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lncRNA RP11-624L4.1 Is Associated with Unfavorable Prognosis and Promotes Proliferation via the CDK4/6-Cyclin D1-Rb-E2F1 Pathway in NPC

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Nasopharyngeal carcinoma (NPC) is one of the most common malignant tumors in southern China and southeast Asia. Emerging evidence revealed that long noncoding RNAs (lncRNAs) might play important roles in the development and progression of many cancers, including NPC. The functions and mechanisms of the vast majority of lncRNAs involved in NPC remain unknown. In this study, a novel lncRNA RP11-624L4.1 was identified in NPC tissues using next-generation sequencing. *In situ* hybridization (ISH) was used to analyze the correlation between RP11-624L4.1 expression and the clinicopathological features or prognosis in NPC patients. RNA-Protein Interaction Prediction (RPISeq) predictions and RNA-binding protein immunoprecipitation (RIP) assays were used to identify RP11-624L4.1's interactions with cyclin-dependent kinase 4 (CDK4). As a result, we found that RP11-624L4.1 is hyper-expressed in NPC tissues, which was associated with unfavorable prognosis and clinicopathological features in NPC. By knocking down and overexpressing RP11-624L4.1, we also found that it promotes the proliferation ability of NPC *in vitro* and *in vivo* through the CDK4/6-Cyclin D1-Rb-E2F1 pathway. Overexpression of CDK4 in knocking down RP11-624L4.1 cells can partially rescue NPC promotion, indicating its role in the RP11-624L4.1-CDK4/6-Cyclin D1-Rb-E2F1 pathway. Taken together, RP11-624L4.1 is required for NPC unfavorable prognosis and proliferation through the CDK4/6-Cyclin D1-Rb-E2F1 pathway, which may be a novel therapeutic target and prognostic in patients with NPC.

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a unique epithelial malignancy with clinical, demographic, and geographic features that are distinct from other head and neck carcinomas.[1,2] Genetic susceptibility, epigenetic variation, ethnic background, environmental factors, and Epstein-Barr virus infection contribute to this malignancy.[3–7] There are apparent regional characteristics of NPC, with a high incidence in North Africa, Southeast Asia, and East Asia, especially Southern China.[8] NPC tends to metastasize and invade other areas of the body; lymph node metastasis occurs early, and most patients had already reached the late stage by the time of the initial diagnosis.[9] Currently, NPC diagnosis relies primarily on its unique clinical and pathological features. The treatment strategy for NPC primarily includes radiotherapy-supplemented chemotherapy.[10,11] Although radiotherapy and chemotherapy have made various advances in the treatment of NPC, these treatments have not significantly improved the 5-year survival rate of patients with NPC.[12–14] The frequent recurrence and poor prognosis of NPC remain huge clinical challenges.[15] Therefore, identifying novel targets and in-depth understanding of the molecular mechanisms underlying NPC may lead to effective therapeutic strategies and improve the overall prognosis of patients with NPC.

Long noncoding RNAs (lncRNAs) are a group of endogenous noncoding transcripts that are >200 nt in length and have no or little functional protein-coding ability in the conventional sense.[16,17] lncRNAs can be divided into the following five categories based on their relationship with protein-coding genes: sense, antisense, intergenic, intronic, and bidirectional lncRNAs. Accumulating research shows that lncRNAs can be used as molecular signals, decoys, guides, scaffolds, and enhancers that affect gene transcription.[18,19] Recently, mounting evidences have indicated that the aberrant expression of lncRNAs participates in various human diseases, including cancers.[20–23] Aberrant expression can regulate tumor cell growth, apoptosis, and metastasis.[24–26] Such regulatory mechanisms include

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regulations at the transcriptional level and posttranscriptional level, such as epigenetic regulation.[27–29] lncRNAs have been increasingly identified in the human genome. Nevertheless, the functions and mechanisms of the vast majority of lncRNAs involved with NPC remain unknown.

Cyclin-dependent kinase 4 (CDK4) is a master regulator of the cell cycle that belongs to the cyclin-dependent kinase family,[30] and it is also an essential protein important for normal cell proliferation as a G1 serine/threonine kinase.[31] CDK4 and CDK6 play key roles in mammalian cell proliferation, where they drive the progression of cells into the DNA synthetic (S) phase of the cell-division cycle.[32] CDK4 activation requires the binding of the regulatory cyclin subunits cyclin D1. The CDK4/cyclin D1 complexes phosphorylate and inactivate a tumor suppressor protein retinoblastoma (RB), leading to the release of E2F transcription factor that regulates expression of genes necessary for entry and progression through the S phase.[33] CDK4 has been identified as the major oncogenic driver among the cell-cycle components.[34,35] Some types of tumors, such as liposarcoma and B cell lymphoma, are present with amplified CDK4 or cyclin D1.[36,37] Melanoma cells are also highly reliant on CDK4 or CDK6.[38] In addition, the CDK4/6 inhibitor could induce cell-cycle arrest and significantly inhibit cell proliferation both *in vitro* and *in vivo*.[39]

In our previous research, a new lncRNA RP11-624L4.1 was identified and was found to be highly expressed in NPC tissues using next-generation sequencing.[40] However, the NPC-causing roles and mechanisms of this novel lncRNA RP11-624L4.1 are unknown. Therefore, we examined the expression levels of RP11-624L4.1 in Chinese NPC tissue samples and further analyzed the clinicopathological significance and the prognosis of RP11-624L4.1 expression in a cohort of NPC patients. Moreover, we explored the potential role of this lncRNA in NPC cell proliferation both *in vitro* and *in vivo*. We also explored whether CDK4 interacts with lncRNA RP11-624L4.1 to promote the proliferation ability of NPC through the CDK4/6-Cyclin D1-Rb-E2F1 pathway. Our discovery may shed new light on the pathogenesis of NPC and provide a novel potential prognostic biomarker and therapeutic target for the treatment of NPC.

RESULTS

Highly Expressed RP11-624L4.1 in NPC Tissues Is Correlated with a Poor Prognosis in NPC Patients

By RNA sequencing, we identified differentially expressed lncRNAs between seven inflammatory nasopharyngeal epithelium (NPE) tissues and seven NPC tissues,[40] and 2,192 lncRNAs were found to be differentially expressed. Among these, 204 lncRNAs were considered to be significantly downregulated, and 306 lncRNAs were considered to be significantly upregulated ($\geq$2-fold change, false discovery rate [FDR] < 0.05). Figure 1A shows the top 40 significantly altered lncRNAs and indicated that RP11-624L4.1 was extremely upregulated (4.35-fold higher expression in NPE tissues) (Figure 1B). By checking the database, RP11-624L4.1, which is located in 15q14 as a transcript of 1,487 bp in length according to the results of 5′ and 3′ RACE (rapid amplification of cDNA ends) (Figures S1 and S2), was revealed as a novel lncRNA that has not been previously reported. We then deposited it in the GenBank database with accession number GenBank: MT490310 (data are to be held confidential until March 31, 2021). Then, we validated RP11-624L4.1 expression in the biopsy tissues of group 2 using quantitative real-time polymerase chain reaction (PCR), and the results are shown in Figure 1C, verifying that RP11-624L4.1 was shown to be exceedingly highly expressed in 20 NPC samples compared with 14 inflammatory NPE samples, which was consistent with the RNA sequencing results.

To identify the RP11-624L4.1 expression levels and its localization in NPC tissues, we performed *in situ* hybridization (ISH) analysis on a tissue array of 130 NPC specimens using a 5′- and 3′-digoxin (DIG)-labeled oligonucleotide probe that was complementary to RP11-624L4.1. The results are shown in Figures 2A–2D, indicating that RP11-624L4.1 is observed to be primarily localized in the cytoplasm of NPC cell. Moreover, we analyzed the correlation between RP11-624L4.1 expression and the clinicopathological features in NPC patients. The results showed that 50.8% of the NPC specimens (66/130) are classified into the high RP11-624L4.1 expression group (Table 1). Moreover, RP11-624L4.1 expression level was significantly correlated with T stage ($p = 0.003$), N stage ($p = 0.004$), M stage ($p < 0.001$), clinical stage ($p = 0.003$), survival state ($p = 0.002$), and relapse ($p = 0.033$) in NPC patients. However, there was no significant relationship between RP11-624L4.1 expression and other clinicopathological features, such as patient age ($p = 0.601$), gender ($p = 0.179$), or World Health Organization (WHO) classification ($p = 0.079$) (Table 1).

Furthermore, Kaplan-Meier survival analysis and the log rank test were used to investigate the correlation between RP11-624L4.1 expression and the prognosis of NPC patients. The results suggest that NPC patients with higher RP11-624L4.1 expression have shorter overall survival (OS) ($p = 0.0048$) and disease-free survival (DFS) ($p = 0.03$) times (Figures 2E and 2F). In the Cox regression analysis, the univariate survival analysis indicates that RP11-624L4.1 expression ($p = 0.009$), T stage ($p < 0.001$), N stage ($p = 0.001$), M stage ($p < 0.001$), and clinical stage ($p = 0.001$) are significantly associated with the OS of NPC patients (Table 2). The multivariate survival analysis showed that T stage ($p = 0.022$) and M stage ($p < 0.001$) are considered independent factors that affect the OS of NPC patients (Table 2).

RP11-624L4.1 Promotes NPC Cell Proliferation and Cycle

To investigate the biological functions of RP11-624L4.1 in the development of NPC, we subjected NPC cells to short interfering RNA (siRNA) transfection and short hairpin RNA (shRNA) lentiviral infection. Before doing so, we first detected the endogenous expression of RP11-624L4.1 in six NPC cell lines (Figure 3A), and we found that RP11-624L4.1 was universally highly expressed in the NPC cell lines compared with NP69 cells (normal human NPE cells). We then selected two cell lines that highly expressed RP11-624L4.1, CNE1, and 5-8F for the functional studies. Three siRNAs targeting

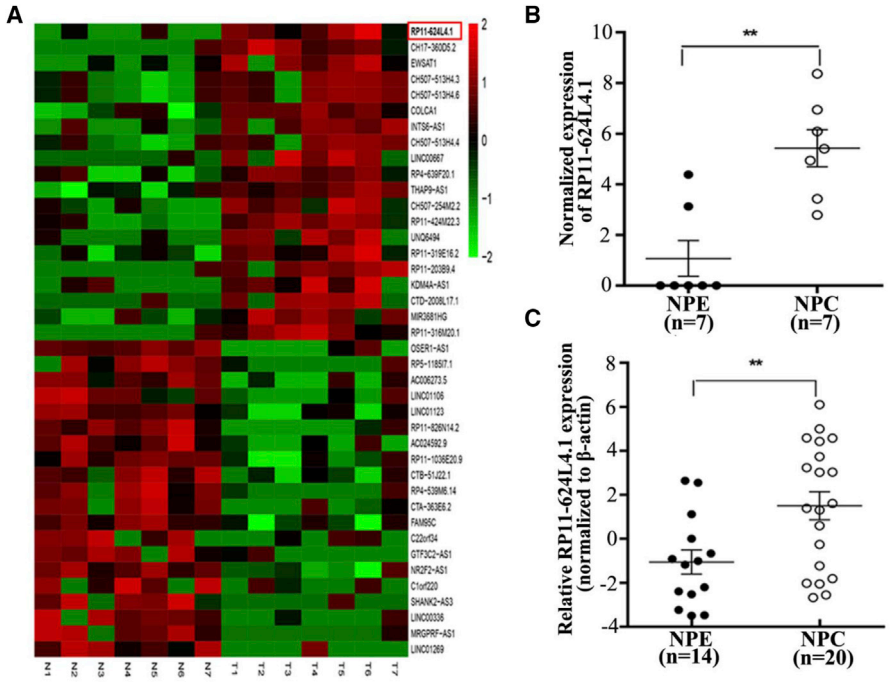

Figure 1. lncRNA RP11-624L4.1 Is Highly Expressed in NPC

(A) Differential gene expression heatmap showing the top 40 altered lncRNAs between inflammatory NPE tissues (N; n = 7) and NPC tissues (T; n = 7). (B) The expression of RP11-624L4.1 in the gene expression profile (NPE = 7; NPC = 7). (C) The expression of RP11-624L4.1 in inflammatory NPE tissues (n = 14) and NPC tissues (n = 20) was determined by qRT-PCR (bottom). The results are expressed as log2 ($2^{-\Delta\Delta Ct}$). Data are presented as the mean ± SD. Statistical significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Student's t test.

RP11-624L4.1 were used to interfere with the expression of RP11-624L4.1 (Figure 3B). The results showed that the siRNAs of si-1 and si-3 efficiently knocked down RP11-624L4.1 expression; the interference efficiency of siRNA si-1, especially, was approximately 65% in both cell lines. Therefore, we designed a shRNA lentiviral system based on the si-1 core target sequences. Then, we used the sh-1 lentiviral infection construct to establish cell lines with stably downregulated RP11-624L4.1 expression, which we examined by quantitative real-time PCR (Figure 3C).

Next, we examined the phenotypic changes in the RP11-624L4.1 stable knockdown NPC cell lines (sh-1). Both Cell Counting Kit-8 (CCK-8) assay and 5-ethynyl-20-deoxyuridine (EdU) assay were conducted to explore the role of RP11-624L4.1 in NPC proliferation levels, and the results demonstrated that cell proliferation is decreased in both CNE1 and 5-8F cells when RP11-624L4.1 expression was knocked down (Figures 3D and 3E). Moreover, cell-cycle assays showed that the knockdown of RP11-624L4.1 induces G1 phase arrest in both CNE1 and 5-8F cells (Figure 3F) but does not affect the apoptosis rates (Figures S3 and S4).

To explore the effect of RP11-624L4.1 overexpression on cell phenotype, we used GV146 plasmid to insert the full length of RP11-624L4.1 sequence for overexpression. First, we examined the overexpression efficiency in the two NPC cell lines. The result showed that RP11-624L4.1 is successfully overexpressed (Figure 4A). A CCK-8 assay (Figures 4B and 4C) and EdU assay (Figure 4D) demonstrated that cell proliferation is increased in the RP11-624L4.1-overexpressed cells.

Taken together, our data clearly demonstrated that RP11-624L4.1 plays important roles for promoting NPC cell proliferation and cell cycle by either RP11-624L4.1 knockdown or overexpression *in vitro*.

RP11-624L4.1 Induces Tumor Growth of NPC Cells *In Vivo*

Given RP11-624L4.1 plays an important role in promoting NPC cell proliferation and cell cycle *in vitro*, the role of RP11-624L4.1 in NPC progression *in vivo* using an NPC xenograft model in BALB/c nude mice was investigated. First, 5-8F cells were infected with a lentiviral vector harboring RP11-624L4.1 shRNA-1 (sh-1) or the control shRNA (sh-NC). The lentiviral-infected cells were injected into the right and left flanks of the nude mice, and tumor growth was measured twice per week. The results showed that knockdown of RP11-624L4.1 expression significantly inhibits the growth of the tumors compared with the control tumors (Figure 5A). Twenty-eight days after the inoculation, the tumor size and mass of the sh-1 group were markedly smaller/lower in comparison with the control group (sh-NC) (Figures 5B and 5C). Moreover, we used a Ki67 (proliferation-related nuclear antigen) immunohistochemistry (IHC) assay to further investigate the *in vivo* effects of RP11-624L4.1 on tumor cell proliferation. The hematoxylin and eosin (H&E)-stained photomicrographs revealed that the tumor

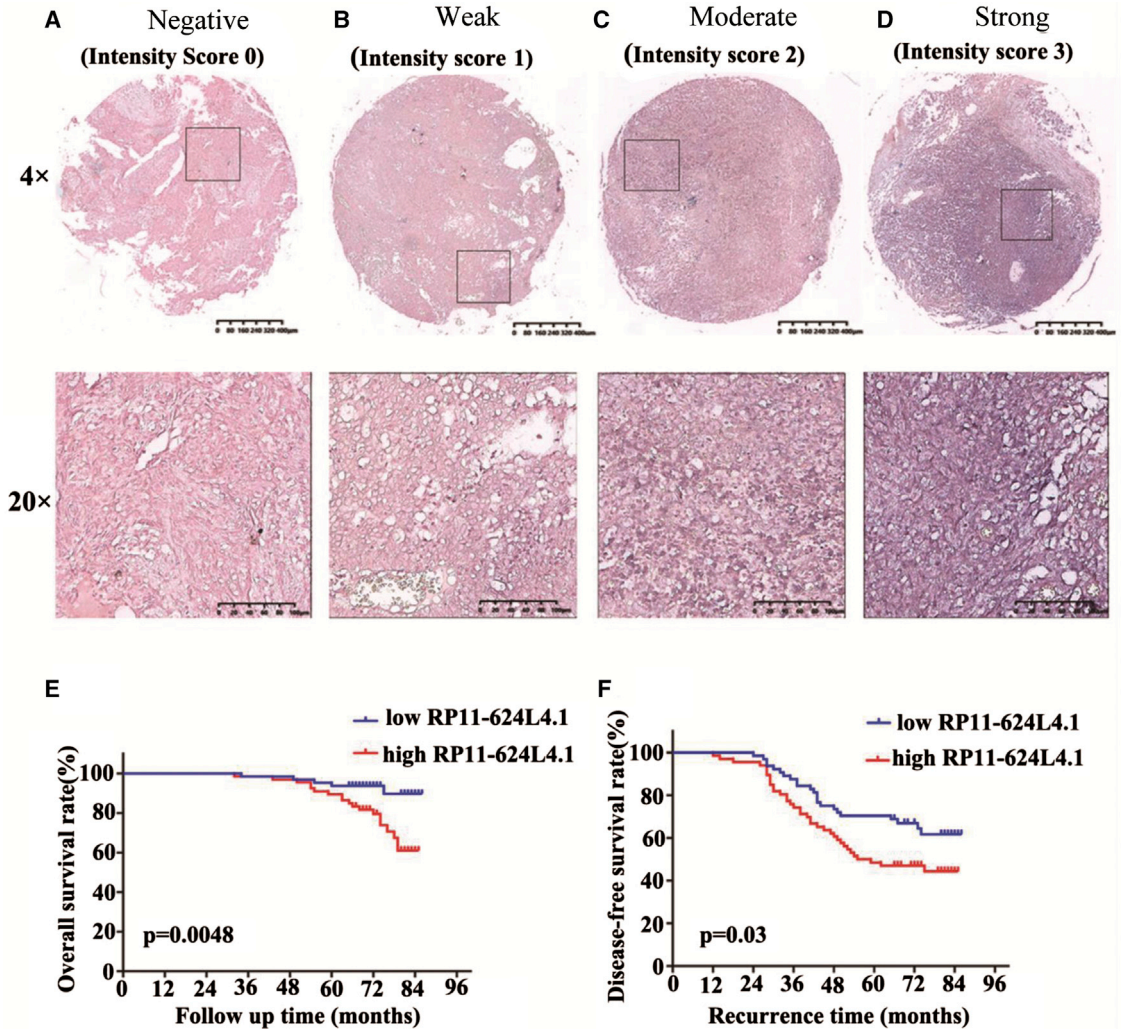

Figure 2. Representative ISH Staining of RP11-624L4.1 and Kaplan-Meier Curve of Overall Survival (OS) and Disease-Free Survival (DFS) in NPC Specimens

(A) Nearly negative expression of RP11-624L4.1; staining intensity level scored as 0. (B) Weak expression of RP11-624L4.1; staining intensity level scored as 1. (C) Moderate expression of RP11-624L4.1; staining intensity level scored as 2. (D) Strong expression of RP11-624L4.1; staining intensity level scored as 3. (A–D) Original magnification 4×, scale bars: 400 μm (top panels); original magnification 20×, scale bars: 100 μm (bottom panels). (E and F) Kaplan-Meier curve of (E) OS and (F) DFS. p values were calculated with the log rank test. RP11-624L4.1 highly expressed is associated with an unfavorable prognosis in NPC patients.

cells of the sh-NC group invade the skin appendages, whereas the tumor cells of the sh-1 group do not (Figure 5D). The results further indicated that the relative number of proliferating (Ki-67-positive) tumor cells in the sh-1 group is significantly lower in the xenografts, compared with the sh-NC tumors (Figure 5E).

RP11-624L4.1 Interacts with CDK4 Physically

We sought to identify the target genes of RP11-624L4.1 to explore the molecular mechanisms by which RP11-624L4.1 exerts its effects on NPC cells proliferation and cell cycle. RNA-Protein Interaction Prediction (RPISeq) predictions were first used to identify RP11-624L4.1 interaction proteins and revealed that RP11-624l4.1 may directly interact with CDK4, CDK6, and Cyclin D1 (Figures S5–S7, with probability of 0.65, 0.6, and 0.65, respectively, using Random Forest classifier, and with probability of 0.98, 0.94, and 0.89, respectively, using support vector machines [SVM]). Because CDK4 has the highest probability of binding to RP11-624L4.1, following this indication, RNA-binding protein immunoprecipitation (RIP) assay was performed, and we successfully verified that RP11-624L4.1 interacts with CDK4 in both 5-8F and CNE1 cells (Figure 6A). Confocal microscopy of RP11-624L4.1 fluorescence ISH (FISH) and CDK4 immunofluorescence (IF) showed that they co-localize at the cytoplasm of NPC cell line 5-8F (Figure 6D).

RP11-624L4.1 Interacts with and Regulates CDK4 in the CDK4/6-Cyclin D1-Rb-E2F1 Pathway for Proliferation in NPC *In Vitro*

Moreover, we knocked down RP11-624L4.1 expression to investigate whether doing so would alter the mRNA or protein levels of CDK4.

Table 1. Relationships between RP11-624L4.1 Expression and the Clinicopathological Characteristics of NPC Patients

		RP11-624L4.1 Expression (n = 130)		
Characteristics	All Cases (n = 130)	Low (n = 64)	High (n = 66)	p Value
Age (Years)				
<48	66	31 (48.4%)	35 (53.0%)	0.601
≥48	64	33 (51.6%)	31 (47%)	
Sex				
Male	99	52 (81.3%)	47 (71.2%)	0.179
Female	31	12 (18.7%)	19 (28.8%)	
T Stage[a]				
T1–2	85	50 (78.1%)	35 (53.0%)	0.003*
T3–4	45	14 (21.9%)	31 (47.0%)	
N Stage[a]				
N0–1	73	44 (68.8%)	29 (43.9%)	0.004*
N2–3	57	20 (31.3%)	37 (56.1%)	
M stage[a]				
M0	109	61 (95.3%)	48 (72.7%)	<0.001*
M1	21	3 (4.7%)	18 (27.3%)	
Clinical Stage				
I–II	70	43 (67.2%)	27 (40.9%)	0.003*
I–IV	60	21 (23.8%)	39 (59.1%)	
WHO Type				
NKUC	116	54 (84.4%)	62 (93.9%)	0.079
NKDC	14	10 (15.6%)	4 (6.1%)	
Survival Status				
Survive	106	59 (92.2%)	47 (71.2%)	0.002*
Death	24	5 (7.8%)	19 (28.8%)	
Relapse				
No	71	41 (64.1%)	30 (45.5%)	0.033*
Yes	59	23 (35.9%)	36 (54.5%)	

NKDC, nonkeratinizing differentiated carcinoma; NKUC, nonkeratinizing undifferentiated carcinoma.
[a]T, N, and M refer to tumor, nodes, and metastasis, based on the *American Joint Committee on Cancer/International Union against Cancer Staging Manual* (7th ed., 2010).
*$p < 0.05$.

As demonstrated by the results, no significant changes were observed in the mRNA levels of CDK4 (Figure 6B), but a significant decrease in the protein levels of CDK4 was observed in both the CNE1 and 5-8F cells with a stable knockdown of RP11-624L4.1 (Figure 6C). Interestingly, we found that the knockdown of RP11-624L4.1 expression also affects protein expressions/levels for CDK6, Cyclin D1, p-Rb (Ser 780), Rb, and E2F1 (Figure 6C). Because CDK4 is a protein-serine kinase involved in the cell cycle, we performed a CDK4 IHC assay, and results showed that the relative expression of CDK4 in the sh-1 group was significantly lower than that in the tumors (Figures 5D and 5F). These results indicated that RP11-624L4.1 regulates CDK4 in the CDK4/6-Cyclin D1-Rb-E2F1 pathway by interacting and stabilizing CDK4.

RP11-624L4.1 Is Required for NPC Proliferation Depending on the CDK4/6-Cyclin D1-Rb-E2F1 Pathway

Given RP11-624L4.1 interacts with and stabilizes CDK4 in the CDK4/6-Cyclin D1-Rb-E2F1 pathway, we then investigated whether RP11-624L4.1 is required for NPC proliferation depending on CDK4/6-Cyclin D1-Rb-E2F1. To do so, we overexpressed CDK4 in shRNA-1 cell with RP11-624L4.1 knocked down, and CCK-8 assay showed that overexpression of CDK4 promotes cell proliferation (Figure 7A) and partially rescues RP11-624L4.1 NPC promotion, thus indicating its role in the RP11-624L4.1-CDK4/6-Cyclin D1-Rb-E2F1 pathway. As we predicted, protein expressions for Cyclin D1, p-Rb (Ser 780), and E2F1 were upregulated, whereas Rb was downregulated (Figure 7B). Surprisingly, the expression level of CDK6 was slightly reduced, mostly likely because of RP11-624L4.1 acting as a scaffold of CDK4, CDK6, and Cyclin D to form a protein complex, which leads to disruption of distinct regions of this lncRNA to affect different effector partners and function. These results clearly indicated that RP11-624L4.1 promoting NPC proliferation is dependent, at least partially, on the CDK4/6-Cyclin D1-Rb-E2F1 pathway.

DISCUSSION

A high metastatic tendency and obvious regional distribution are two important characteristics of NPC. Currently, radiotherapy is the most common treatment for NPC, and radiotherapy combined with chemotherapy can improve the treatment efficacy.[41] However, the OS rate of patients has not been significantly improved because of the frequent recurrence.[42] Therefore, identification of early diagnostic markers and therapeutic targets is urgently needed.

In recent years, increasing numbers of lncRNAs have been proved to play an important role in cancers. In this present study, we showed that RP11-624L4.1 is significantly upregulated in NPC tissues, which had not been previously reported. We determined that a high expression level of RP11-624L4.1 was significantly associated with numerous clinicopathological characteristics, including T stage, N stage, M stage, American Joint Committee on Cancer (AJCC) clinical stage, frequent recurrence, and cancer-related death. Kaplan-Meier survival analysis and univariate analysis indicated that high RP11-624L4.1 expression is associated with a poor prognosis. These data suggest that RP11-624L4.1 may be a potential prognostic indicator for NPC. Therefore, RP11-624L4.1 may play an oncogenic role during the development of NPC. Supporting this hypothesis, knockdown of RP11-624L4.1 inhibited proliferation and G1-S progression in NPC cells. Conversely, overexpressing RP11-624L4.1 in NPC cells had the opposite effect. Moreover, the tumorigenic experiment in nude mice demonstrated that upregulation of RP11-624L4.1 promotes NPC cell tumorigenicity and tumor growth *in vivo*. Overall, these data indicate that RP11-624L4.1 may play an oncogenic role in human NPC progression.

lncRNAs normally exert their biological functions through direct or indirect interactions with regulatory proteins, miRNAs, or other

Table 2. Univariate and Multivariate Analyses of OS in 130 NPC Patients by Cox Regression Analysis

	Univariate Analysis			Multivariate Analysis		
Variable	HR	95% CI	p Value	HR	95% CI	p Value
RP11-624L4.1 expression (high/low)	3.731	1.392–9.995	0.009*			
Age (≥48/<48 years)	1.019	0.458–2.269	0.963			
Sex (female/male)	0.918	0.343–2.460	0.865			
T stage[a] (T3–4 versus T1–2)	68.205	9.146–508.657	<0.001*	14.3	1.479–138.276	0.022*
N stage[a] (N2–3 versus N0–1)	33.729	4.554–249.829	0.001*			
M stage[a] (M1/M0)	49.559	16.691–147.147	<0.001*	11.62	3.437–39.290	<0.001*
Clinical stage (III–IV/I–II)	31.608	4.267–234.149	0.001*			
WHO type (NKDC/NKUC)	0.04	0.000–8.013	0.234			

CI, confidence interval; HR, hazard ratio; OS, overall survival.
[a]T, N, and M refer to tumor, nodes, and metastasis, based on the *American Joint Committee on Cancer/International Union against Cancer Staging Manual* (7th ed., 2010).
*$p < 0.05$.

cellular factors.[43,44] Several studies have shown that lncRNAs play important roles in the regulation of target protein expression.[45-47] In the present study, we found that knockdown of RP11-624L4.1 induces G1 phase arrest and cell-cycle inhibition in NPC cells. RPISeq prediction and RIP assay indicated that RP11-624L4.1 interacts with CDK4 directly. CDK4 is a member of the CDK family and has been reported to be an oncogenic gene in various tumors. The oncogenic mechanism of CDK4 primarily focuses on cell proliferation because it is essential for the G1-to S-phase transition during the cell cycle.[48] The qRT-PCR and western blotting (WB) results confirmed that RP11-624L4.1 regulates CDK4 protein expression at the post-transcriptional level. It has been reported that the CDK4/CDK6-Cyclin D1-Rb-E2F1 pathway normally appears to be abnormal in many tumors. The change of this pathway accelerates the process of G1 phase and promotes the proliferation of tumor cells to gain a survival advantage.[49] Rescue assays indicated that RNA RP11-624L4.1 is required for proliferation in the CDK4/6-Cyclin D1-Rb-E2F1 pathway. In this study, we then used WB to detect the key molecules in the CDK4/CDK6-Cyclin D1-Rb-E2F1 pathway. In the process of cell proliferation, the complex of Cyclin D1 and CDK4/6 can phosphorylate Rb. Once Rb is phosphorylated, it can release the transcription factor E2F, which is tightly bound without phosphorylation. The activation of E2F further promotes the cell-cycle transition from G1 phase to S phase, thus entering the cell proliferation cycle.[50] Therefore, RP11-624L4.1 might promote proliferation of NPC cells partly through regulating the CDK4/CDK6-Cyclin D1-Rb-E2F1 pathway.

There are few studies on the mechanism of lncRNA control of CDK4. One mechanism is that lncRNAs can act as decoys to attenuate small RNA regulation through sequestration of proteins or RNA-dependent effectors. The competing endogenous RNA (ceRNA) hypothesis is based on this hypothesis.[28] Studies have shown that MALAT1,[51] HAGLR,[52] and lncRNA PCAT-1[53] acted as a ceRNA, which increased CDK4 expression though binding to miR-124, miR-185-5p, and miR-149-5p, respectively, in some cancers. In our study, knocked down RP11-624L4.1 had no significant changes in the mRNA levels of CDK4 in NPC cells, indicating that RP11-624L4.1 was not involved the regulation of mRNA of CDK4. lncRNA also participates in transcriptional regulation as a signaling molecule in a straightforward one-to-one relationship.[54] lncRNAs showed cell-type-specific expression and responded to diverse stimuli, suggesting that their expression is under considerable transcriptional control. In this way, lncRNAs can serve as molecular signals because transcription of individual lncRNAs occurs at a very specific time and place to integrate developmental cues, interpret cellular context, or respond to diverse stimuli. RP11-624L4.1 might not function at the transcriptional level as a molecular signaling in NPC cells because knocked down RP11-624L4.1 have no significant effects in the mRNA levels of CDK4. The second mechanism is that lncRNA also participates in transcriptional regulation as a signaling molecule in a straightforward one to one relationship. The third mechanism of lncRNA is the guide: RNA binds protein(s), which can direct the localization of the ribonucleoprotein complex to specific targets. In principle, lncRNAs can guide chromatin change *in cis* in a cotranscriptional manner (tethered by RNA polymerase) or as a complementary target for small regulatory RNAs. Guidance *in trans* can occur by lncRNA binding to target DNA as a RNA:DNA heteroduplex, RNA:DNA:DNA triplex, or RNA recognition of a complex surface of specific chromatin features.[55,56] In the present study, there is no evidence that RP11-624L4.1 brings transcription factors to the CDK4 promoter or binds to DNA of the CDK4-forming complex. Therefore, the possibility of RP11-624L4.1 as the guide to CDK4 is very low.

The last but most important mechanism (fourth mechanism of action) of lncRNA acts as scaffolds. lncRNAs can serve as central platforms upon which relevant molecular components are assembled.[54] In many diverse biological signaling processes, this characteristic is vital to the precise control of the specificity and dynamics of intermolecular interactions and signaling events.[57] This is perhaps the most functionally intricate and complex class where the lncRNA possesses different domains that bind distinct effector molecules. The lncRNA would bind its multiple effector partners at the same time, and consequently spatially and temporally assemble the effectors, which may have transcriptional activating or repressive activities. Key predictions for this

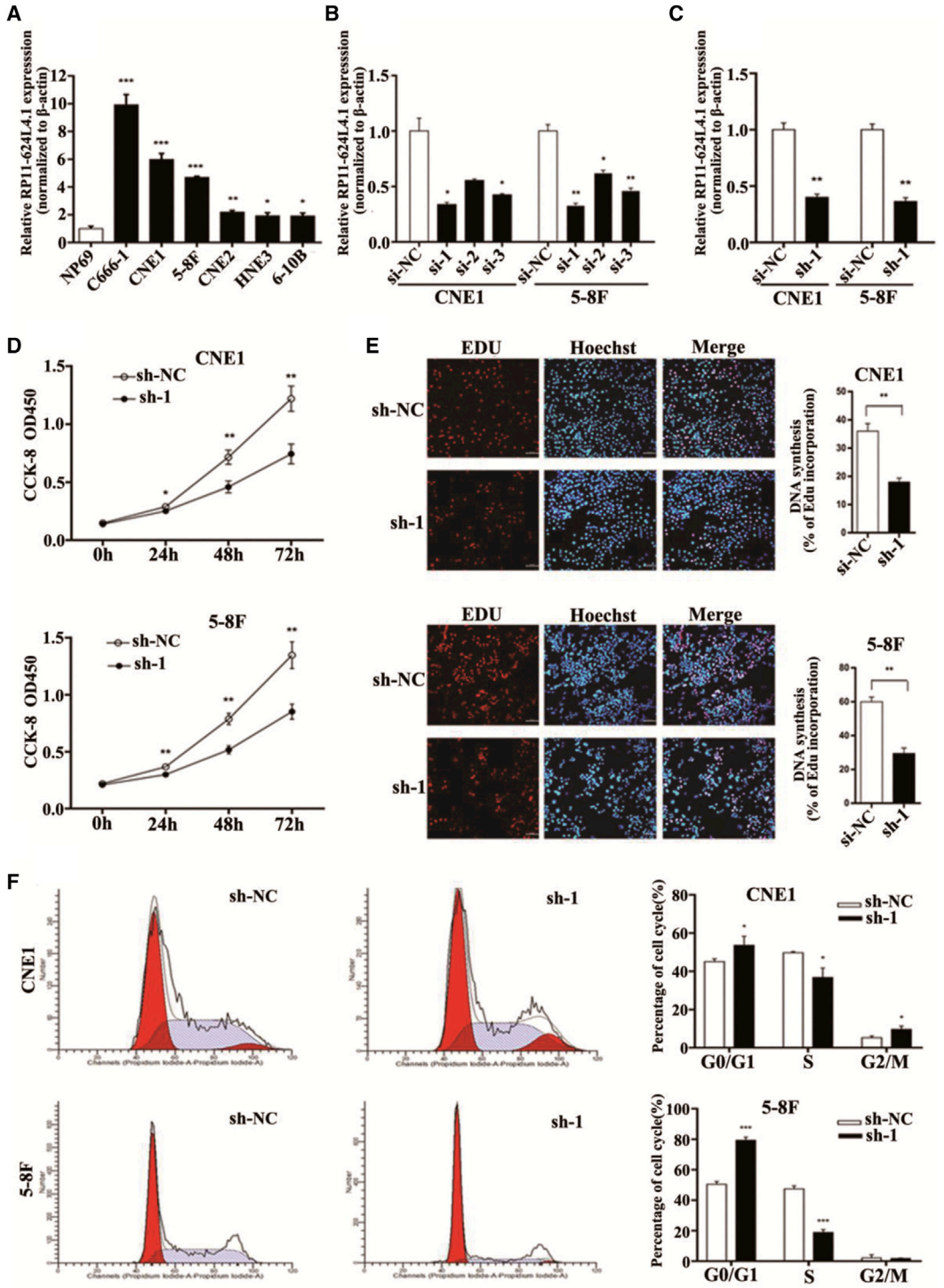

Figure 3. Knockdown of RP11-624L4.1 Inhibits the Proliferation and Cycle of NPC Cells

(A) The expression of RP11-624L4.1 was significantly higher in NPC cell lines (C666-1, CNE1, 5-8F, CNE2, 6-10B, and HNE3) compared with that in NP69. (B) The knockdown efficiency in the CNE1 and 5-8F cell lines was examined by quantitative real-time PCR. (C) Establishment of cell lines with the stable knockdown of RP11-624L4.1 confirmed by quantitative real-time PCR. sh-1, cell lines with the stable knockdown of RP11-624L4.1; sh-NC, cell lines with the stable infection of the negative control lentivirus. (D) The line

(legend continued on next page)

mechanism of lncRNAs would include the following: knockdown of lncRNA would change or interfere with the proper localization of the effector molecule, or may phenocopy loss of function of the component effector itself, dismantling the lncRNA-effector scaffold such that the components no longer assemble together. Moreover, the disruption of distinct regions of the lncRNA should affect different effector partners and function.[54] In our study, RPISeq predictions found that RP11-624L4.1 may act as a scaffold for CDK4, CDK6, and Cyclin D1 to form a protein complex. When overexpressed, CDK4 in the shRNA-1 cell with RP11-624L4.1 knocked down would not distinctly rescue the expression of CDK6, which further confirms the role of RP11-624L4.1 as a scaffold in the NPC cell. Wang et al.[58] reported that SKP2, an E3 ubiquitin ligase, interacts with the C-terminal lobe of CDK4 and enhances polyubiquitination and degradation of CDK4. We propose that highly expressed RP11-624L4.1 might increase the CDK4 expression level through competitive binding of SKP2 to inhibit ubiquitin-dependent degradation of CDK4. The specific mechanism needs to be further validated in future studies.

Conclusions

We found that RP11-624L4.1 is hyper-expressed in NPC tissues and predicts a poor prognosis. Knockdown of RP11-624L4.1 suppresses the proliferation and cycle of NPC cells. The opposite results are obtained in overexpression of NPC cell lines. RP11-624L4.1 promotes NPC cell tumorigenicity and tumor growth *in vivo*. Moreover, we provide evidence regarding the interaction of RP11-624L4.1 and CDK4, which further influences the CDK4/CDK6-Cyclin D1-Rb-E2F1 pathway. Upregulation of lncRNA RP11-624L4.1 is associated with unfavorable prognosis and promotes proliferation through the CDK4/CDK6-Cyclin D1-Rb-E2F1 pathway. Thus, lncRNA RP11-624L4.1 is required for NPC unfavorable prognosis and proliferation through the CDK4/6-Cyclin D1-Rb-E2F1 pathway, which may be a novel therapeutic target and prognostic in patients with NPC.

MATERIALS AND METHODS

Clinical Tissue Samples and Ethics Statements

The following three groups of clinical tissue samples of Chinese nasopharyngeal tissue were used in this study: group 1 included a total of seven NPC and seven nontumor NPE biopsy tissues for RNA sequencing (Illumina HiSeq 2500 instrument, the TruSeq PE Cluster Kit v.3-cBot-HS; Illumina, USA), group 2 included 20 NPC and 14 nontumor NPE biopsy tissues to analyze the expression of RP11-624L4.1 by quantitative real-time PCR, and group 3 included a tissue array comprising 130 paraffin-embedded NPC specimens (Shanghai Outdo Biotech, P.R. China) that were subjected to ISH to confirm RP11-624L4.1 expression. The NPC biopsy tissues in groups 1 and 2 were confirmed by histopathological examination at Xiangya Hospital of Central South University (patients had not undergone radiotherapy, chemotherapy, or surgery). NPE biopsy tissues with chronic inflammation of the nasopharyngeal mucosa were also collected as controls. All fresh tissues were immersed in RNAlater solution (Ambion, USA), then immediately immersed in liquid nitrogen and transferred to a −80°C laboratory freezer. All subjects gave their informed consent for inclusion in the study before they participated. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Xiangya Hospital, Central South University (project identification code: 201612797).

Cell Lines

The human immortalized nasopharyngeal epithelial cell line NP69 and the NPC cell line C666-1 were obtained from Cell Biology Laboratory, Modern Analysis and Testing Center, Central South University (Changsha, P.R. China). NPC cell lines, including CNE1, CNE2, 6-10B, 5-8F, and HNE3, were maintained in our laboratory. NP69 was cultured in a keratinocyte/serum-free medium (Invitrogen, USA) supplemented with bovine pituitary extract. CNE1, CNE2, 6-10B, 5-8F, HNE3, and C666-1 were cultured in RPMI-1640 medium (GIBCO, USA) with 10% fetal bovine serum (Biowest, France) and 1% penicillin-streptomycin (GIBCO, USA). All of the cell lines were cultured in a humidified incubator with 5% CO_2 at 37°C.

RNA Extraction, cDNA Synthesis, and Quantitative PCR (qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. Subsequently, first-strand cDNA was synthesized from 1 μg total RNA using the GoScript Reverse Transcription System (Promega, USA), followed by qPCR using the SYBR Green PCR master mix (Promega, USA) and a LightCycler 480 II System (Roche, USA).[40] Data were normalized to the expression level of β-actin and further normalized to the negative control. The relative expression of each target gene was calculated using the $2-\Delta\Delta Ct$ method. Primers were synthesized by Shanghai Sangon Biotech (P.R. China), and the sequences were as follows: RP11-624L4.1 forward (5′-GCATCCACTGTCTGGCACTC-3′) and reverse (5′-GATGGCCGAATAGGAACAGC-3′); CDK4 forward (5′-AGC TCCCGAAGTTCTTCTGC-3′) and reverse (5′-CATCTCGAGGCCA GTCATCC-3′); and β-actin forward (5′-AGGGGCCGGACTCGTCA TACT-3′) and reverse (5′- GGCGGCACCACCATGTACCCT-3′).

5′ and 3′ RACE Assay

We used 5′ and 3′ RACE (rapid amplification of the cDNA ends) to determine the full-length RP11-624L4.1, including the transcriptional initiation and termination sites with a GeneRacer Kit (Invitrogen, USA) according to the manufacturer's instructions. In brief, total RNA from CNE1 cells was extracted with an RNeasy Mini Kit (QIAGEN), and gDNA was removed by on-column deoxyribonuclease I (ribonuclease [RNase] free; New England Biolabs) digestion. The poly(A) tail detection assay was performed using total RNA from CNE1 cells, which were treated with or without poly(A) polymerase (TAKARA). The relative abundance of TROJAN in poly(A) polymerase-treated or untreated RNA was determined by qPCR.

diagram of the absorbance as determined by CCK-8 cell proliferation assay. (E) A representative photomicrograph and a column chart of EdU assays. Original magnification 10×, scale bars: 100 μm. (F) The representative images and quantification column chart of cell-cycle assays were measured using flow cytometry. Data are presented as the mean ± SD. Statistical significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Student's t test.

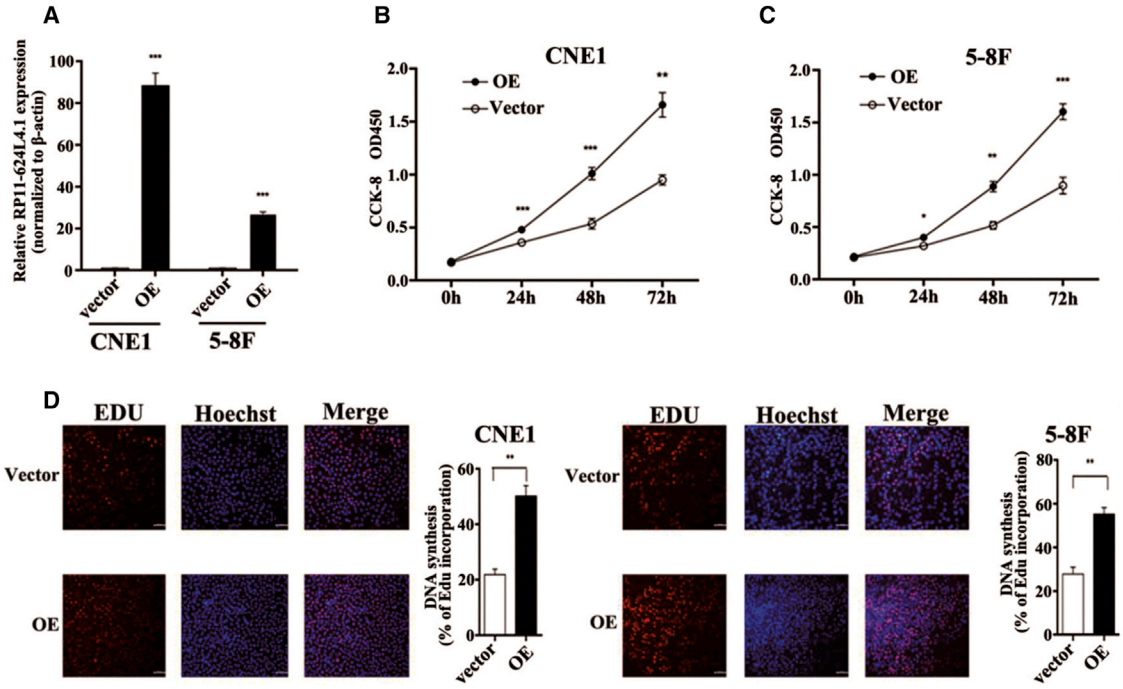

Figure 4. RP11-624L4.1 Overexpression Promotes NPC Cell Proliferation

(A) The overexpression efficiency in the CNE1 and 5-8F cell lines was evaluated by quantitative real-time PCR. OE, cell lines with the overexpression of RP11-624L4.1; vector, cell lines with the transfection with empty vector DNA. (B and C) The line diagram of the absorbance is determined by CCK-8 cell proliferation assay in OE and vector groups in CNE1(B) and 5-8F(C) cell line, respectively.. (D) A representative photomicrograph and a column chart of EdU assays. Original magnification 10×, scale bar: 100 μm. Data are presented as the mean ± SD. Statistical significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Student's t test.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (containing poly(A)) and U6 (without poly(A)) were used as reference genes. 5′ RACE and 3′ RACE products were amplified with their respective lncRNA-specific primers and cloned into the pGM-T Easy Vector (TIANGEN, P.R. China) for sequencing, and the spliced full-length lncRNAs were obtained using a new pair of primers. The sequences for the gene-specific PCR primers used for 5′ and 3′ RACE analysis are given in the Supplemental Information.

Transfection with siRNAs and Plasmids

siRNAs targeting human RP11-624L4.1 and negative control RNAs were provided by Guangzhou RuiboBio (P.R. China). In addition, to overexpress RP11-624L4.1, we amplified the full-length RP11-624L4.1 sequence and cloned it into the GV146 plasmid (GeneChem, P.R. China). To overexpress the sequence, we amplified the full-length CDK4 sequence and cloned it into the GV141 plasmid (GeneChem, P.R. China). The transfection of cells with siRNAs and plasmids was performed using Lipofectamine 3000 (Life Technologies, USA) or Polyplus-transfection (jetPRIME, France) reagent according to the manufacturer's instructions. siRNAs and the negative control RNAs were transfected into cells at a density of 50 nM/well of a six-well plate. In addition, the overexpression plasmids and the empty vector DNA plasmids were transfected into cells with 2 μg/well. Cells were harvested after being transfected for 48 h and used for the subsequent analyses.

The siRNA sequences were as follows: siRNA1 forward (5′-UCUG GAUUCGGCAUUACAUdTdT-3′) and reverse (5′-AUGUAAUG CCGAAUCCAGATdTd-3′); siRNA2 forward (5′-GGACCUAACU GAACUAAGAdTdT-3′) and reverse (5′-UCUUAGUUCAGUU AGGUCCTdTd-3′); and siRNA3 forward (5′-GGGUGUGGUG AUAAGGGAAdTdT-3′) and reverse (5′- UUCCCUUAUCACCAC ACCCTdTd-3′).

Infection with Lentiviral Vector Containing shRNA

To construct the RP11-624L4.1 stable knockdown cell lines, we generated a lentiviral vector containing hU6-MCS-Ubiquitin-EGFP-IRES-puromycin. The shRNA lentiviral system was designed according to the siRNA core target sequences, and the effects of the siRNA were validated. The shRNA sequence harbored on the lentiviral vector was as follows: shRNA-1 (5′-UCUGGAUUCGGCAUUACAU-3′). The lentivirus shRNA plasmids were transfected with a multiplicity of infection (MOI) of 50. Cell lines that exhibited stably knocked down expression of RP11-624L4.1 were selected for 14 days with 4 mg/mL puromycin after infection for 72 h. The infected cells were cultured with 2 mg/mL puromycin in the subsequent experiments.

Then, overexpression of CDK4 was performed in the RP11-624L4.1 stable knockdown cell lines in GV141 vector (GeneChem, P.R. China), and cell proliferation assays and WB were performed as described below.

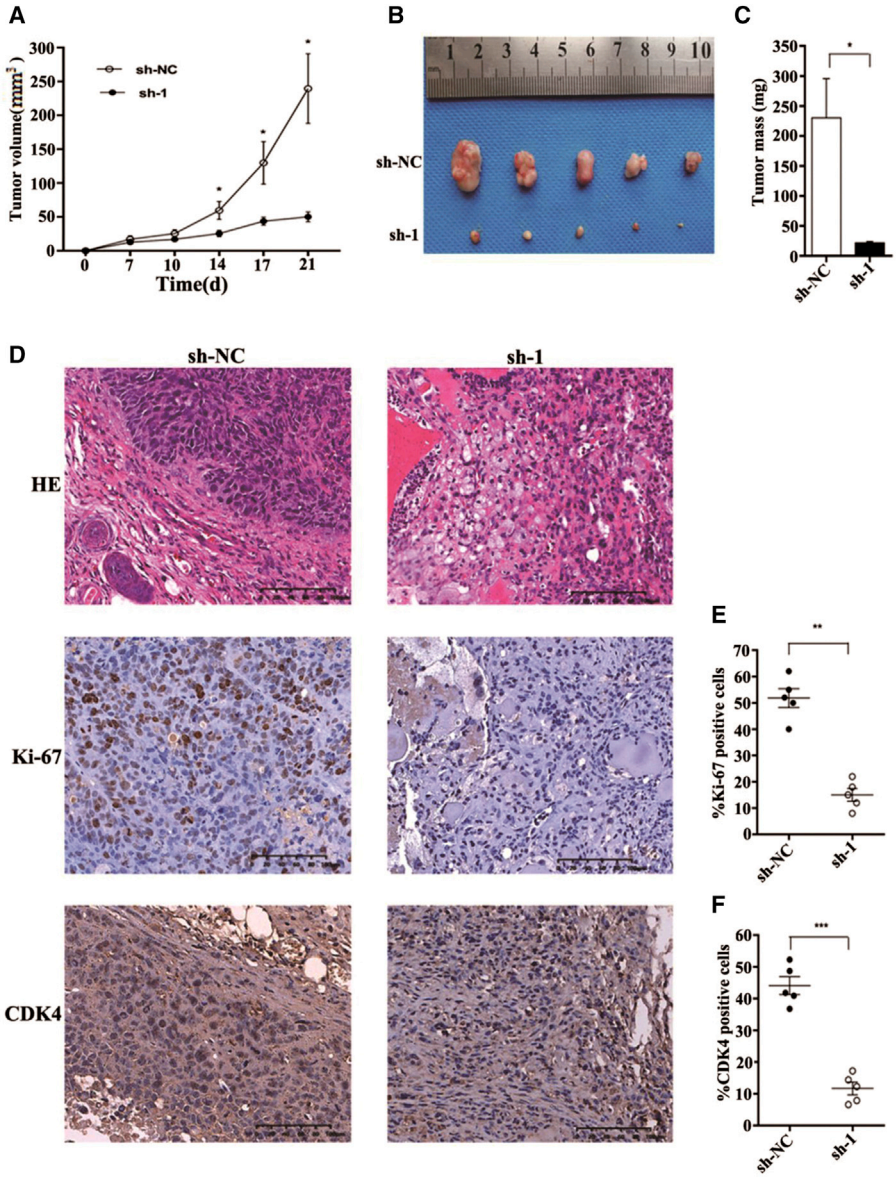

Figure 5. Knockdown of RP11-624L4.1 Suppresses the Growth of NPC Xenografts

(A) Lentiviral-infected tumor cells were injected into the nude mice, and the volume of the tumors was measured twice per week in the sh-NC and sh-1 groups (n = 5 in each groups). (B) Image of the fresh tumors. (C) The final xenograft weights of the sh-NC and sh-1 groups. Data are presented as the mean ± SEM; *$p < 0.05$, Student's t test. (D) The histomorphology of the tumor sections was analyzed in hematoxylin and eosin (H&E)-stained tumor tissues. Ki-67 and CDK4 were detected in the tumor tissues by IHC. Original magnification, 20×; scale bars: 100 μm. (E) The quantification charts indicated by the expression of Ki-67-positive cells in all tumor sections (n = 5 in both groups). (F) The quantification charts indicated by the expression of CDK4-positive cells in all tumor sections (n = 5 in both groups). Data are presented as the means ± SEM. **$p < 0.01$, Mann-Whitney *U* test.

ISH and Semiquantitative Scoring Criteria

For the *in situ* detection of RP11-624L4.1, the formalin-fixed paraffin-embedded tissue (4-mm-thick sections) array was hybridized with a 5′- and 3′-DIG-labeled oligonucleotide (Exiqon, USA) complementary to RP11-624L4.1. Hybridization was visualized using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color substrate (Roche, Basel, Switzerland). The probe sequence was designed to be complementary to RP11-624L4.1 as follows: 5′-AGACATGTA ATGCCGAATCCA-3′.

The intensity level of the stained probe and the positive staining levels were determined using semiquantitative scoring. Staining intensity was classified as follows: 0, no cell staining; 1, light blue staining; 2, moderate blue-purple-colored staining; and 3, strong purple-colored

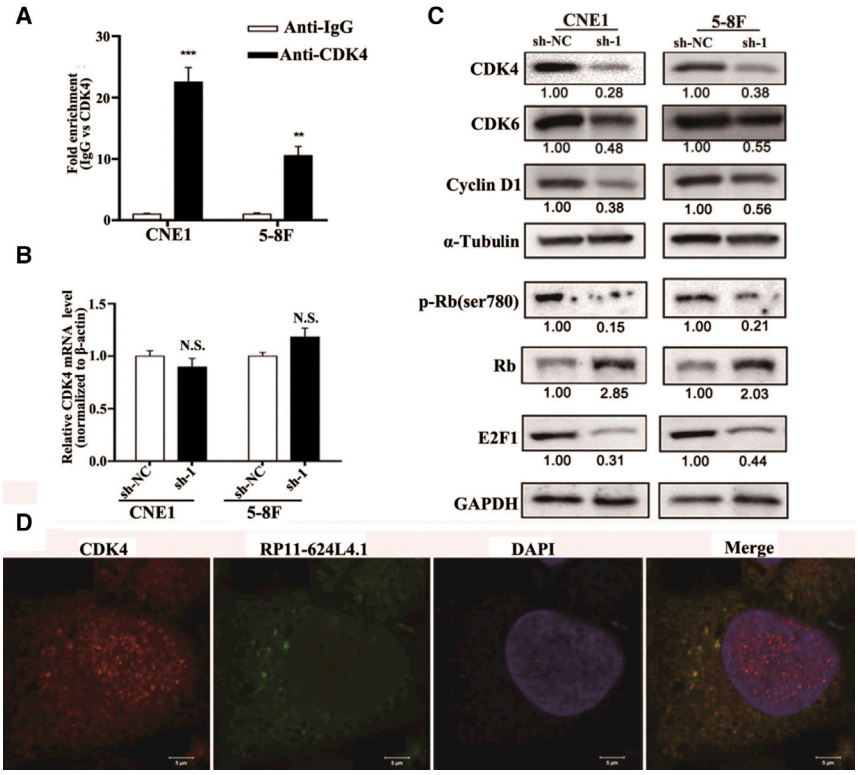

Figure 6. RP11-624L4.1 Interacts with CDK4 and Affects CDK4, CDK6, Cyclin D1, p-Rb (Ser 780), Rb, and E2F1 Protein Levels

(A) RIP assays of the interaction between RP11-624L4.1 and CDK4 by CDK4 antibody in CNE1 and 5-8F cells. The level of RP11-624L4.1 was determined by quantitative real-time PCR and normalized by the input levels. (B) The mRNA expression of CDK4 was determined by qRT-PCR in sh-NC and sh-1 cells. The results are expressed as log2 ($2-\Delta\Delta Ct$). Data are presented as the means ± SD; $^{N.S.}p > 0.05$, Student's t test. (C) The effects of RP11-624L4.1 on the protein expression levels of CDK4/6, cyclin D1, p-Rb (Ser 780), Rb, and E2F1 were detected by western blot (from three independent experiments). GAPDH and α-Tubulin were used as loading controls. The band intensity was quantified by ImageJ. (D) Confocal RNA fluorescence ISH and immunofluorescence images are shown as the colocalization of CDK4 and RP11-624L4.1. Scale bars: 5 μm. DAPI, 4′,6-diamidino-2- phenylindole.

staining. Positive staining was evaluated using the proportion of positive cells within the entire tumor: 0, no positive cells; 1, 0%–25% positive cells; 2, 26%–50% positive cells; 3, 51%–75% positive cells; and 4, 76%–100% positive cells. The final score was calculated by multiplying the two scores. All scores were determined independently by two pathology specialists who were double blinded. The median of the final score was determined as the cutoff value as follows: a final score ≤ 4 was defined as low RP11-624L4.1 expression, whereas a final score >4 was defined as high RP11-624L4.1 expression.

Cell Proliferation Assays

To measure cell proliferation levels, we conducted CCK-8 (Dojindo Laboratories, Japan) assays.[59] The cells (3×10^3 per well) were seeded into 96-well plates with 5-repetition wells and allowed to grow overnight. Then the medium was aspirated and replaced with 100 μL of fresh serum-free medium containing 10 μL CCK-8 solution (diluted 1:10). After incubation for 2 h, the 96-well plate was placed into an ultraviolet spectrophotometer (Bio Tek, USA), and the optical density value (absorbance) was detected at 450 nm. The treatments mentioned above were conducted at 0, 24, 48, and 72 h.

EdU assays were performed to detect the levels of cell proliferation. The cells (1×10^4 per well) were seeded into 96-well plates with 5-repetition wells and then incubated with EdU (Guangzhou RuiboBio, P.R. China) for 2 h followed by incubation with 100 μL 1× Apollo dyeing reaction solution for 30 min. Then, the DNA replication activity of the cells was detected with Hoechst 33342 stain for 30 min. The number of EdU-positive cells was determined under a fluorescence microscope in five random fields. All experiments were performed in triplicates.

Flow Cytometry Analysis

CNE1 and 5-8F cells were cultured in six-well plates for 48 h, trypsinized, centrifuged at 1,000 rpm for 5 min, and washed twice with cold PBS to measure cell cycle. ~70%–80% of ice-cold ethanol was added, incubated at 4°C for overnight, and washed twice with PBS. Propidium iodide (PI)/RNase staining buffer (BD, USA) was added and incubated at room temperature for 15 min in the absence of light. Results were measured by an FACSCalibur system (Cytek NL3000) and analyzed using FlowJo software. All assays were performed in triplicates, and representative data were provided.

To measure apoptosis, we stained CNE1 and 5-8F cells using the Annexin V kit according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). Cells were harvested by trypsinization. Cells were washed once with PBS, and then 1×10^6–10^7 cells were resuspended in 100 μL of 1× binding buffer. Fluorescein isothiocyanate (FITC)-labeled Annexin V and PI were added to samples and incubated in the dark for 15 min at room temperature (18°C–22°C). Subsequently, cells were subjected to the Cytek NL3000 FACSCalibur system. The results were analyzed using FlowJo software. All assays were performed in triplicates.

Mouse Xenograft Model, H&E Staining, and IHC

BALB/c nude male mice (4 weeks old) were obtained from the Laboratory Animal Department of Central South University (Changsha, P.R. China) and maintained under specific pathogen-free conditions. This animal study was approved by the Institutional Animal Ethics Committee of Central South University (project identification code: 2018sydw0111, date of approval: April 12, 2018). For the tumor

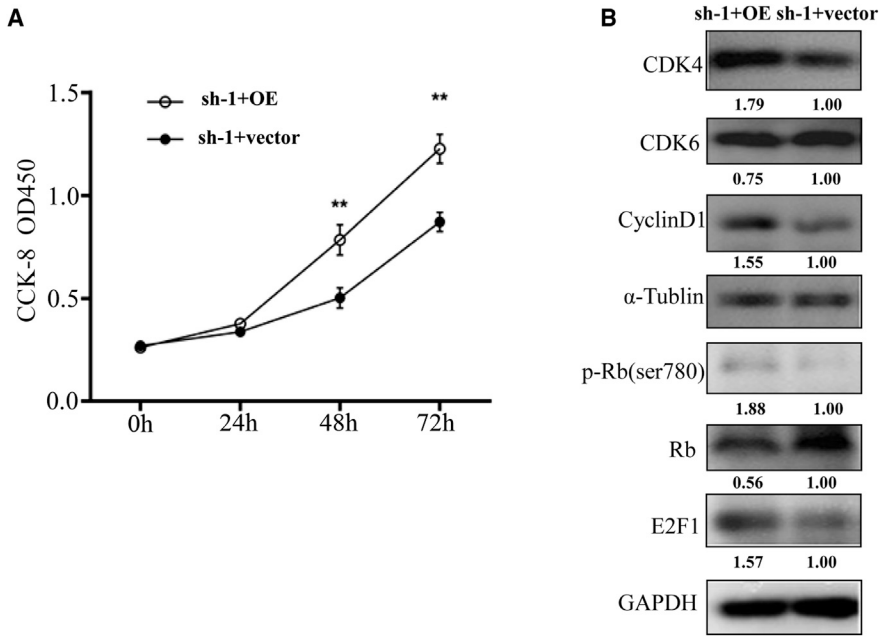

Figure 7. Rescue Assay Results by Overexpressing CDK4 in the Knocked Down lncRNA RP11-624L4.1 Cell sh-1

(A) The line diagram of CCK-8 cell proliferation assay. (B) Changes in proteins by western blotting in the CDK4/CDK6-Cyclin D1-Rb-E2F1 pathway. Sh-1+OE, overexpression of GV141-CDK4 in sh-1, which knocked down lncRNA RP11-624L4.1; Sh-1+vector, without overexpression of CDK4 (GV141 vector only) in sh-1, which knocked down lncRNA RP11-624L4.1.

xenograft experiments, nude mice were randomly divided into two groups (n = 5/group), including the sh-NC group and sh-1 group. Lentiviral-infected tumor cells (5×10^6 cells) were resuspended in 100 μL of free culture medium and injected subcutaneously into the right and left flanks of the nude mice. The tumor width (W) and length (L) were monitored twice per week, and the tumor volume was calculated using the following formula: $V = 1/2 (L \times W^2)$. Four weeks after the injection, the mice were killed by cervical dislocation, and the tumors were isolated, weighed, and immersed in formalin.

Next, the tumors were embedded in paraffin and cut into 4.0-μm sections for H&E staining and IHC. H&E staining and IHC were performed according to described protocols.[60,61] Anti-Ki-67 antibody (Beijing Zsbio, P.R. China) and anti-CDK4 antibody (Proteintech, P.R. China) were used for IHC assay. Images were captured using a Leica DMI3000B inverted microscope (Germany).

RIP Assay

RIP assays were performed using the Magna RIP RNA-binding protein immunoprecipitation kit (Millipore, USA). Anti-CDK4 antibody (Proteintech, P.R. China) and rabbit IgG purified (Millipore, USA) were used for RIP assay.[62] In brief, 2×10^7 cells were lysed in 0.1 mL of complete RIP lysis buffer containing RIP lysis buffer with protease inhibitors and RNase Inhibitor, and then centrifuged at 14,000 rpm for 10 min at 4°C. The supernatants and magnetic beads were incubated with 5 μg of CDK4 antibody or negative control rabbit IgG at 4°C overnight with gentle rotation. The beads were washed six times with ice-cold RIP wash buffer. Then, the RNA was purified, and quantitative real-time PCR was performed as described above.

WB

Proteins were extracted, and a WB assay was performed according to previously described methods.[60,63,64] Anti-CDK4 antibodies, anti-CDK6 antibodies (Proteintech, P.R. China), anti-Cyclin D1 antibodies (Proteintech, P.R. China), anti-Rb antibodies (Cell Signaling Technology, USA), anti-Phospho-Rb (Ser780) antibodies (Cell Signaling Technology, USA), goat anti-rabbit IgG (Millipore, USA), goat anti-mouse IgG (Millipore, USA), pre-stained protein ladder (Thermo Fisher Scientific, the USA), anti-α-Tubulin antibodies (Cell Signaling Technology, USA), and anti-GAPDH antibody (Proteintech, P.R. China) were used for the WB. The blots were visualized with the chemiluminescence detection system (FluorChem FC3; Bio-Tek, USA) using an enhanced chemiluminescence detection reagent (Beyo ECL Plus, P.R. China). The band intensity was quantified by densitometry using ImageJ software (https://imagej.nih.gov/ij). GAPDH or α-Tubulin served as internal controls. The experiment was performed three times.

IF and RNA FISH

For IF, cells were fixed on coverslips and permeabilized with 4% paraformaldehyde in PBS at room temperature for 10 min, and blocked with 5% normal goat serum in PBS at room temperature for 10 min. Cells were then incubated with primary antibodies at room

temperature for 1 h, followed by incubating with secondary antibodies at room temperature for 1 h. Primary antibody for IF was anti-CDK4 antibodies (1:100). Red fluorescent secondary antibody for IF was DyLight 594 goat anti-rabbit IgG (Abbkine, USA). The samples were then processed using RNA FISH protocol. RNA FISH experiments were done using Custom LNA Detection Probe (0.25), 5′ and 3′ TYE563 kit (QIAGEN, Germany), and ViewRNA ISH Kit (Boster Biological Technology, P.R. China), according to manufacturers' instructions. The samples were counterstained with DAPI and observed using laser scanning confocal microscopy (Carl Zeiss, Germany).

Bioinformatics Analysis and Statistical Analysis

RPISeq software (from website http://pridb.gdcb.iastate.edu/RPISeq/) was used to predict the interaction probability between RP11-624L4.1 and proteins of the CDK4/6-Cyclin D1-Rb-E2F1 pathway.[65]

Statistical analyses were performed using SPSS 18.0, GraphPad Prism 7.0, and Microsoft Excel. The relationships between RP11-624L4.1 expression level and the clinicopathological features of NPC patients were analyzed with a 2×2 χ^2 test. Differences between two groups were assessed using a two-tailed Student's t test and a Mann-Whitney *U* test. Survival curves were plotted by Kaplan-Meier survival analysis and evaluated through the log rank test.[66] Univariate and multivariate regression analyses were performed according to the Cox proportional hazards regression model. All data were obtained from three independent experiments and expressed as the mean ± SD or mean ± SEM. A p value < 0.05 was used as the threshold for significance.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2020.10.017.

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AUTHOR CONTRIBUTIONS

Conceptualization, P.Z.; Methodology, P.Z. and J.F.; Software, L.Z.; Validation, L.Z., R.L., and W.B.; Formal Analysis, L.Z. and S.Z.; Investigation, R.L., W.B., M.Y., and Y.H.; Resources, S.L. and X.Y.; Data Curation, S.Z.; Draft Preparation, L.Z.; Manuscript – Revising & Editing, P.Z. and J.F.; Visualization, L.Z.; Supervision, P.Z.; Project Administration, P.Z. and M.Y.; Project Design, P.Z. and J.F.

DECLARATION OF INTEREST

The authors declare no competing interests.

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