RNA extraction, reverse transcription and quantitative RT-PCR (qRT-PCR): Total RNA was extracted by TRIzol reagent (Invitrogen) and cDNA was synthesized using ReverTra Ace® qPCR RT Kit (FSQ-101, Toyobo, Japan). Real-time quantification was performed with Bestar® SYBR Green qPCR Master Mix (DBI Bioscience). The relative expression of LAMB1 was normalized to GAPDH mRNA levels by the $2^{-\Delta\Delta Ct}$ method. The primers were synthesized by Sango Biotech (Shanghai, China), whose sequences are shown in Supplementary Table 1.

Western blotting: NPC cells were lysed with RIPA lysis buffer (Thermo Fisher Scientific) containing protease and phosphatase inhibitors. The protein lysates were separated by 5% SDS-PAGE gel electrophoresis and blotted onto PVDF membranes (Millipore). The membranes were sealed with 5% skimmed milk and incubated overnight at 4°C with the primary antibodies against LAMB1 (1:100, 17763, Santa Cruz Biotechnology), N-cadherin (1:1,000, A01577-3, Boster, Beijing, China), E cadherin (1:1,000, BM4166, Boster), Vimentin (1:1,000, PB9395, Boster) and GAPDH (1:5,000, #HRP-60004, Proteintech, Wuhan, China). The membranes were then incubated with secondary antibodies for 1 hr at room temperature and developed by ECL illuminating solution (Millipore). The results of the protein blots were analyzed using ImageJ software.

Plasmid infection and transient transfection: Lentivirus carrying cDNA for LAMB1 and control were purchased from Shanghai Genechem Company. To knock down LAMB1 expression in NPC cells, LAMB1 shRNA (LAMB1-shRNA) or scrambled shRNA (sh-NC) was synthesized, shown in Supplementary Table 2 and subcloned into the lentivirus vector pLKO.1 (Genechem, Shanghai, China). To investigate the roles of LAMB1 in NPC, human NPC cells 6-10B and 5-8F were infected with lentiviruses carrying LAMB1 cDNA using Lipofectamine 3000 reagent (Invitrogen) or LAMB1 shRNA using RNAiMAX Reagent (Invitrogen), respectively. Puromycin (Sigma) was used to select cells after 48 hrs of incubation at 37°C with 5% CO_2.

Cell counting kit-8 (CCK-8) assay: At the density of 10^3 cells per well, 6-10B and 5-8F cells were inoculated in 96-well plates, incubated with CCK-8 (C0038, Beyotime, China) for 2 hrs and measured for the absorbance at 450 nm for 4 days.

Wound healing assay: In six-well plates, NPC cells were cultured until 80% confluence and then scraped using a plastic pipette tip. The wound healing was observed under a phase-contrast microscope (Olympus).

Invasion assay: Transwell assays were carried out in a transwell chamber (Corning, USA) with matrix gel (BD Biosciences). 1×10^5 NPC cells suspended in serum-free medium were inoculated in the upper chamber and a medium supplemented with 10% FBS was placed in the lower chamber. After 24 hrs, cells on the underside of the membrane were fixed with 6% paraformaldehyde, stained with 0.5% crystalline violet (Beyotime) and counted in 5 random fields of view under a phase-contrast microscope (Olympus).

Statistical analysis: The data were analyzed using GraphPad 9.0.0 and R 3.6.3 software and expressed as Mean±SD. When variances were similar between groups, differences were analyzed by t-test or one-way ANOVA, with a $p<0.05$ considered statistically significant.

RESULTS

Screening for DEGs of NPC: The microarray dataset GSE64634 was retrieved, consisting of 12 NPC and 4 normal healthy nasopharyngeal tissue specimens. First, a Principal Component Analysis (PCA) was carried out and scatter plots revealed the distinction between NPC and normal specimens (Fig. 1a). Based on the cutoff criteria ($|\log_2(FC)|>1$, $p<0.05$), the significant DEGs are presented in different colours in the volcano plot, with the 316 highly expressed genes in

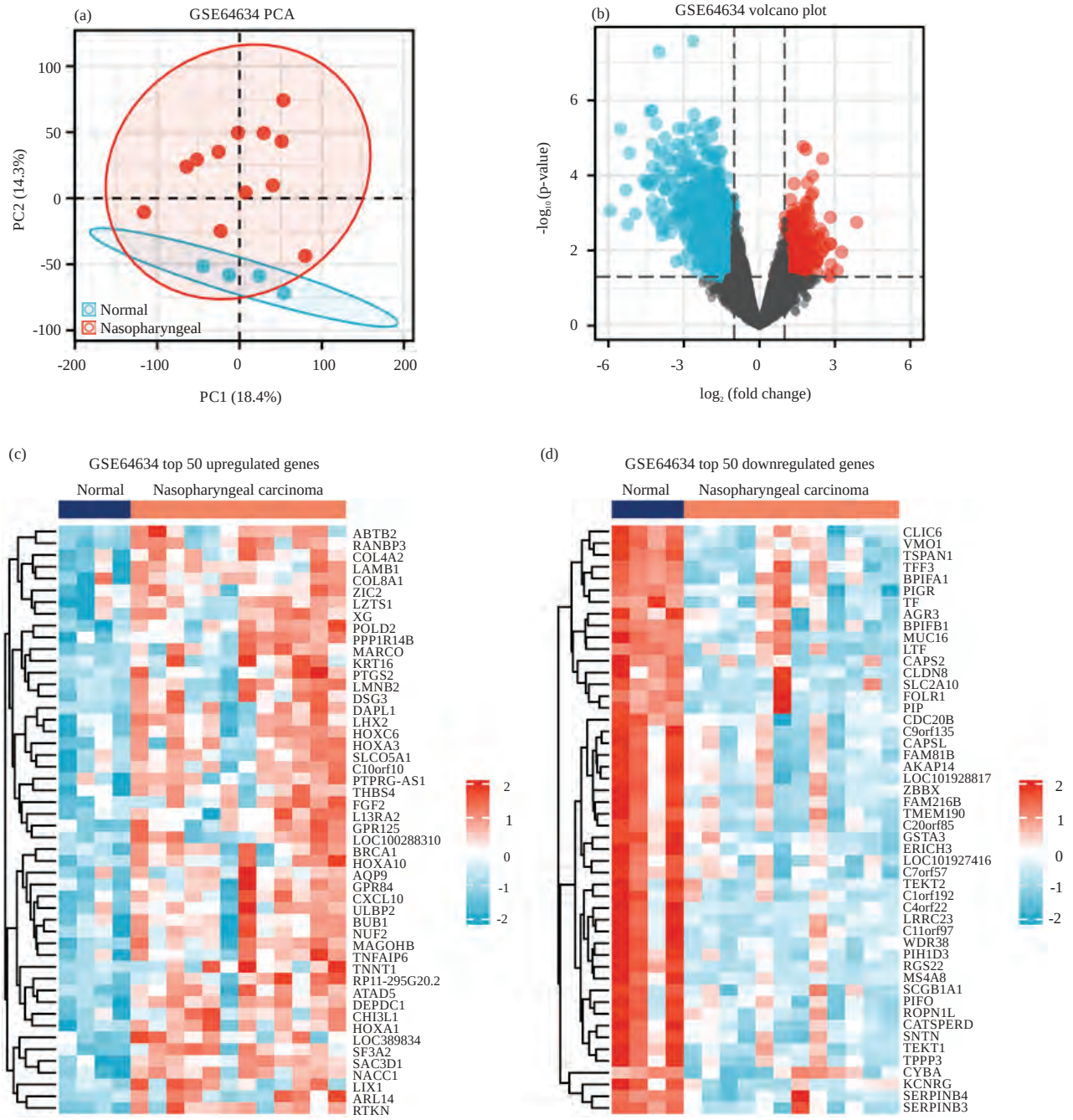

Fig. 1(a-d): Analysis of DEGs in the GSE64634 microarray dataset, (a) PCA analysis plot of GSE64634 microarray dataset, (b) Volcano map of GSE64634 microarray dataset, (c) Heatmap of top 50 up-regulated and (d) Down-regulated DEGs in the GSE64634 microarray dataset

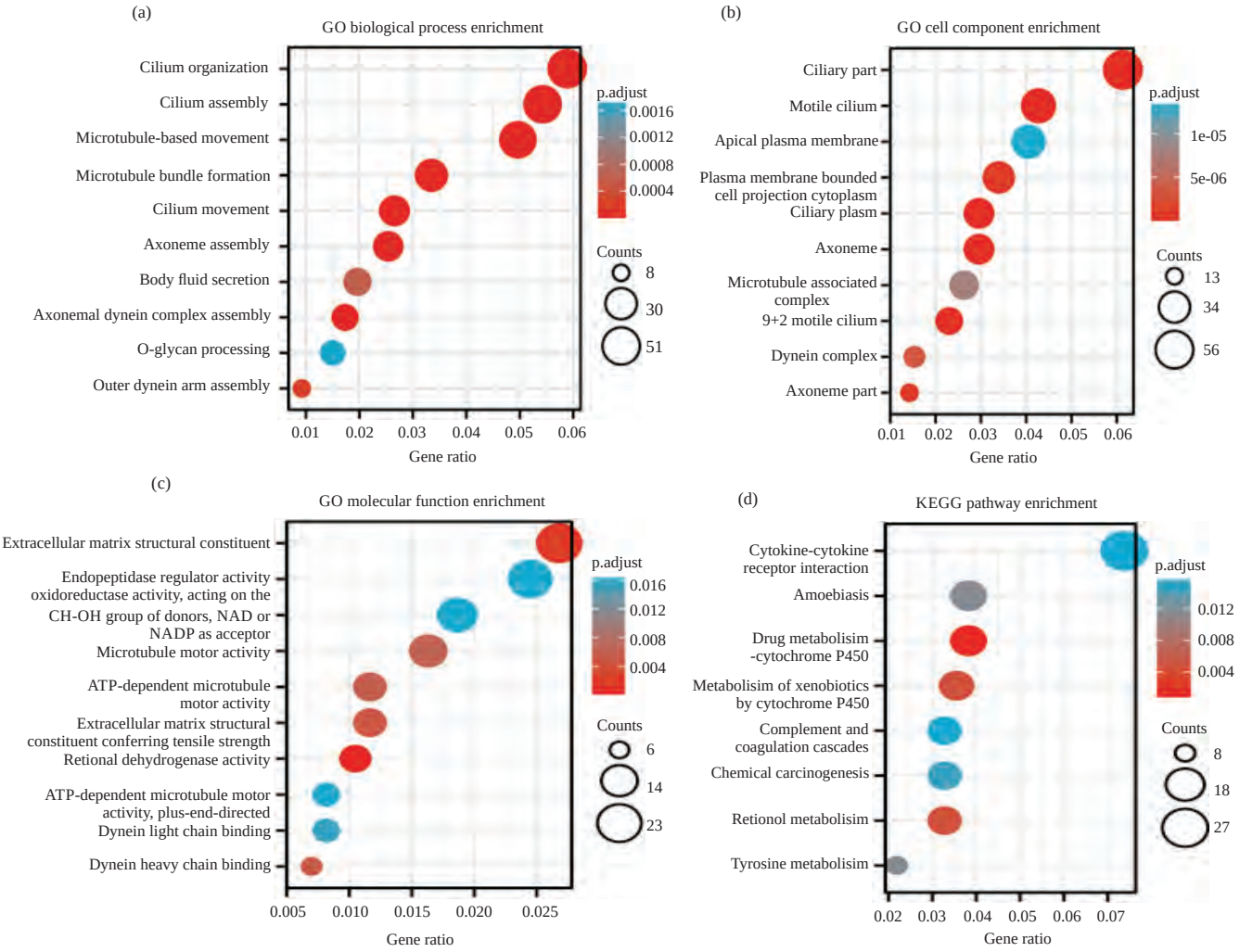

Fig. 2(a-d): GO and KEGG enrichment analysis of DEGs, (a) GO-BP enrichment, (b) GO-CC enrichment, (c) GO-MF enrichment and (d) KEGG pathway enrichment

red and the 967 lowly expressed genes in blue (Fig. 1b). The expression of the top 50 up-and down-regulated DEGs in each sample is presented by heat map (Fig. 1c-d).

GO and KEGG enrichment analysis for NPC-related DEGs: The enrichment analysis of the screened DEGs was performed using the cluster profiler 3.14.3 package of the R language, of which the species was limited as "Homo sapiens". For BP, DEGs were mainly enriched for cilium organization, outer dynein arm assembly, O-glycan processing, axonemal dynein complex assembly, body fluid secretion, axoneme assembly, microtubule bundle formation, cilium movement, cilium assembly, microtubule-based movement (Fig. 2a). For CC, DEGs were mainly related to the ciliary part, motile cilium, apical plasma membrane, plasma membrane-bounded cell projection cytoplasm, 9+2 motile cilium, ciliary plasm, axoneme, microtubule-associated complex, dynein complex, axoneme part (Fig. 2b). For MF, DEGs were mainly involved in extracellular matrix structural constituent, endopeptidase regulator activity, acting on the CH-OH group of donors, oxidoreductase activity, NAD or NADP as acceptor, ATP-dependent microtubule motor activity, microtubule motor activity, extracellular matrix structural constituent conferring tensile strength, retinol dehydrogenase activity, plus-end-directed, ATP-dependent microtubule motor activity, dynein heavy chain binding, dynein light chain binding (Fig. 2c). Moreover, KEGG terms were mainly focused on Retinol metabolism, complement and coagulation cascades, chemical carcinogenesis, Metabolism of xenobiotics by cytochrome P450, cytokine-cytokine receptor interaction, drug metabolism-cytochrome P450, amoebiasis, tyrosine metabolism (Fig. 2d).

Module analysis for PPI network of DEGs: The DEGs were uploaded to the online tool STRING for PPI construction and then the results obtained were visualized in cytoscape

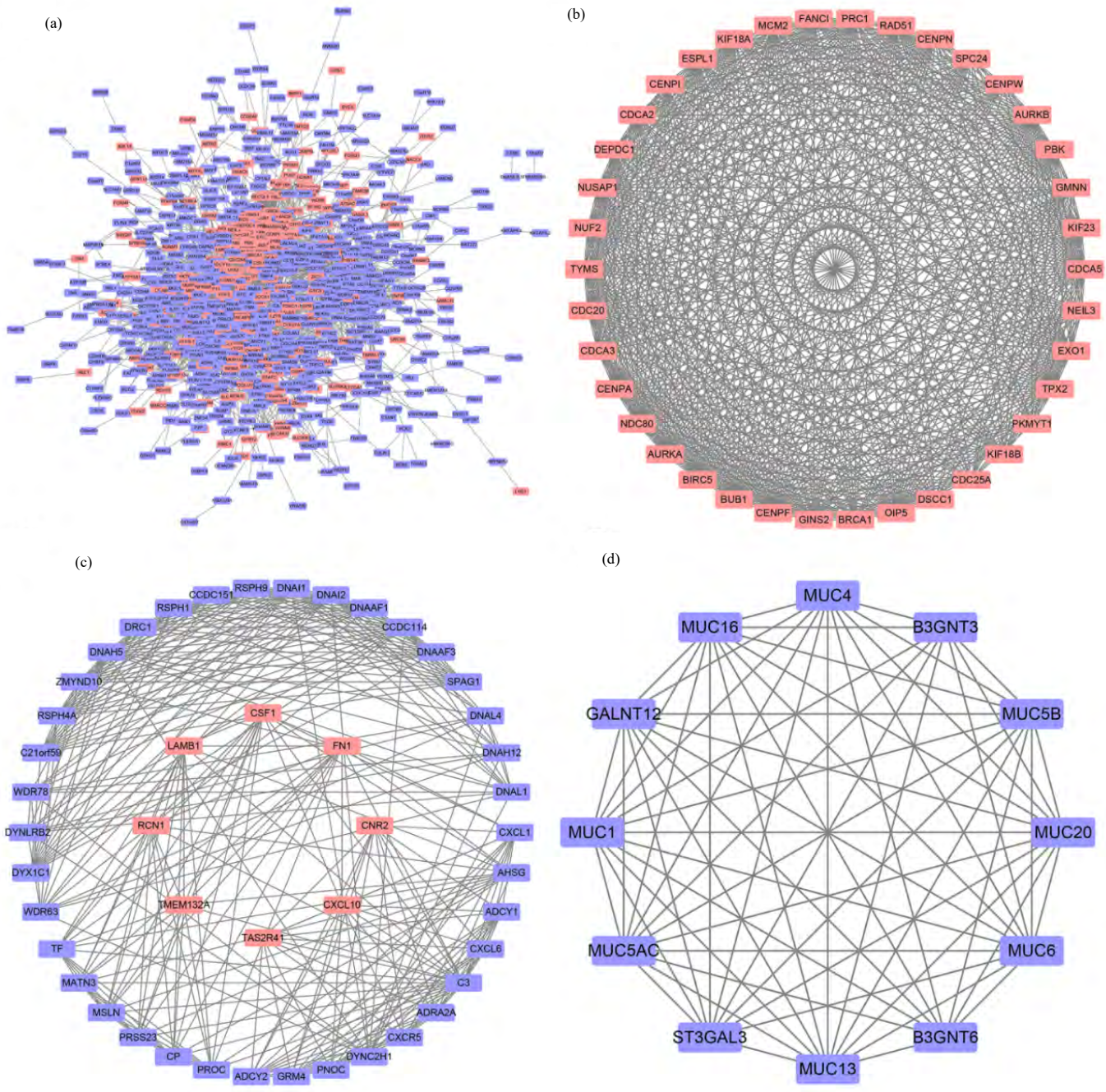

Fig. 3(a-d): PPI network construction and core module screening, (a) PPI network of DEGs, (b) Module 1 contained 38 gene nodes and 597 edges, with an MCODE score of 32.27, (c) Module 2 contained 8 upregulated genes nodes, 38 down regulated genes nodes and 307 edges, with an MCODE score of 13.644 and (d) Module 3 contained 12 downregulated genes nodes and 63 edges, with an MCODE score of 11.455

Downregulated and upregulated genes were presented as blue-violet and pink nodes, respectively

software, where pink nodes were upregulated genes and blue-violet nodes were down regulated genes (Fig. 3a). Cluster analysis of all nodes in the PPI performed through MCODE plugin with default parameters yielded three modules with top scores, including module 1 (38 nodes, score 32.27, Fig. 3b), module 2 (46 nodes, score 13.644, Fig. 3c) and module 3 (12 nodes, score 11.455, Fig. 3d).

LAMB1 was predicted to promote NPC metastasis: Considering that upregulated genes are more valuable for

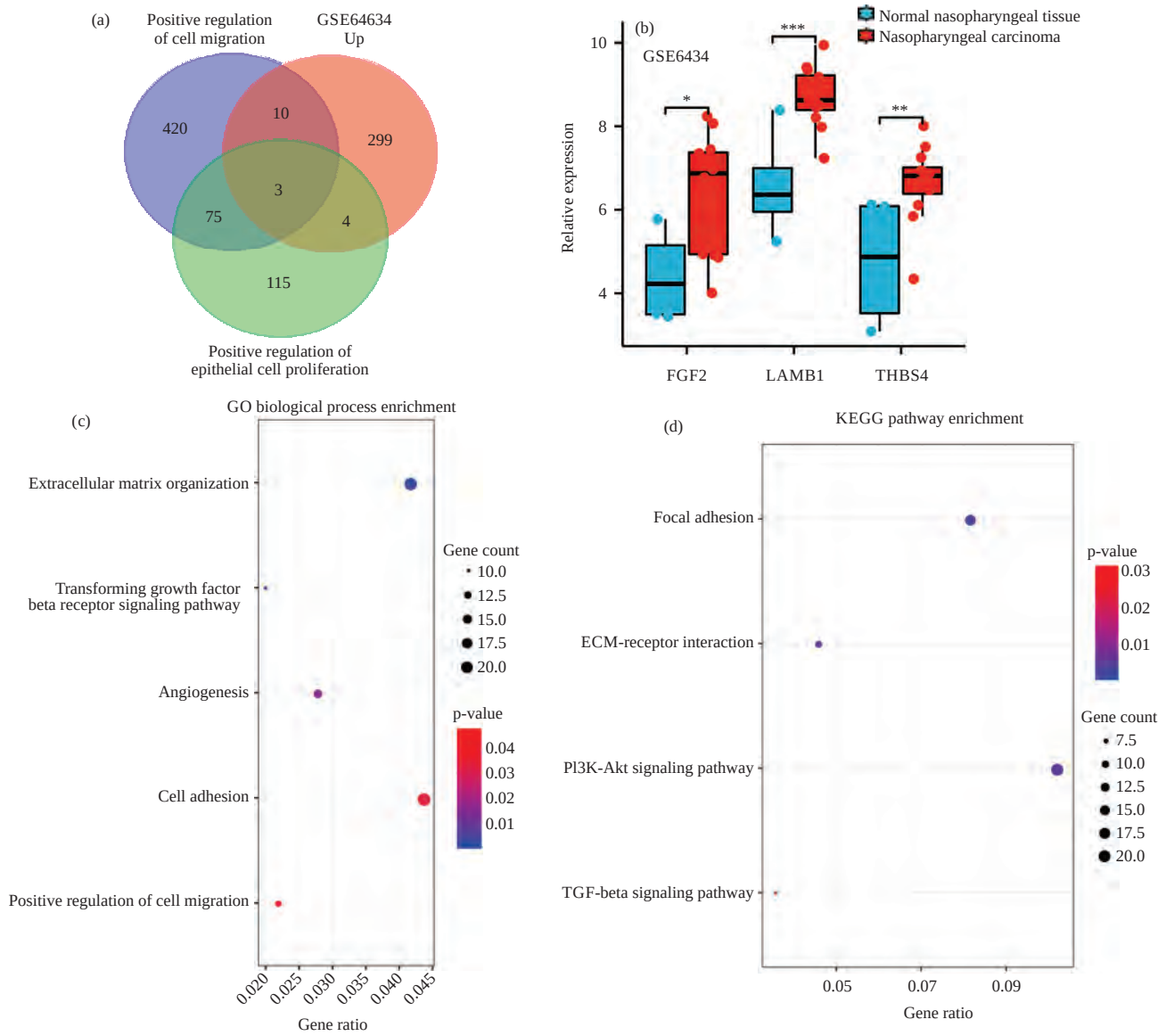

Fig. 4(a-d): LAMB1 was screened out to promote NPC metastasis, (a) Venn diagram of 3 potential pro-metastatic genes, (b) Expression of FGF2, LAMB1 and THBS4 in the GSE64634 microarray dataset, (c) 5 GO-BP terms and (d) 4 KEGG terms enriched in NPC samples of GSE64634 with LAMB1 highly expressed

Table 1: Information of 3 key genes related to positive regulation of cell migration and proliferation

Gene names	Protein names	logFC	Enriched significant modules
FGF2	Fibroblast growth factor 2	1.927	None
LAMB1	Laminin subunit beta-1	2.125	Module 2
THBS4	Thrombospondin-4	1.905	None

research, we selected upregulated genes for further analysis. Because NPC metastases affect prognosis and treatment decisions, we extracted genes related to positive regulation of cell migration and proliferation from upregulated DEGs and obtained 3 genes, namely FGF2, LAMB1 and THBS4 (Fig. 4a, Table 1). Notably, these 3 genes, were found in the top 50 upregulated genes. Among these, LAMB1 has the highest fold change and was enriched in module 2, while FGF2 and THBS4 were not shown in the top 3 modules (Fig. 4b). To identify the potential function of LAMB1 in NPC, we divided the NPC samples in GSE64634 into a high and low group according to LAMB1 expression and then performed GO-BP and KEGG analysis of DEGs between the 2 groups to search the enriched functions and pathways. As shown in Fig. 4c-d,

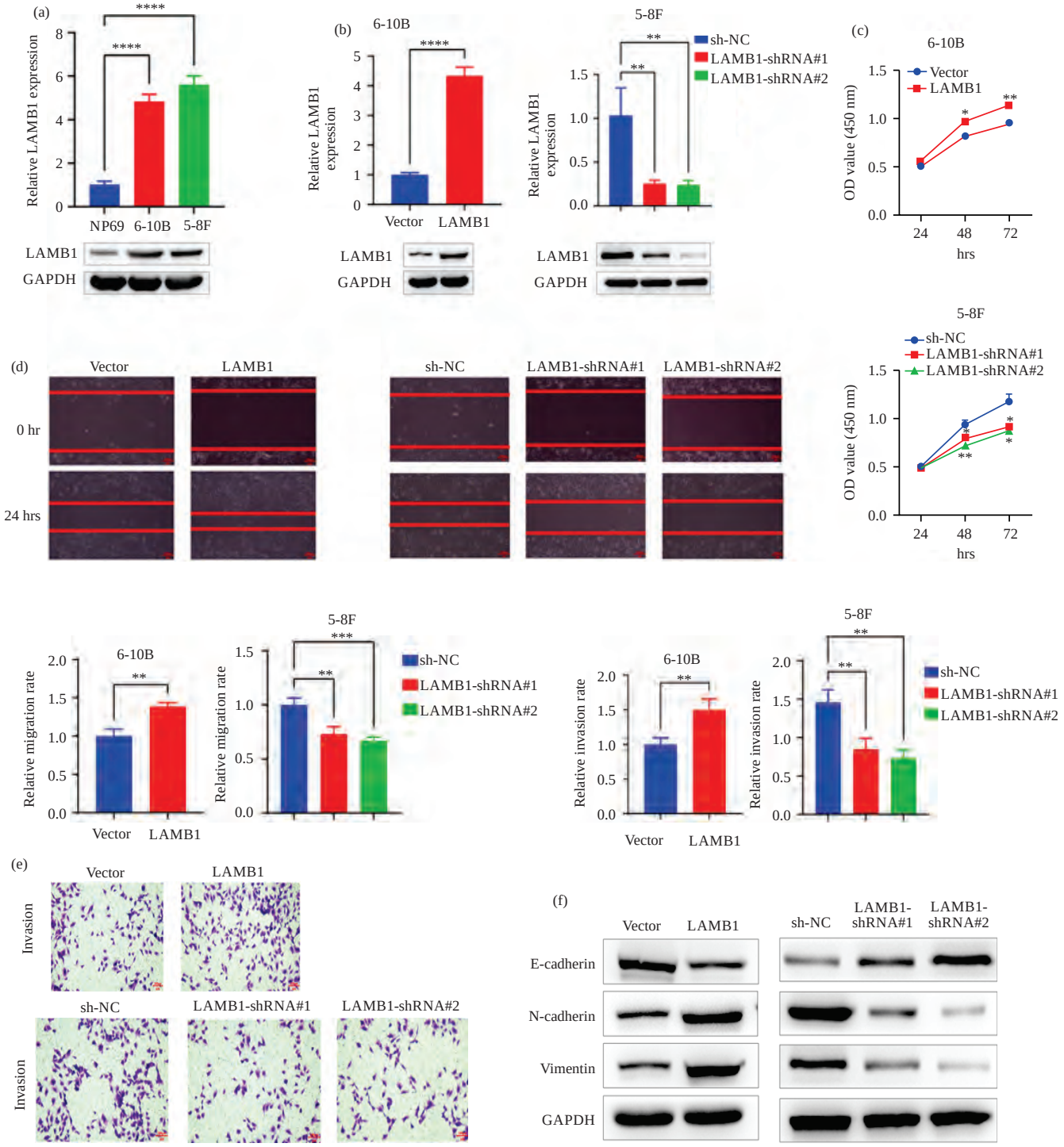

Fig. 5(a-f): LAMB1 promoted proliferation, migration, invasion and EMT of NPC cells, (a) mRNA and protein expression of LAMB1 in NP69, 5-8F and 6-10B cells, (b) mRNA and protein expression of LAMB1 in 6-10B cells upon overexpression lentivirus and 5-8F cells upon LAMB1-shRNA #1, #2, (c) 6-10B cell viability upon overexpression lentivirus and 5-8F cell viability upon LAMB1-shRNA #1, #2, (d) 6-10B cell migration upon overexpression lentivirus and 5-8F cell migration upon LAMB1-shRNA #1, #2, (e) 6-10B cell invasion upon overexpression lentivirus and 5-8F cell invasion upon LAMB1-shRNA #1, #2 and (f) EMT-related protein expression in 6-10B cells upon overexpression lentivirus and 5-8F cells upon LAMB1-shRNA #1, #2

*p<0.05, **p<0.01 and ***p<0.001, all experiments were repeated 3 times

high LAMB1 expression was associated with positive regulation of migration, which is consistent with the results shown in Fig. 4a.

LAMB1 accelerated proliferation, migration, invasion and EMT in NPC cells: First, LAMB1 expression levels were examined in NPC cells. Figure 5a, compared to NP69 cells, LAMB1 was highly expressed in NPC cells both at the mRNA and protein levels. To investigate the role of LAMB1 in NPC, we transfected 6-10B cells with LAMB1 overexpression lentivirus and 5-8F cells with LAMB1 silence lentivirus (Fig. 5b). CCK8 assay showed that LAMB1 overexpression enhanced cell proliferation in 6-10B cells, while LAMB1 silence reduced cell proliferation in 5-8F cells (Fig. 5c). To verify whether LAMB1 could promote NPC cell migration, we did scratch assays (Fig. 5d) and transwell assays (Fig. 5e) and the results were consistent with those predicted by bioinformatics analysis. Moreover, the results of Western blot exhibited that upregulated N-cadherin and Vimentin and downregulated E-cadherin were induced by LAMB1 overexpression, suggesting that LAMB1 may promote NPC metastasis through EMT (Fig. 5f).

DISCUSSION

In this study, we identified a potential NPC biomarker LAMB1 for the 1st time based on bioinformatics analysis on GSE64634 microarray and *in vitro* assays. NPC is one of the common tumours in southern China because of ineffective control[1]. As currently, the main treatment for NPC, radiotherapy treatment effect is not ideal, contributing to NPC progression[25]. Ionizing Radiation (IR) promotes cancer cell survival and invasion in different ways, including promoting EMT, promoting tumour stem cells maintenance and altering the tumour microenvironment[26]. This study might provide an alternative therapeutic target.

In this study, we screened for metastasis-related genes in GSE64634 and found that ECM organization, ECM-receptor interaction, Transforming Growth Factor-beta (TGF-beta) receptor signalling pathway, TGF-beta signalling pathway, cell adhesion, angiogenesis, positive regulation of cell migration, focal adhesion and PI3K-Akt signalling pathway enriched in NPC samples with high LAMB1 expression. These biological processes and signalling pathways were reportedly associated with cancer progression as well as metastasis. EMT requires the disruption of cell-cell contact and cell-matrix adhesion, which lead to migration and invasion acquisition[27,28]. Cell adhesion to the ECM is fundamental to tissue integrity[29], whose attenuation facilitate metastasis of tumour cells from their primary site[30]. EMT sometimes coincide with angiogenesis as some angiogenesis-inducing factors can also promote EMT, such as hypoxia, which has been reported to accelerate EMT[31] and TGF-beta, which activates angiogenesis[32]. In addition, previous studies have found that over-activation of the TGF-beta signalling pathway[33,34] and PI3K-Akt signalling pathway[35,36] stimulates EMT and invasion in NPC cells. As we know, FAK can promote phosphorylation of β-catenin during EMT, thereby enhancing the expression of EMT-associated transcription factors[37]. FAK has also been reported to promote EMT in NPC[38,39].

Laminins, secreted and incorporated into the ECM are a major component of the basal lamina[11]. Binding to other cell membrane and ECM molecules is essential for the maintenance of the tissue. Except that, Laminins can affect cell differentiation[40], migration, adhesion[41] and promote tumour cell proliferation and invasion[42]. Laminin is a heterotrimeric protein-containing alpha, beta and gamma-chains. Recently, several studies have reported overexpression of LAMB1 in patients with various cancers, such as colorectal cancer[16], gastric cancer[43] and Glioblastoma multiforme[17]. However, it has not been reported in NPC. In this study, we found for the 1st time that overexpression of LAMB1 accounted for the augmentation of proliferation, migration, invasion and EMT in NPC cell lines, consistent with the predicted results from bioinformatics.

However, there are some limitations to this study. First, our study is based on previous data with a relatively small sample size. Secondly, although we have predicted some of the key biological processes and pathways regulated by LAMB1, we have not further validated them in cells. In the future, we will verify the findings on clinical specimens and animal models and explore underlying mechanisms.

In total, we analyzed the NPC microarray dataset, screened out upregulated genes and possible regulatory pathways associated with NPC cell metastasis and identified LAMB1 as a potential biomarker and therapeutic target of NPC, increasing the understanding of the underlying mechanisms of NPC metastasis.

CONCLUSION

Within the GSE64634 dataset, LAMB1 was screened out by intersecting positive regulation of proliferation and migration-related genes and upregulated genes in GSE64634. GO and

KEGG enrichment analysis showed that extracellular matrix organization, transforming growth factor-beta receptor signalling pathway, angiogenesis, cell adhesion, positive regulation of cell migration, focal adhesion, ECM-receptor interaction, PI3K-Akt signalling pathway, TGF-beta signalling pathway were enriched, which were reportedly involved in cancer development. Importantly, LAMB1 was verified to promote NPC cell growth and motility *in vitro*, which might provide novel insights into the treatment of NPC.

SIGNIFICANCE STATEMENT

This study discovers the possible NPC biomarker LAMB1 that can contribute to NPC cell growth and motility. This study will help the researcher to uncover the critical area of NPC progression that many researchers were not able to explore. Thus, a new theory and therapeutic target on NPC may be arrived at.

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Supplementary Table 1: Sequences of PCR primers

LAMB1	Forward (5′-3′)	AGGAACCCGAGTTCAGCTAC
	Reverse (5′-3′)	CACGTCGAGGTCACCGAAAG
GAPDH	Forward (5′-3′)	CCTCTGACTTCAACAGCGAC
	Reverse (5′-3′)	TCCTCTTGTGCTCTTGCTGG

Supplementary Table 2: Sequences of shRNA against specific targets

sh-NC	5′-3′	CAACAAGATGAAGAGCACCAA
LAMB1-shRNA#1	5′-3′	CCCAAGGATACAGAATTTATT
LAMB1-shRNA#2	5′-3′	CGCAGGTAGAAGTGAAATTAA