MYCT1 represses apoptosis of laryngeal cancerous cells through the MAX/miR-181a/NPM1 pathway

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Introduction

Laryngeal carcinoma is a malignant tumor originated from the epithelial cells of laryngeal mucosa; it is also the second most common malignant head and neck tumor in the world [1,2]. Both environmental and genetic factors contribute to laryngeal carcinogenesis and the incidence of laryngeal cancer has been increasing steadily [3,4]. Despite various advanced treatment interventions, the 5-year survival rate of laryngeal cancer patients has not been significantly improved over the past several decades, with a 5-year survival rate of 50–60%, and the molecular mechanism underlying laryngeal cancer occurrence and development remains unclear [5,6]. Inhibition of apoptosis is a common event in carcinogenesis [7]. Laryngeal carcinoma is an aggressive and lethal malignant tumor resistant to various apoptosis stimulating factors and its poor prognosis is also related to inhibition of apoptosis [8,9]. Studies have revealed that expression of BCL2 (B-cell lymphoma 2)

Abbreviations
BCL-2, B-cell lymphoma 2; bHLHZip, helix-loop-helix leucine zipper; CCK8, Cell Counting Kit-8; ChIP, chromatin immunoprecipitation assay; Co-IP, co-immunoprecipitation; IRF1, interferon regulatory factor 1; KLF6, Krüppel like factor 6; KRAS, Kirsten rat sarcoma 2 viral oncogene homolog; LSCC, laryngeal squamous cell cancer; miRNA, microRNA; NC, negative control; NPM, nucleophosmin; TNF, tumor necrosis factor; UTR, untranslated region.
and Survivin in laryngeal squamous cell carcinoma is associated with pathological grade and recurrence rate of laryngeal cancer, indicating that these are potential biomarkers for predicting the survival of laryngeal cancer patients [10–12]. Livin expression is significantly higher in laryngeal squamous cell carcinoma than in vocal cord polyp, and its overexpression has been correlated with laryngeal pathological stages [13]. Mutant P53 shows differential expression in different stages of laryngeal carcinoma and precancerous lesions, and is also associated with laryngeal cancer cell viability and prognosis [14,15]. In addition, the expression of Caspases-3, -8 and -9 has also been related to the clinical prognosis of laryngeal cancer patients and thus, can be potential biomarkers for laryngeal cancer treatment and prognosis [16–18]. Available data all imply that apoptosis plays an important role in the occurrence, development, and prognosis of laryngeal carcinoma.

We previously identified and cloned human MYCT1, which we named as MTLC standing for myc-target from laryngeal carcinoma [19]. Our data have demonstrated that MYCT1 inhibits laryngeal cancer cell viability and promotes apoptosis as a tumor suppressor [20,21]. However, the molecular mechanism through which MYCT1 modulates laryngeal cancer cell viability and apoptosis still remains unknown. NPM1 gene encodes a shuttle protein that travels between the nucleus and cytoplasm [22]. In addition to constituting the nucleolus, NPM1 is also involved in the regulation of cancer cell viability and apoptosis via both extracellular death receptor pathway and intracellular mitochondrial pathway [23]. Studies have shown that NPM1 inhibits apoptosis by blocking the translocation of P53 from nucleus to mitochondria [24,25]. In acute promyelocytic leukemia, NPM1 represses apoptosis by preventing TNF (tumor necrosis factor)-induced extracellular apoptosis pathways [26]. NPM1 was also reported to maintain the resistance of NIH-3T3 cells to UV-induced apoptosis by binding IRF1 (interferon regulatory factor 1) [27]. Although NPM1 has been studied in the other cancers, whether and how NPM1 is involved in the pathogenesis of laryngeal cancer is yet to be investigated.

In our subsequent study using next-generation RNA deep sequencing (RNA-seq) analyses, we identified NPM1 as the most dysregulated apoptosis-related genes in Hep2 cells with stable overexpression of MYCT1. This prompted us to explore whether MYCT1 directly controls NPM1 expression, which, in turn, affects laryngeal cancer apoptosis. Here, we show that MYCT1 may interact synergistically with MAX as a co-transcription factor or a component of MAX transcript complex, which, in turn, directly enhances the transcriptional activity of miR-181a. Moreover, NPM1 is a direct target of miR-181a. Thus, MYCT1 appears to participate in the regulation of laryngeal cancer cell viability and apoptosis through the MAX/miR-181a/NPM1 pathway.

Results

MYCT1 is a negative regulator of NPM1 in laryngeal cancer cells

To identify the downstream effectors of MYCT1, we performed RNA-seq to compare the gene expression profiles between laryngeal cancer cells stably transfected with MYCT1 expression plasmids and control cells with the empty vector. Raw data were archived in the Gene Expression Omnibus under accession number GSE75544. Our transcriptomic analyses identified 326 down-regulated genes and 1 up-regulated gene (≥2-fold) (Fig. 1A). Gene ontology (GO) analyses identified that these genes were involved in a wide variety of biological processes and cellular functions (Fig. 1B). Among these dysregulated genes, NPM1 was down-regulated by ~3-fold (Data S1). To validate the RNA-seq results, we conducted both quantitative real-time PCR (qRT-PCR) and western blot assays using stable MYCT1-expressing Hep2 cells. MYCT1 mRNA was indeed significantly overexpressed in Hep2 cells transfected with MYCT1 expression plasmids, as compared to the control cells transfected with empty vectors (Fig. 1C). In contrast, both mRNA and protein levels of NPM1 significantly decreased in Hep2 cells with stable MYCT1 overexpression (Fig. 1D,E). These results suggest that MYCT1 down-regulates NPM1 in laryngeal cancer cells.

NPM1 plays an oncogenic role in laryngeal cancer cells

We then examined NPM1 levels in 45 cases of laryngeal cancer tissues (T) and paired nontumorous tissues (R). NPM1 mRNA levels were up-regulated in 37 out of 45 cases (82%) of laryngeal cancer tissues (Fig. 2A) and the average levels were significantly greater in laryngeal cancer tissues than in paired nontumorous tissues (P < 0.001, Fig. 2B). Moreover, NPM1 mRNA levels were significantly higher in Hep2 cells than in HEK293T cells (P < 0.05, Fig. 2C). NPM1 protein was overexpressed in 14 out of 16 laryngeal cancer cases (87.5%) and the average levels were significantly higher in laryngeal cancer tissues than in controls (Fig. 2D).
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A. Gene expression level (Control-VS-MYCT1)
   Define: Regulated (FDR <= 0.001 & abs(log2(Y/X)) >= 1)

B. Go Functional classification (Control-VS-MYCT1)

C. Relative MYCT1 mRNA expression

D. Relative NPM1 mRNA expression

E. Western Blotting
   Stable MYCT1-overexpressing vs Control
   NPM1
   β-actin
   Relative NPM1 protein expression
We then assayed the effects of NPM1 on laryngeal cancer cell viability and apoptosis. Three NPM1 sh-RNAs were used to knock down the NPM1 gene. Both NPM1 mRNA and protein levels significantly decreased in Hep2 cells transfected with the three sh-RNAs compared to those in Hep2 cells transfected with a scrambled shRNA (Fig. 2E,F). Since sh-NPM1-2# displayed the highest knockdown efficiency, it was used in the subsequent experiments. Hep2 cells were transfected with the expression plasmids and sh-RNA 2# of NPM1, and the effect on cell viability was measured 48 h after transfection. Cell Counting Kit-8 (CCK8) and colony-formation assays revealed that NPM1 knockdown significantly inhibited laryngeal cancer cell survival and colony formation, as compared to empty vector-transfected and untransfected cells. In contrast, both survival and colony formation were enhanced significantly after the laryngeal cancer cells were transfected with NPM1 expression plasmids (Fig. 2G,H). We also examined the effect of NPM1 overexpression on Hep2 cell apoptosis. For the apoptosis assay, Hep2 cells were harvested 48 h after transfection, stained with Annexin V-PE and 7-AAD and subsequently analyzed with flow cytometry. As a result, early apoptosis significantly increased in the NPM1 knockdown Hep2 cells compared to the empty vector-transfected and untransfected cells. In contrast, there was no significant effect of NPM1 overexpression on early apoptotic event in laryngeal cancer cells (Fig. 2I). Together, these data suggest that NPM1 has an oncogenic role in laryngeal cancer cells.

MAX may interact synergistically with MYCT1 in Hep2 cells

Based on online-software STRING prediction, we found four potential MYCT1-interacting proteins including MAX (Fig. 3A). Co-immunoprecipitation (Co-IP) assays revealed that both anti-GFP (for MYCT1) and anti-MAX antibodies could precipitate (Co-IP) assays revealed that both anti-GFP (for MYCT1) and anti-MAX antibodies could precipitate the MYCT1-MAX protein interaction in Hep2 cells (Fig. 3B). Immunofluorescence analyses revealed enhanced co-localization of MYCT1 (green) and MAX (red) proteins in the nuclei of Hep2 cells overexpressing MYCT1, as compared to the controls (Fig. 3C), suggesting that nuclear translocation of MAX was promoted by overexpression of MYCT1, leading to the enhanced co-localization of MYCT1 and MAX in Hep2 cell nuclei. These results suggest that there was a protein-protein interaction between MYCT1 and MAX which may be direct by combining with each other or indirect by depending on a third protein.

MAX positively regulates miR-181a transcription in laryngeal cancer cells

qRT-PCR analyses indicated that endogenous miR-181a levels were down-regulated in the majority (85%, 40 of 47) of the laryngeal cancer cases (Fig. 4A), and its average levels were significantly lower in laryngeal cancer tissues than that in paired noncancerous tissues (P < 0.001, Fig. 4B). qRT-PCR analyses also showed that endogenous miR-181a levels decreased significantly in Hep2 cells compared to HEK293T cells (P < 0.001, Fig. 4C). These findings suggest that miR-181a might act as a cancer suppressor gene in laryngeal cancer. Compared to the control cells, miR-181a was significantly up-regulated in Hep2 cells stably expressing MYCT1 (P < 0.01, Fig. 4D), indicating that miR-181a is also a potential downstream target of MYCT1.

Based on bioinformatics prediction, we found two potential-binding sites of MAX in the miR-181a gene promoter region (Fig. 4E). Three specific small interfering RNAs against MAX mRNA, si-MAX 1#, si-MAX 2#, and si-MAX 3#, significantly repressed MAX expression at both mRNA and protein levels in Hep2 cells as compared to negative control-treated and untransfected Hep2 cells (Fig. 4F,G), suggesting that MAX was successfully knocked down. Meanwhile, we found that miR-181a was significantly down-regulated in Hep2 cells when MAX was suppressed compared to negative control-treated and untransfected Hep2 cells (P < 0.01, Fig. 4H).

To explore whether miR-181a was regulated by MAX in Hep2 cells, we examined the miR-181a core
promoter region for two putative MAX-binding sites at the regions -292 to -283 bp (R1) and -611 to -602 bp (R2) in the miR-181a promoter (Fig. 4E). PCR products spanning two putative MAX-binding sites of the miR-181a promoter region were detected from chromatin fragment precipitated by anti-MAX antibody in our chromatin immunoprecipitation assays (ChIP) (Fig. 4I). The result showed that endogenous MAX indeed bound to miR-181a promoters. Furthermore, MAX knockdown significantly reduced levels of MAX on the miR-181a promoter compared to the negative controls (P < 0.01, Fig. 4J). These results imply that MAX binds directly to the miR-181a promoter region in vivo and promotes its expression as the transcriptional factor.
To corroborate this notion, we then explored the binding of MAX to miR-181a in Hep2 cells by luciferase reporter assays. The fragments of miR-181a promoter regions containing one (-292 bp) or two (-611 bp) MAX-binding sites were cloned into GV148 vector and transiently transfected into Hep2 cells along with pRL-TK. Luciferase results revealed ~7-fold and ~5-fold increases in transcriptional activities.

Fig. 2. NPM1 is an oncogenic gene in laryngeal carcinoma. (A) Endogenous NPM1 mRNA levels in 45 laryngeal cancer tissues and their paired adjacent noncancerous tissues, as determined by qRT-PCR. (B) Levels of endogenous NPM1 mRNA expression in 45 laryngeal cancer tissues (T) and their adjacent nontumorous tissues (R) with β-ACTIN as a loading control. Left, western blot results showing NPM1 protein levels in laryngeal cancer tissues. Right, quantitative analyses of NPM1 protein levels. (E) Transfection efficiency of NPM1 overexpressing plasmid and sh-RNA in Hep2 cells, as determined by qRT-PCR analyses of mRNA levels. Hep2 cells treated with a nontargeting sh-RNA were used as controls. (F) Transfection efficiency of overexpression plasmid and sh-RNA of NPM1 in Hep2 cells, as determined by western blot analyses of protein levels using β-ACTIN as a loading control. Hep2 cells transfected with a nontargeting sh-RNA were used as controls. Left, western blot results showing NPM1 protein levels in Hep2 cells. Right, quantitative analyses of NPM1 protein levels. (G) Effects of NPM1 on laryngeal cancer cell viability by CCK8 assays. Each sample was assayed in triplicates for 3 consecutive days. Hep2 cells transfected with a nontargeting sh-RNA were used as controls. The blank samples were untransfected Hep2 cells. (H) Effects of NPM1 on laryngeal cancer cell viability by colony formation assays. Representative photographs are shown, and the number of colonies was counted. The control group was Hep2 cells transfected with a nontargeting sh-RNA. The blank group was untransfected Hep2 cells. (I) Effects of NPM1 on laryngeal cancer cell apoptosis using Annexin V PE/7-AAD by flow cytometry. The control group was Hep2 cells transfected with a nontargeting sh-RNA. The blank group was untransfected Hep2 cells. Left, the vertical and horizontal axes stand for 7-AAD- and Annexin V PE-positive areas, respectively. Identified by flow cytometry, cells were divided into four sections: Q1: Annexin V PE-/7AAD+, was representative of mechanical error; Q2: Annexin V PE+/7AAD-, was representative of late apoptosis or necrosis cells; Q3: Annexin V PE-/7AAD-, was representative of living cells; Q4: Annexin V PE+/7AAD-, was representative of early apoptosis cells. Right, bar graphs show the percentage of early apoptosis cell population after transfection for 48 h. Data are presented as mean ± SEM, n = 3. *,**,*** and ns represent P < 0.05, P < 0.01, P < 0.001 and no significance, respectively.

Fig. 3. MYCT1 interacts with MAX. (A) Prediction of proteins that interact with MYCT1 using STRING (http://string-db.org/). The combined score between MYCT1 and MAX was 0.90. The thickness of lines between molecules indicates the strength of data support. (B) Binding of MYCT1 to MAX in Hep2 cells by co-immunoprecipitation followed by western blot. Upper panel, co-IP using anti-GFP antibody for immunoprecipitation and anti-MAX antibody for western blot detection in MYCT1-overexpressing Hep2 cells. Lower panel, co-IP using anti-MAX antibody for immunoprecipitation and anti-MYCT1 antibody for western blot detection in wild-type Hep2 cells. (C) Confocal microscopic images of Hep2 cells with and without transfection of MYCT1. The control group was Hep2 cells without MYCT1 overexpression. The nucleus was stained blue with DAPI, MYCT1 was shown in green, MAX was shown in red, and MYCT1 co-localized with MAX are yellow in the merged image. Bar: 50 μm. Data were from at least three independent experiments.
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Fig. 4. MAX directly represses miR-181a transcription in laryngeal cancer cells. (A) Relative endogenous expression levels of miR-181a levels in 47 pairs of laryngeal cancer samples. (B) Levels of miR-181a in 47 pairs of laryngeal cancer samples. T and R represent cancer tissues and paired noncancerous adjacent tissues, respectively. (C) Levels of endogenous miR-181a in Hep2 and HEK293T cells, as determined by qRT-PCR. (D) Levels of endogenous miR-181a in laryngeal cancer cells stably expressing MYCT1 or the empty vector, as determined by qRT-PCR. Here, U6 was used as the internal control. (E) Predicted MAX-binding sites in miR-181a promoter. (F) Transfection efficiency of MAX siRNAs in Hep2 cells at mRNA levels, as determined by qRT-PCR. The Blank group was untransfected Hep2 cells. (G) Transfection efficiency of MAX siRNAs in Hep2 cells, as determined by western blot analyses of protein levels using β-actin as a loading control. The Blank group was untransfected Hep2 cells. Left, western blots showing β-actin protein levels in Hep2 cells. Right, quantitative analyses of β-actin protein levels. (H) Effects of MAX knockdown on expression of miR-181a in Hep2 cells by qRT-PCR. The Blank group was untransfected Hep2 cells. (I) Binding of endogenous MAX to the miR-181a gene promoter region in Hep2 cells by ChIP-qPCR. Antibody products containing MAX-binding sites are 128 bp and 179 bp in length. Input DNA and epitope-matched anti-IgG antibody were used as controls. (J) Effects of MAX knockdown on binding ability of MAX to the miR-181a gene promoter regions in Hep2 cells by ChIP-qPCR. Antibody enrichment was quantified relative to amount of input DNA. IgG was used as a negative control. (K) Effects of MAX/MYCT1 knockdown on miR-181a transcription activity by a luciferase activity assay. The luciferase reporter vectors of GV148-miR-181a R1 (-292)/R1 + R2 (-611) promoter were co-transfected with a renilla luciferase vector (pRL-TK) for normalization to Hep2 cells, which were treated with or without si-MAX/si-MYCT1, respectively. The relative luciferase activities were represented by the ratio of firefly to renilla signals. Data are presented as mean ± SEM, n = 3. *,**, *** and ns represent P < 0.05, P < 0.01, P < 0.001 and no significance, respectively.

miR-181a down-regulates laryngeal cancer cell viability and apoptosis via directly targeting NPM1

Using several miRNA target prediction programs (TargetScan, miRanda, and picTar), we found NPM1 as a potential target gene of miR-181a (Fig. 5A). However, miR-181a mimics significantly up-regulated, whereas miR-181a inhibitor repressed miR-181a expression in Hep2 cells as compared to negative control-treated and untransfected Hep2 cells, indicating that transfection is successful (P < 0.01, Fig. 5B). Here, qRT-PCR and western blot analyses showed that both NPM1 mRNA (Fig. 5C) and protein (Fig. 5D) levels were significantly down-regulated in Hep2 cells treated with miR-181a mimics, but up-regulated in Hep2 cells treated with the inhibitor, as compared to negative control-treated cells and untransfected Hep2 cells. A negative correlation was identified between miR-181a and NPM1 mRNA levels (r = -0.4185, P < 0.01, Fig. 5E). We also performed luciferase reporter assays with the wild-type and mutant 3′UTRs of NPM1. Our results demonstrated that miR-181a significantly decreased the relative luciferase activity of the wild-type NPM1 3′UTR compared to the controls (P < 0.01, Fig. 5F). However, miR-181a had no significant effect on mutant 3′UTR of NPM1, suggesting that miR-181a indeed targets NPM1.

CCK8 and colony formation assays showed that miR-181a mimics and inhibitor significantly suppressed and promoted Hep2 cell survival and colony formation compared to negative control-treated cells and untransfected Hep2 cells, respectively (Fig. 5G,H). For apoptosis assays, Hep2 cells were harvested 48 h after incubation, stained with Annexin V-PE and 7-AAD for flow cytometry. As a result, miR-181a overexpression and knockdown significantly enhanced and inhibited laryngeal cancer cell apoptosis, respectively (Fig. 5I). Moreover, the effects of miR-181a on laryngeal cancer cell viability and early apoptosis were rescued by NPM1 expression (Fig. 5G-I). Collectively, these results suggest that miR-181a suppresses cell viability and promotes early apoptosis directly via NPM1 in laryngeal cancer cells.

MYCT1 promotes cell viability and inhibits apoptosis through miR-181a and NPM1

MYCT1 significantly inhibited laryngeal cancer cell survival and colony formation compared to controls (empty vector-transfected cells and untransfected Hep2 cells) (Fig. 6A,B). Both NPM1 overexpression and
miR-181a knockdown rescued the effects of MYCT1 on laryngeal cancer cell survival and colony formation compared to controls. Flow cytometry suggested that MYCT1 significantly promoted laryngeal cancer cell early apoptosis compared to controls of empty vector-transfected cells and untransfected Hep2 cells, and

Fig. 5. miR-181a affects the viability of laryngeal cancer cells via targeting NPM1. (A) Predicted targeting sites by miR-181a in NPM1 3′UTR. Mutations were generated in the NPM1 3′UTR sequence at the complementary sites for the seed regions in miR-181a. (B) Transfection efficiency of miR-181a mimics and inhibitor in Hep2 cells by qRT-PCR 48 h post-transfection. U6 was used as an internal control. The blank group was untransfected Hep2 cells. (C) Effects of miR-181a on NPM1 mRNA expression in Hep2 cells after transfection with miR-181a mimic and inhibitor, as determined by qRT-PCR. The blank group was untransfected Hep2 cells. (D) Effects of miR-181a on NPM1 protein levels in Hep2 cells after transfection with miR-181a mimic and inhibitor, as determined by western blot analyses using β-ACTIN as a loading control. The blank group was untransfected Hep2 cells. Upper panel: a representative western blot result. Lower panel: quantitative analyses of NPM1 protein levels. (E) Correlations between miR-181a and NPM1 mRNA levels in laryngeal cancer tissues by Pearson’s product-moment correlation coefficient. (F) Validation of the targeting relationship between miR-181a and NPM1 using luciferase assays in Hep2 cells. The reporter vector was co-transfected with a renilla luciferase vector (pRL-TK) for normalization to Hep2 cells, which were treated by miR-181a mimics or NC. The 3′UTR of NPM1 was mutated on the predicted binding site as shown in (A), and was tested in the luciferase assays as described above. The relative luciferase activities were represented by the ratio of firefly to renilla signals. (G) miR-181a affects laryngeal cancer cell viability via targeting NPM1. Each sample was assayed in triplicates for 3 consecutive days. The blank group represented untransfected Hep2 cells. (H) miR-181a affects laryngeal cancer colony formation via targeting NPM1. Representative photographs are shown, and the number of colonies was counted. The blank group was untransfected Hep2 cells. (i) Effects of miR-181a on laryngeal cancer cell apoptosis via targeting NPM1 using Annexin V PE+/AAD+, was representative of late apoptosis or necrosis cells; Q2: Annexin V PE+/AAD+, was representative of late apoptosis or necrosis cells; Q3: Annexin V PE+/AAD+, was representative of late apoptosis or necrosis cells; Q4: Annexin V PE+/AAD+, was representative of early apoptosis cells. Right, bar graphs show the percentage of early apoptosis cell population 48 h after transfection. Data are presented as mean ± SEM, n = 3. *,**, and *** represent P < 0.05, P < 0.01 and P < 0.001, respectively.
**Fig. 6.** The function of MYCT1 in Caspase-mediated apoptosis in laryngeal cancer cells. (A) NPM1 and miR-181a inhibitor rescued the effects of MYCT1 on Hep2 cell viability. Each sample was assayed in triplicates for 3 consecutive days. The Control group was empty vector-transfected Hep2 cells. The Blank group was untransfected Hep2 cells. (B) NPM1 and miR-181a inhibitor rescued the effects of MYCT1 on Hep2 cell colony formation. Representative photographs are shown, and the number of colonies was counted. The Control group was empty vector-transfected Hep2 cells, whereas the Blank group was untransfected Hep2 cells. (C) NPM1 and miR-181a inhibitor rescued the effects of MYCT1 on Hep2 cell apoptosis. Effects of MYCT1 on laryngeal cancer cell apoptosis using Annexin V PE/7-AAD and flow cytometry. The Control group was empty vector-transfected Hep2 cells. The Blank group was untransfected Hep2 cells. Left, the vertical and horizontal axes stand for 7-AAD- and Annexin V PE-positive area, respectively. Based on flow cytometry, cells were divided into four sections: Q1: Annexin V PE-/7AAD+, was representative of mechanical error; Q2: Annexin V PE+/7AAD+, was representative of late apoptosis or necrosis cells; Q3: Annexin V PE-/7AAD-, was representative of living cells; Q4: Annexin V PE+/7AAD-, was representative of early apoptosis cells. Right, bar graphs showing the percentage of early apoptosis cell population 48 h after transfection. (D) NPM1 and miR-181a inhibitor rescued the effects of MYCT1 on Caspases-3, -8, and -9 activities in Hep2 cells. The Blank group was untransfected Hep2 cells. (E) NPM1 overexpression and miR-181a inhibitor rescued the effects of MYCT1 on Caspases-3, -8, and -9 protein levels in Hep2 cells after 48 h transfection, as determined by western blot using β-ACTIN as a loading control. The Blank group was untransfected Hep2 cells. Left, western blot results showing Caspase-3, -8, and -9 protein levels in Hep2 cells. Right, quantitative analyses of Caspase-3, -8 and -9 protein levels. Data are presented as the mean ± SEM, n = 3. *, ** and ns represent P < 0.05, P < 0.01 and no significance, respectively.
both NPM1 overexpression and miR-181a knockdown significantly rescued the effects of MYCT1 on laryngeal cancer cell early apoptosis compared to controls (Fig. 6C). These results suggest that MYCT1 regulates laryngeal cancer cell viability and apoptosis via miR-181a/NPM1.

As NPM1 acts as a regulator in mitochondrial-dependent and -independent apoptotic pathways, we explored the effects of MYCT1 on protein levels and activities of Caspases-3, -8, and -9 in laryngeal cancer cells. MYCT1 significantly increased the activities of Caspases-3, -8, and -9 compared to untransfected Hep2 cells and both NPM1 overexpression and miR-181a knockdown rescued the effects of MYCT1 on Caspases-3, -8, and -9 activities in Hep2 cells compared to controls. (Fig. 6D). Similarly, MYCT1 significantly promoted Caspases-3, -8, and -9 cleavages compared to untransfected Hep2 cells and both NPM1 overexpression and miR-181a knockdown significantly rescued the effects of MYCT1 on Caspases-3, -8, and -9 activities in Hep2 cells compared to controls (Fig. 6E). Our data suggest that MYCT1 regulates laryngeal cancer cell apoptosis mediated by NPM1 in mitochondrial-dependent and -independent manners.

**Discussion**

The imbalance between cell viability and apoptosis represents an important cause of carcinogenesis [28,29]. Apoptosis is a unique form of cell death essential for many physiological processes [30]. Apoptosis is controlled by both the extracellular death receptor pathway and the intracellular mitochondrial pathway [31,32]. Apoptotic activators, Caspases-8 and -9 are necessary for the extracellular death receptor pathway and intracellular mitochondrial pathway, respectively, both of which have the same effector Caspase-3 [33].

MAX is a ubiquitously expressed and highly conserved transcription factor that regulates various aspects of cell behaviors including cell viability, differentiation, and apoptosis via the MYC/MAX/MAD network of basic helix-loop-helix leucine zipper (bHLHZip) transcription factors. Max was originally discovered based on its ability to associate with c-Myc and is required for Myc to bind DNA and activate transcription [34]. MAX has been viewed as a central component of the transcriptional network, forming homodimers as well as heterodimers with other members of the Myc and Mad families [35]. MYCT1 is mainly associated with the cytoplasmic membrane, endoplasmic reticulum, and Golgi body. MYCT1 has three forms of protein’s secondary structure: alpha-helix, extended strand, and random coil [36]. Consistent with the notion that MYCT1 is not a transcriptional activator, we did not find any potential MYCT1-binding site in miR-181a promoter region (data not shown). Instead, we discovered that MYCT1 interacts with MAX, which binds the miR-181a gene promoter and promotes its transcription. Thus, it appears that MYCT1 acts as a co-transcription factor or a component of MAX transcript complex in the regulation of miR-181a transcription. We will conduct more studies in the future to identify the specific mechanism and relationship between MYCT1 and MAX.

Nucleophosmin (NPM/B23) family contains three subtypes, including NPM1, NPM2, and NPM3 [37]. NPM1 is a multifunctional protein that modulates ribosome biosynthesis, centrosome replication, cell viability, and apoptosis through a variety of signaling pathways [38]. Studies have shown that NPM1 is overexpressed in multiple solid tumors, and accelerates malignant transformation and viability and inhibits apoptosis [39]. Furthermore, NPM1 overexpression has been positively correlated with cancer progression and thus, a valuable biomarker for many types of cancer, including gastric cancer, colon cancer, liver cancer, and ovarian cancer [40–43]. Similar to the above findings, we also found overexpression of NPM1 in laryngeal carcinoma tissues and cells, suggesting that NPM1 is involved in laryngeal tumorigenesis. We reveal for the first time the key roles of NPM1 in laryngeal cancer. Our data here suggest that NPM1 is one of the downstream targets of MYCT1, and MYCT1 regulates laryngeal cancer cell viability and apoptosis by NPM1 through the extracellular death receptor pathway and the intracellular mitochondrial pathway to at least maintain the balance between viability and apoptosis.

**Here, miR-181a** is an important member of the miR-181 family and has been shown to take part in cancer cell viability, apoptosis, invasion, and migration by targeting different genes [44]; miR-181a is also abnormally expressed in lots of malignancies such as gastric cancer, myeloma and breast cancers, suggesting that it is an important gene in carcinogenesis [45–47]. Interestingly, miR-181a is considered tumor-specific depending on different targets. For instance, miR-181a enhances cell viability and diminishes apoptosis in clear cell renal cell carcinoma by targeting Krüppel-like factor 6 (KLF6) [48]. In contrast, miR-181a reduces cell viability and inhibits apoptosis of cutaneous squamous cell carcinoma by down-regulating Kirsten rat sarcoma 2 viral oncogene homolog (KRAS) [49]. Although these various observations for the roles of miR-181a may be highly dependent on the cellular and disease context, we provide strong
evidence that miR-181a-targeting NPM1 inhibits laryngeal cancer cell viability and promotes apoptosis in vitro for the first time. The restoration of NPM1 rescued the effects of miR-181a on laryngeal cancer cell viability and apoptosis. Our finding that NPM1 is a direct target of miR-181a and miR-181a is the target of MYCT1-MAX complex revealed a complex network operating in laryngeal cancer cells (Fig. 7).

Taken together, our data, for the first time, revealed the roles of NPM1 and miR-181a in laryngeal cancer and the association with MYCT1. We conclude that MYCT1 decreases NPM1 expression via MAX-regulated miR-181a expression and the net effects lie in the inhibition of cell viability and promotion of apoptosis through extracellular and intracellular apoptotic pathways. The factors involved in this pathway may serve as biomarkers for early diagnosis and treatment of laryngeal cancer. Further investigations to gain insights into the functional and clinical implications of the pathway in vivo will be pivotal in the prevention and treatment of laryngeal cancer.

**Experimental procedures**

**Specimens and cell culture**

Cancerous and paired adjacent tissues were obtained from patients with laryngeal squamous cell cancer (LSCC), who underwent surgery at the NO. 463 Hospital of PLA (Shenyang, China). The study was approved by the ethics committee of China Medical University and written informed consents were obtained from each patient. No patient had received radiotherapy or chemotherapy prior to surgery. Pathological verification of these tissues was conducted by a pathologist and the samples were stored at −80 °C immediately after been removed from the patients.

Hep2 cell lines (human laryngeal cancer) and HEK293T cell lines (human embryonic kidney) were obtained from the Cell Biology Company of Shanghai, Chinese Academy of Science. Hep2 cells and HEK293T cells were maintained in complete RPMI-1640 medium (GIBCO, LA, CA) and Dulbecco’s high glucose-modified Eagle’s medium (DMEM) (GIBCO), respectively, supplemented with 10% new-born calf serum (SERANA, SA, AG), 100 units·mL⁻¹ penicillin, and 100 µg·mL⁻¹ streptomycin in a humidified atmosphere at 37 °C with 5% CO₂.

**Gene transfection and stable cell lines**

For transient transfection, pGFP-MYCT1 expression vector, pGFP-NPM1 expression vector, NPM1-shRNAs, and empty vector were purchased from GENECHEM (Shanghai, China). Here, miRNA duplex, small interfering RNAs, and negative control RNA were obtained from GenePharma (Shanghai, China). Target sequences of the NPM1-shRNAs used were as follows: sh-NPM1 1#: AAAGGAT GAGTTGACATT; sh-NPM1 2#: TAGCAAGGTTCA CAGAAA; sh-NPM1 3#: GATGAAAATGAGCACCA AGT. Sequences of the small RNAs used were as follows: si-MAX 1#-5’-GAGCAACCGAGGUUCAAUTT-3’; si-MAX 2#-5’-GUAGGGACCACAUCAAAGATT-3’; si-MAX 3#-5’-GCCACAGAAUAUAUCCAGUTT-3’; si-MYCT1-5’-GUGUGAAAGUCGAAGCAATT-3’; miR-181a-5p mimics-5’-AACAUUCAACGCUGUCGGUGAGU-3’; miR-181a-5p inhibitor-5’-ACUCACCGACGCGUGAGU-3’; negative control RNA-5’-UUCUCCGAACGUGUCACGU-3’. The miRNA duplex (or siRNAs) and plasmids with the concentration of 100 nM or 2 µg were transfected into Hep2 cells using the jetPRIME® in vitro DNA & siRNA transfection kit (Polyplus Transfection, Illkirch, France) according to the manufacturer’s instructions. The medium was replaced at 6 h and cells were harvested for analyses 48 h after transfection.

Lentiviral GV358-MYCT1 and control, AgeI, were constructed by GENECHEM, to overexpress MYCT1 and to establish stable cell lines. The lentiviruses were transduced...
into Hep2 cells following the manufacturer’s instruction using Lipofectamine® 2000 reagent (Invitrogen, Carlsbad, CA, USA). After 2 weeks of puromycin antibiotic (2 µg·mL⁻¹) selection, transduction results were validated by western blot and quantitative real-time PCR (qRT-PCR).

RNA-sequencing and analysis

RNA samples were sequenced by the Beijing Genomics Institute (BGI, Shenzhen) with the HiSeq™ 4000 SE50 platform (for small RNA) and Hiseq™ 4000 PE101 platform (for large RNA). After the total RNA extraction and DNase I treatment, magnetic beads with Oligo (dT) are used to isolate mRNA (for eukaryotes). Mixed with the fragmentation buffer, the mRNA is fragmented into short fragments. Then cDNA is synthesized using the mRNA fragments as templates. Short fragments are purified and resolved with EB buffer for end reparation and single nucleotide A (adenine) addition. Thereafter, the short fragments are connected with adapters. After agarose gel electrophoresis, the suitable fragments are selected for the PCR amplification as templates. During the QC steps, Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System are used in quantification and qualification of the sample library. At last, the library could be sequenced using Illumina HiSeq™ 4000.

We used cuffdiff for quantitative and differential analysis. Based on the quantitative results, NOIseq was used to analyze the differences between groups, and the subsequent model expression cluster analysis. Primary sequencing data that was produced by Illumina HiSeq™ 4000, called as raw reads, was subjected to quality control (QC) that determines if a resequencing step is needed. After QC, raw reads were filtered into clean reads which were aligned to the reference sequences. QC of alignment was performed to determine if resequencing is needed. The alignment data was utilized to calculate distribution of reads on reference genes and mapping ratio. When the alignment result passed QC, we proceed with downstream analysis, including gene and isoform expression, deep analysis based on gene expression (PCA/correlation/screening differentially expressed genes and so on), exon expression, gene structure refinement, alternative splicing, novel transcript prediction and annotation, SNP detection, Indel detection, gene fusion. Further, we also performed deep analysis based on DEGs, including Gene Ontology (GO) enrichment analysis, Pathway enrichment analysis, cluster analysis, protein–protein interaction network analysis, and finding transcription Factor (Data S2).

We made a multi-hypothesis test correction for the P-values of the difference test, and determined the domain values of the P-value by controlling the FDR. We used ‘FDR ≤ 0.001 and the absolute value of Log2 Ratio ≥ 1’ as the threshold to judge the significance of gene expression difference.

RNA isolation and Poly(A)-tailed, quantitative real-time PCR

Total RNA was extracted from cultured cells and corresponding tissues using Trizol (Takara, Dalian, China) according to the manufacturer’s instructions. Concentrations of total RNA were measured by reading the absorbance at OD260/280 nm. Quantitative real-time PCR was performed using SYBR® Premix Ex Taq™ II (Takara, Dalian, China) according to the manufacturer’s instructions using the 7500 real-time RT-PCR system (Applied Biosystems, Foster City). Briefly, poly(A) polymerase was used to add poly(A) tails to the 3’ ends of the miRNAs, and M-MLV Reverse Transcriptase and a unique oligo-dT adaptor primer were used to transcribe the poly(A)-tailed miRNAs to generate a cDNA library. Reverse transcription for miRNA and mRNA was performed by using the One Step PrimeScript® miRNA cDNA Synthesis Kit (Takara, Dalian, China) and the Prime-Script® RT reagent Kit (Takara) in accordance with the manufacturer’s instructions, respectively. Primer sequences are as follows: MYCT1 Forward-5’-GCCCAGAAACCTTTTTGGAGGA-3’; and Reverse-5’-ATCCAGTTCCGTTGAGCGCCG-3’; miR-181a Forward-5’-ACATTTCAACGCTGGCTTGG-3’; and Reverse-5’-TTCCACTGCGCCTTTTCT-3’; U6 Forward-5’-CTCGCTTCGCAAGCCACATCA-3’; and Reverse-5’-AACGGTTACGAAATGCTGCTT-3’; GAPDH Forward-5’-CATGTTCGTCATGCGTTAAGAC-3’; and Reverse-5’-GGTCGATGTCCTCCACGATAC-3’. However, mRNA and miRNA PCR products were normalized to GAPDH mRNA and endogenous U6 levels and quantified using the 2⁻ΔΔCt method in relation to the control.

Western blot analysis and antibodies

Protein was extracted from cells using RIPA cell lysis buffer (Beyotime, Shanghai, China) in the presence of PMSF (Beyotime) and protease inhibitor cocktail (Biotool, Houston, TX, USA) according to the manufacturer’s instructions. Protein concentration was measured by the BCA protein assay (Beyotime). A quantity of 70 µg of the extract was separated by 10% SDS/PAGE and transferred to the PVDF membrane. The membrane was then blocked with 5% nonfat milk and incubated overnight with the following primary antibodies, respectively, which are anti-NPM1 (1 : 3000 dilution; Proteintech, Wuhan, China), anti-MYCT1 (1 : 300 dilution; Abcam, Cambridge, UK), anti-GFP (1 : 1000 dilution; Proteintech), anti-MAX (1 : 1000 dilution; Abcam), anti-caspase-8 and -9 (1 : 1000 dilution; Wanleibio), anti-caspase-3 (1 : 600 dilution; Proteintech), anti-β-actin (1 : 1000 dilution; Proteintech) primary antibodies, and horseradish peroxidase (HRP) conjugated with rabbit or mouse IgG (1 : 2000 dilution; Zhongshan, Guangdong, China) appropriate secondary antibodies, respectively. Hybridization signals were detected by ECL Plus (Beyotime) according to the manufacturer’s instructions.
Co-immunoprecipitation

Hep2 cells were lysed in IP lysis buffer (Beyotime) containing 1% PMSF for 30 min on ice and centrifuged at 12,000 g for 20 min at 4 ºC. Extracts (containing 1 mg of total protein) were pre-cleared with 100 μL of protein G Plus/protein A agarose beads at 4 ºC for 10 min and centrifuged at 12,000 g for 15 min to remove the beads for supernatant. Since no commercial IP-grade antibody for MYCT1 is available at present, here anti-GFP antibody for MYCT1 was used. And considering the insufficient of GFP antibody, we selected the IP-grade antibody for MAX to perform co-IP experiment. A quantity of 2μg anti-GFP antibody or 2μg anti-MAX antibody together with 50 μL protein G Plus/protein A agarose beads were added to the immunoprecipitate and incubated by rotation at 4 ºC overnight. Then the mixtures were centrifuged at 12,000 g for 1 min to remove the precipitates and the beads containing immune complexes were washed for three times with cold PBS buffer following centrifugation at 300 × g for 2 min. Normal mouse IgG was used as a negative control. Then western blotting assay was performed with another related primary antibody.

Immunofluorescence analysis

Hep2 cells were grown on glass coverslips in 12-well culture plates for 4 h, respectively, and Hep2 cells were transiently transfected with MYCT1 overexpression plasmid. After 24 hours post-transfection, cells were washed with PBS, fixed for 15 min at room temperature with 4% paraformaldehyde. After being washed in PBS with 0.5% Triton X-100 at room temperature for three times, cells were blocked in PBS with 1% bovine serum albumin for 1 h. Then, cells were incubated with the desired primary antibody (1:100) overnight at 4 ºC, followed by incubation with a specific fluorescently labeled IgG for 1 h in the dark. Subsequently, cells were stained with DAPI (blue), mounted for 5 min and examined using fluorescence microscopy.

Cell viability assay

Hep2 cells (5 × 10^3 cells per well) were seeded into 96-well plates. Cell viability was measured at specified time points using the Cell Counting Kit-8 (KeyGEN, Nanjing, China) according to manufacturer’s instruction. After incubating for 2 h, viable cells were counted and growth curve was constructed using OD450 nm as the ordinate axis.

Colony formation assay

Hep2 cells (5 × 10^3 cells per well) were seeded into 6-well plates and allowed to grow until visible colonies formed. After 4–6 days, colonies were fixed with methanol and stained with hematoxylin. Cell colonies were photographed under a microscope and determined by counting the number of colonies.

Cell apoptosis assay

Hep2 cells were grown in six-well plates to reach about 70% confluence. After 48 hrs transfection, cells were digested and collected. Thereafter cells were harvested and stained with Annexin V-PE/7-AAD Apoptosis Detection Kit (KeyGEN, Nanjing, China) according to the manufacturer’s protocol, and finally analyzed by Flow Cytometer (FACS Calibur, Becton Dickinson, USA).

Chromatin immunoprecipitation assay

Hep2 cells were cross-linked with 1% formaldehyde at 37 ºC for 10 min in growth medium when cell density was estimated to reach about 85% confluence. ChIP assay was performed using EZ-Magna ChIP™ kit (Millipore, Billerica, MA, USA) according to the manufacturer’s instructions. In brief, DNA–protein complex of chromatin fragments was treated with anti-MAX antibody (1:1000 dilution; Abcam) or anti-IgG antibody (1:2000 dilution; Santa Cruz, CA, USA). DNA was then eluted and extracted with phenol-chloroform and subjected to quantitative-PCR or PCR. miR-181a promoter-specific primer sequences amplifying the MAX-binding regions are as follows: primer1, Forward-5’-ATACCCAGCTGATGTTCT; primer 2, Reverse-5’-GACATGACCACAGTTA ATATTCCA-3’; and reverse-5’-CAGTATTATGAGTG TAAGCTGGT-3’. PCR products were separated by electrophoresis on 2% agarose gel, stained with ethidium bromide and photographed.

Luciferase reporter assay

GV272-NPM1-3’UTR (XbaI/XbaI), GV272-NPM1-3’UTR-mut (XbaI/XbaI), GV148-miR-181a promoter-R1 (KpnI/XhoI), and GV148-miR-181a promoter-R1 + R2 (KpnI/XhoI) plasmids, were obtained from GENECHEM. Hep2 cells seeded in 24-well plates were cotransfected with appropriate plasmids and miR-181a mimics, si-MAX or nonrelative control RNA duplex (NC duplex; GenePharma) by using polyplus in accordance with the manufacturer’s instructions, respectively. pRL-TK (Promega, Madison, WI, USA) was transfected as a normalization control. After 24 h transfection, cells were collected and lysed, and the luciferase activity was detected using the Dual-Luciferase® Reporter Assay System (Promega) and recorded by Chemiluminescence Meter (Promega) according to manufacturer’s instruction.

Caspase activity assay

Caspase-3, caspase-8, and caspase-9 activities were determined using the Colorimetric Assay Kits (KeyGEN, Nanjing, China) according to the manufacturer’s instruction. Briefly, Hep2 cells were harvested after treatment with...
MYCT1, NPM1 plasmids and miR-181a inhibitor for 24 h. Cells were incubated on ice in 100 μL lysis buffer containing 1 μL DTT for 60 min and proteins were then isolated by centrifugation at 11 200 g for 1 min. Protein concentration was measured by the BCA Protein Assay kit (Byotime) and 200 μg protein was diluted in 50 μL reaction buffer containing 0.5 μL DTT for each assay. A quantity of 5 μL of Caspase-3, -8 or -9 substrates was added, respectively. The reaction mixture was incubated at 37 °C for 4 h in the dark. Levels of caspase activities were measured by OD microplate reader, and were calculated by ODexperiment/ODcontrol.

Statistical analysis
All data were subjected to statistical analysis using r software program (IBM, SPSS, New York, NY, USA) and shown as mean ± standard error of the mean (SEM). Each experiment was executed at least three times. NPM1 and miR-181a expression levels were analyzed by nonparametric t-test. Correlation analysis between of miR-181a and NPM1 mRNA expression was performed by Pearson correlation analysis. The student’s t-test and one-way ANOVA were used in estimating the significance of differences in mean values. Symbols *, **, *** and ns represent P < 0.05, P < 0.01, P < 0.001 and no significance, respectively.

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Conflict of interest
The authors declare no conflict of interest.

Author contributions
HTW performed the experiments and prepared the draft manuscript. XT performed the molecular experiments. YYS and ZXZ conducted the bioinformatics analyses. ZMX collected the cancer tissues. WNF and WY conceived the study and edited the manuscript.

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### Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Data S1.** Control-VS-MYCT1.GeneDiffExpFilter.

**Data S2.** Novel transcripts analysis report.

**Appendix S1.** Technical figures, graphs or tables of analysis.