**MYCT1-TV, A Novel MYCT1 Transcript, Is Regulated by c-Myc and May Participate in Laryngeal Carcinogenesis**

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**Abstract**

**Background:** MYCT1, a putative target of c-Myc, is a novel candidate tumor suppressor gene cloned from laryngeal squamous cell carcinoma (LSCC). Its transcriptional regulation and biological effects on LSCC have not been clarified.

**Methodology/Principal Findings:** Using RACE assay, we cloned a 1106 bp transcript named Myc target 1 transcript variant 1 (MYCT1-TV) and confirmed its transcriptional start site was located at 140 bp upstream of the ATG start codon of MYCT1-TV. Luciferase, electrophoretic mobility shift and chromatin immunoprecipitation assays confirmed c-Myc could regulate the promoter activity of MYCT1-TV by specifically binding to the E-box elements within –886 to –655 bp region. These results were further verified by site-directed mutagenesis and RNA interference (RNAi) assays. MYCT1-TV and MYCT1 expressed lower in LSCC than those in paired adjacent normal laryngeal tissues, and overexpression of MYCT1-TV and MYCT1 could inhibit cell proliferation and invasion and promote apoptosis in LSCC cells.

**Conclusions/Significance:** Our data indicate that MYCT1-TV, a novel MYCT1 transcript, is regulated by c-Myc and down-regulation of MYCT1-TV/MYCT1 could contribute to LSCC development and function.

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**Introduction**

Laryngeal squamous cell carcinoma (LSCC) is the second main upper respiratory tract tumor behind lung cancer in incidence and mortality rates [1], and represents the vast majority (approximately 96%) of laryngeal malignancies [2]. The mortality of LSCC shows an increasing trend in China, especially in the northeast part. The etiology of LSCC is considered to be multifactorial. The main predisposing factors are tobacco and alcohol abuse [3]. Despite many advances achieved in the diagnosis and treatment of the disease, its overall survival rate has remained unchanged (at approximately 35–70%) over the past several decades [1]. Therefore, it is necessary to develop new diagnostic and therapeutic targets for LSCC.

Although much work is presently focused on the research about the relationship between LSCC and oncogenes, such as BCL2 [4], c-Myc [4,5] and EGFR [6], or tumor suppressor genes (TSGs), such as P53 [7], Rb [8], P16 [8] and P21 [8], no information about gene cloning related to laryngeal carcinoma is reported except human Myc target 1 (MYCT1) [9].

MYCT1 (Gene ID: 80177) is a novel candidate TSG cloned using in silicon hybridization and molecular methods from LSCC by our team, which was previously named MTLC (c-Myc target from laryngeal cancer cells, GenBank accession No. AF_527367). The full length of this gene is about 21 kb. It contains two exons and produces a 1006 bp transcript coding a protein with 235 amino acid residues. Our previously study demonstrated that MYCT1 existed in various human tissues and was down-regulated in gastric carcinoma tissues. The 5′ flanking sequence of MYCT1 contains two binding sites of oncoprotein c-Myc [9,10]. But whether it is also down-regulated in LSCC or is really regulated by c-Myc and its biological effects on LSCC have not been systematically analyzed. In this present work, we have studied this candidate c-Myc target gene in greater detail. We cloned a new MYCT1 transcript variant (MYCT1-TV, GenBank accession No. GU997693), confirmed its transcriptional start site (TSS) for the first time, identified a 230 bp region that mediated basal transcription by two E-box elements and explored the role of MYCT1 and its variant in the development and aggression of LSCC by comparing the expression or biological characteristics between MYCT1 and its isoform in LSCC tissues or cells, which will lay a foundation for the further exploration on understanding the function of MYCT1 in c-Myc regulatory subnetwork and provide us a novel LSCC diagnostic and therapeutic target for the future study.
Figure 1. cDNA characteristics of MYCT1-TV. The 1106 bp cDNA, obtained by 5' and 3' RACE, contains a putative open reading frame that encodes a polypeptide of 187 amino acid residues. Transcriptional start site is marked in bold and italic. Initiator codon ATG is boxed.

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Figure 2. Comparison of MYCT1-TV and MYCT1. A. The full-length cDNA clones of MYCT1-TV and MYCT1 are 1106 bp and 1006 bp, respectively. MYCT1-TV has a shorter 5' flanking sequence and a longer 3' flanking sequence compared to MYCT1. B. The amino acids sequence comparison of MYCT1-TV and MYCT1.

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Results

Cloning and characterization of MYCT1-TV

We obtained a novel 1106 bp transcript variant of MYCT1 named myc target 1 transcript variant 1 (MYCT1-TV, GenBank accession No. GU997693) using RACE and cDNA cloning methods. Our sequencing results indicated a potential MYCT1-TV TSS was located at 140 bp upstream of the ATG start codon of MYCT1-TV (Fig. 1). Meanwhile, the only TSS we confirmed was also located at 12 bp downstream of the start nucleotide of the published MYCT1 mRNA sequence (GenBank accession No. AF_527367, Fig. 1). MYCT1-TV has a 140 bp 5' untranslated leader and a 370 bp 3' untranslated region with a 32 bp poly (A) tail. As predicted using ExPASy proteomics server, the 564 bp open reading frame codes for a putative protein of 187 amino acid residues with a predicted molecular mass of 20835Da and pl 10.26 (Fig. 1). Using NCBI blast, we compared the protein and nucleotide sequences of MYCT1-TV with those of MYCT1 (GenBank accession No. AF_527367). The analysis results revealed that MYCT1-TV cDNA has a shorter 5' flanking sequence and a longer 3' flanking sequence than MYCT1 (Fig. 2A). MYCT1-TV protein has a shorter 48 amino acid N-terminus than MYCT1 protein and the other 187 amino acid protein of them are much the same (Fig. 2B). ExPASy proteomics server analysis demonstrated the shorter 48 amino acid protein contains no obviously structural or functional motif.

Identification and analysis of MYCT1-TV promoter region

Using web software BDGP, Promoter 2.0 and Promoter Scan, we analyzed the proximal 1033 bp (−981/+52) promoter sequence of MYCT1-TV and found two core promoter sequences in this region (Fig. 3A). In order to determine which region play an important role in promoter activity, we generated a number of consecutive 5' deletions of MYCT1-TV promoter (Fig. 3B). The generated fragments were cloned into pGL3-Basic and transiently co-transfected into Hep2 and HEK293 cells along with pRL-TK. Luciferase results revealed a 7.26-fold increased transcriptional activity of P852 as compared to the empty vector in Hep2 cells, indicating that we obtained an active MYCT1-TV promoter (p<0.01, Fig. 3B). Removal of 53 bp at 5' end from P852 caused a

Figure 3. Identification and functional characterization of human MYCT1-TV promoter. A. Nucleotide sequence of the promoter region (from −981 to +52) of human MYCT1-TV. The putative binding sites for transcriptional factors are underlined and marked as E-box A and B. The transcriptional start site is marked in bold and italic at −140 bp. The start nucleotide of the published MYCT1 mRNA sequence is marked in bold at −152 bp. The positions of putative core promoter sequences are marked with gray color. The numbering of the nucleotides starts at initiator codon ATG (+1) which are boxed. B. Analysis of human MYCT1-TV promoter activities detected by luciferase assay. Hep2 (black bars) or HEK293 (gray bars) cells are transiently transfected with 0.8 μg of the deletion constructs together with 16 ng pRL-TK. The relative activities of a series of deletion constructs are determined by luciferase assay (**, p<0.01; *, p<0.05).

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2.76-fold decrease in luciferase activity (p<0.05, Fig. 3B). However, a further deletion of 132 bp at 5’ end from P799 caused a drastically drop about 7.16-fold of MYCT1-TV promoter activity compared to P799 (p<0.01, Fig. 3B). The similar results were also obtained in HEK293 cells (Fig. 3B). These results suggested that the basal regulatory elements important for maximal promoter activity are present between −852 bp and −667 bp region, and P652 (−852/+12) acts as the proximal promoter.

Importance of two E-boxes in MYCT1-TV promoter activity
C-Myc is known as a transcription factor through forming heterodimers with its binding partner Max specifically binding to a canonical consensus DNA sequence CACGTG, termed the E-box [11]. And it has also been reported to bind to several other noncanonical DNA motifs, such as CATGTG, CATGGG, CACGGG, CACAGG, and CACCTG [12–14]. In luciferase assay, we confirmed the promoter region between −852 bp and −667 bp was important for basal regulatory elements binding. And two noncanonical E-box elements, CGCATG (the reverse complement of CATGGG) and CAGCG, as we reported before [9], were just present in this region (Fig. 3A). To analyze whether these putative c-Myc/Max-binding sites mediate transcriptional activity of MYCT1-TV core promoter, we introduced three point mutations, P652-mutA, P652-mutB and P652-mutAB. Luciferase assay showed that compared to the wild-type promoter P652 in Hep2 cells, mutated only E-box A (P652-mutA) or B (P652-mutB) or mutated both E-box A and B (P652-mutAB) caused a 5.40-fold, 5.28-fold or 5.52-fold reduction of transcriptional activity (p<0.05, Fig. 4). And there were no significant difference between these mutants (p>0.05, Fig. 4). Similar results were also obtained in HEK293 cells (Fig. 4). These results indicated that both of the identified motifs are essential for the basal activity of MYCT1-TV core promoter.

Regulation of MYCT1-TV promoter by c-Myc
To assess the importance of E-box elements for c-Myc/Max binding in vitro, we performed EMSA using biotin-labeled, synthetic double-stranded oligonucleotides corresponding to the E-box A and B. These probes were incubated with the nuclear extracts isolated from Hep2 or HEK293 cells. As shown in Fig. 5A (lanes 2, 7, 13 and 18), both oligonucleotides specific to E-box A and B were retarded significantly by cell nuclear proteins. Quantitative competitions for bindings were found at the presence of nearly 100-fold excess of unlabeled oligonucleotides (lanes 3, 8, 14 and 19), whereas no competition were observed with a nearly 100-fold excess of unlabeled mutated oligonucleotides (lanes 4, 9, 15 and 20). Furthermore, the bands were supershifted by specific c-Myc antibodies (lanes 5, 10, 12 and 17). The interaction between c-Myc/Max and MYCT1-TV promoter was further confirmed by ChIP assay. Using primers to amplify both c-Myc/Max binding regions of MYCT1-TV promoter, we observed strong PCR products based on the precipitate by c-Myc antibody from Hep2 and HEK293 cells, but not from negative control immunoprecipitations using anti-rabbit IgG antibody (Fig. 5B).

Reduced MYCT1-TV promoter activity with silencing of c-Myc
To further confirm the role of c-Myc/Max in regulation of MYCT1-TV promoter activity, we silenced c-Myc translation via RNA interference. The efficiency of the siRNAs directed against c-Myc was confirmed by qRT-PCR. As compared to the Mock control (no siRNA) in Hep2 and HEK293 cells, transfection of c-Myc specific siRNA effectively knocked down c-Myc mRNA levels (p<0.05, Fig. 6A) whereas transfection of nonspecific control siRNA (NC siRNA) showed no evidence of silencing (p>0.05, Fig. 6A). We then cotransfected the luciferase reporter plasmid P652 containing MYCT1-TV promoter together with siRNA. Compared with NC siRNA group, luciferase activity of P652 displayed significant reduction after transfected with c-Myc siRNA (p<0.01, Fig. 6B). Thus, c-Myc can strongly enhance transcriptional activity of MYCT1-TV promoter.

Extensive expression of MYCT1-TV in human cells
To explore whether MYCT1-TV is widely expressed or specific expression in human cells, we performed RT-PCR in Hep2, HeLa, BGC823, SGC7901, MKN1, Bel7402, GE1 and HEK293 cells compared with normal human blood (positive control). The result showed that the MYCT1-TV extensively expressed in all cells examined, but MYCT1-TV mRNA levels in normal cells of GE1 and HEK293, which shared no difference with normal human blood (p>0.05, Fig. 7A), were significantly higher than those in tumour cells of Hep2, HeLa, BGC823, SGC7901, Bel7402 and MKN1 (p<0.01, Fig. 7A). There was also no significant difference in MYCT1-TV mRNA levels among the tumour cells above (p>0.05, Fig. 7A).

Reduced expression of MYCT1-TV or MYCT1 in LSCC tissues
Since MYCT1 was cloned from LSCC and down-regulated in gastric carcinoma tissues [9,10], we are interested in exploring whether it is also down-regulated in LSCC and existed the expression differences between MYCT1-TV and MYCT1 in LSCC. RT-PCR results showed that both MYCT1-TV and MYCT1 mRNA levels in LSCC tissues were significantly lower than that in paired adjacent normal laryngeal tissues (p<0.01, Fig. 7B). Moreover the mRNA expression levels of these two transcripts showed no significant difference in LSCC or paired adjacent normal laryngeal tissues (p>0.05, Fig. 7B).

We assessed the expression levels of MYCT1-TV/MYCT1 with respect to clinical characteristics (age, sex and cancer stage). No
differences were identified in mRNA levels of MYCT1-TV/MYCT1 with respect to patient age, sex, or patient clinical stage (p > 0.05, Table 1).

Function of MYCT1-TV and MYCT1 in Hep2 and HEK293 cells

To elucidate the function of MYCT1-TV and MYCT1 in human cells, MYCT1-TV-GFP and MYCT1-GFP expression vectors were generated and transiently transfected into Hep2 and HEK293 cells. Forty-eight hours after transfection, Hep2 cells in MYCT1-TV-GFP and MYCT1-GFP groups showed a significant increase at mRNA levels compared to those in blank and GFP groups (p < 0.01, Fig. 8A). MYCT1-TV and MYCT1 protein expression were detected by confocal immunofluorescence microscopy (Fig. 8B). These results revealed that the transfection is successful. Similar results were also obtained in HEK293 cells (Fig. 8A and B).

Cell viability assay showed that the proportion of viable cells in MYCT1-TV-GFP or MYCT1-GFP group was significantly fewer than that in GFP group (p < 0.05, Fig. 9A). The percentages of viable cells in MYCT1-TV-GFP, MYCT1-GFP and GFP groups relative to that in blank group were 41.46 ± 6.00%, 46.91 ± 5.56% and 81.27 ± 8.61%, respectively. In addition, there was no significant difference between MYCT1-TV-GFP and MYCT1-
Figure 6. Silencing of endogenous c-Myc reduces MYCT1-TV promoter activity. A. Detecting of c-Myc expression level in cells by qRT-PCR. The expression of GAPDH was used as an internal control. Hundred nanogram of no siRNA (Mock), nonspecific control siRNA (NC) or c-Myc specific siRNA (c-Myc) were transfected into Hep2 (black bars) or HEK293 (gray bars) cells. B. Luciferase activity of MYCT1-TV promoter after transfected with c-Myc siRNA. Luciferase activity was measured in Hep2 (black bars) or HEK293 (gray bars) extracts 48 h after transfection. pGL3-Basic, cells cotransfected with pGL3-Basic and pRL-TK; P852, cells cotransfected with P852 and pRL-TK; P852+NC, cells cotransfected with P852, NC siRNA and pRL-TK; P852+c-Myc, cells cotransfected with P852, c-Myc siRNA and pRL-TK. Data are indicated as the means ± SEM of three independent experiments.

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Figure 7. Expression of MYCT1-TV and MYCT1 in human cells and tissues. A. MYCT1-TV mRNA levels in human cells. The gray-scale ratios of MYCT1-TV to β-actin mRNA levels in Hep2, HeLa, BGC823, SGC7901, Bel7402, GES1, HEK293, human blood and MKN1 cells are 0.2890±0.0521, 0.3113±0.0138, 0.2985±0.0130, 0.2964±0.0427, 0.3512±0.0407, 1.0522±0.0808, 1.1159±0.1467, 1.1641±0.0665 and 0.2348±0.0147, respectively. B. MYCT1-TV and MYCT1 mRNA levels in LSCC and paired adjacent normal laryngeal tissues. PCR produce a 929 bp DNA fragment for MYCT1-TV, a 726 bp DNA fragment for MYCT1 and a 511 bp DNA fragment for β-actin. The gray-scale ratios of MYCT1-TV to β-actin mRNA levels (black bars) in LSCC and paired adjacent normal laryngeal tissues are 0.4172±0.0324 and 0.8073±0.0478, and the counterpart gray-scale ratios of MYCT1 to β-actin mRNA levels (gray bars) are 0.4304±0.0304 and 0.8416±0.0499. M, T and R indicate DNA marker, LSCC tumor tissue and paired adjacent normal tissue, respectively.

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GFP groups (p>0.05, Fig. 9A). However, similar results were not obtained in HEK293 cells, and the counterparts were 64.47±3.40%, 63.14±5.08% and 72.29±5.62% (p>0.05, Fig. 9A).

Cell apoptosis assay indicated that the proportion of early apoptotic Hep2 cells was significantly higher in MYCT1-TV-GFP or MYCT1-GFP group than those in blank and GFP groups (p<0.01, Fig. 9B). The percentages of early apoptotic Hep2 cells in blank, GFP, MYCT1-TV-GFP and MYCT1-GFP groups were 0.92±0.25%, 1.19±0.08%, 4.37±0.96% and 3.63±0.43%, respectively. In addition, there was no obvious difference between MYCT1-GFP-TV and MYCT1-GFP groups (p>0.05, Fig. 9B). However, similar results were not detected in HEK293 cells (p>0.05, Fig. 9B) and the counterparts were 0.86±0.09%, 0.97±0.27%, 0.74±0.20% and 0.93±0.25%, respectively.

Cell invasion assay displayed that the transmembrane Hep2 cells in MYCT1-TV-GFP or MYCT1-GFP group were significantly fewer than those in blank and GFP groups (p<0.01, Fig. 9C). The numbers of invasive Hep2 cells in blank, GFP, MYCT1-TV-GFP and MYCT1-GFP groups were 31.33±1.67, 28.33±1.28, 11.50±0.76 and 13.83±1.45, respectively. Moreover, there was no obvious difference between MYCT1-TV-GFP and MYCT1-GFP groups (p>0.05, Fig. 9C). Meanwhile similar results were not observed in HEK293 cells (p>0.05, Fig. 9C) and the counterparts were 12.33±1.45, 10.00±0.58, 8.67±1.76 and 9.33±1.20, respectively.

The results above imply that MYCT1-TV and MYCT1 have the abilities in depressing proliferation and invasiveness, and promoting apoptosis in Hep2 cells, but not in HEK293 cells.

Discussion
c-Myc, together with L-Myc, and N-Myc in the family of c-Myc genes, was discovered as the cellular homolog of the retroviral v-myc oncogene 30 years ago [15]. It is also known as a transcription factor through forming heterodimers with its binding partner Max either binding to the canonical E-box sequence CACGTG or to several other noncanonical E-box motifs such as CATGTG, CATGGC, CACCGG, CACGAG, CGCGAG and CACCGTG [11–14]. Dysregulated expression or function of c-Myc is one of the most common abnormalities in human malignancy [16,22].

as breast cancer [17], colorectal cancer [18,19], prostate cancer [20,21], and hepatocellular carcinoma [22,23]. Overexpression of the c-Myc oncogene has also been observed in LSCC [3,24]. In mammalian cells, c-Myc expression is highly regulated and closely tied to cell proliferation, growth, apoptosis and differentiation [16,22].

Given the functions of c-Myc in various biological processes, identification of c-Myc molecular targets has become a hotspot in molecular genetics and oncology research. To date, a large number of c-Myc target genes which could recapitulate c-Myc functions have been confirmed, such as Cdh4, Hmg11/1Y, Cad, Cdk25, Odc, Ldh-a, rel, Gadd45, P53, Tnp4 and mt-ncl [12,25–28]. For example, mt-ncl, rel and Ldh-a can promote cell transformation and tumorigenesis [28,29], mt-ncl, Cdk25, P53 and Gadd45 can induce apoptosis [28,30–32], P53, Gadd45 and Cdh4 can arrest cell cycle [31–33]. These genes have one common feature, in which they all have E-box elements in their promoter regions for c-Myc binding.

Our previously study characterized that there are two putative E-box elements in the 5’ flanking sequence of MYCT1 [9], but it has not been proved by experiment. To address this question, we cloned a 864 bp promoter ranges from position −852/+12 relative to the identified TSS. This only TSS is located at 140 bp upstream of the ATG start codon of MYCT1-TV. Luciferase reporter assays showed that this promoter (−852/+12) which efficiently drove luciferase expression in transiently transfected Hep2 and HEK293 cells was functional and exhibited greater transcriptional activity in the presence of two E-box elements. The consistent transcriptional activities of this 864 bp promoter in tumor and normal cells we tested indicated that ubiquitous E-box elements are involved in regulation of MYCT1-TV gene transcription. EMSA and ChIP assays showed that c-Myc protein extracted from Hep2 or HEK293 cells can specifically bind to either E-box element between −852 bp and −667 bp region. Furthermore, transcriptional activity of MYCT1-TV promoter can be reduced either by site-directed mutagenesis or by c-Myc siRNA transfection, which means c-Myc can specifically enhance MYCT1-TV promoter activity. These results provide the support that c-Myc is the important transcription factor for MYCT1-TV promoter.

By bioinformatics analysis we found that MYCT1-TV encodes a 187 amino acid protein which is part of 235 amino acid protein encoded by MYCT1 and the shorter 48 amino acid protein contains no obviously structural or functional motif. Since MYCT1-TV and MYCT1 have no structural difference, we are interested in whether they have functional differences. We then detected the expression and biological variation of MYCT1-TV and MYCT1 by RT-PCR, cell proliferation, apoptosis and invasion assays.

Invasion and metastasis are the characteristics of malignant tumours, which are responsible for the primary cause of death in cancer patients. The migration and invasion mechanisms of cancer cells begin with the growth of cancer cells at the primary site of development, followed by decreased local invasion through the surrounding tissue. The larger the primary tumour grows, the more likely that the tumour will be able to acquire traits that lead to a metastatic phenotype. Therefore, inhibition of the invasion and metastasis of tumour cells is a crucial step which would be a useful pathway in the treatment of cancer patients [34–39]. Our present study showed that overexpression of MYCT1-TV/MYCT1 in Hep2 cells could promote cells apoptosis and inhibit cells proliferation and invasion. We speculated this MYCT1-TV/ MYCT1 properties in inhibiting the invasiveness of Hep2 cells probably result from their function on promotion of apoptosis and

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Table 1. Analysis of the relationship between MYCT1-TV/ MYCT1 and clinical characteristics (Gray-scale ratio, mean ± SEM).

| Characteristic | Response | MYCT1-TV | MYCT1 |
|---------------|----------|----------|-------|
| Age           | ≤62 y    | 0.4367±0.0562 | 0.4480±0.0524 |
|               | ≥62 y    | 0.3995±0.0361 | 0.4144±0.0143 |
| Sex           | Male     | 0.4438±0.0425 | 0.4433±0.0372 |
|               | Female   | 0.6695±0.1051 | 0.3940±0.0558 |
| Cancer stage  | I        | 0.5795±0.0954 | 0.4755±0.0730 |
|               | II       | 0.3889±0.0518 | 0.4872±0.0843 |
|               | III      | 0.3977±0.0776 | 0.4175±0.0597 |
|               | IV       | 0.3695±0.0400 | 0.3875±0.0453 |

P = 0.069 P = 0.405

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Figure 8. Transfection of MYCT1-TV and MYCT1 in Hep2 and HEK293 cells. A. mRNA levels of MYCT1-TV/MYCT1 in Hep2 and HEK293 cells transfected with MYCT1-TV-GFP/MYCT1-GFP. PCR produce a 929 bp DNA fragment for MYCT1-TV, a 726 bp DNA fragment for MYCT1 and a 511 bp DNA fragment for β-actin. In Hep2 cells, the gray-scale ratios of MYCT1-TV to β-actin mRNA levels (black bars) in blank, GFP and MYCT1-TV-GFP.
inhibition of proliferation. In other words, the increased proliferation in Hep2 cells by suppressing cells apoptosis may promote the migration and invasion abilities of Hep2 cells. However, both MYCT1-TV and MYCT1 displayed lower expression in cancer tissues than that in adjacent normal tissues. We also found that MYCT1-TV and MYCT1 do not affect the proliferation, apoptosis and invasion of HEK293 cells. These findings imply that MYCT1-TV and MYCT1 display TSGs-like properties and the down-regulation of them in LSCC perhaps bring about increased proliferation, decreased apoptosis and stepped up invasiveness of tumour cells which may facilitate laryngeal carcinogenesis.

The oncogene c-Myc which is overexpressed in LSCC [5,24] can specifically activate MYCT1-TV and MYCT1 promoter activities, but MYCT1-TV and MYCT1 which appear TSGs-like properties are down-regulated in LSCC. Thus we inferred that MYCT1-TV/MYCT1 may silence through MYCT1-TV/MYCT1 promoter methylation in the absence of c-Myc overexpression, which is associated with cancer progression. Alternatively, other transcription factors could be involved in MYCT1-TV/MYCT1 regulation. This may be the reason for the lower expression and TSGs-like properties of MYCT1-TV/MYCT1 in LSCC tissues or cells. The specific mechanism need to be further verified.

Results from cells viability and apoptosis assays demonstrated that the overexpression of MYCT1-TV/MYCT1 could decrease proliferation of Hep2 cells, but increase apoptosis of Hep2 cells. Given these findings, we suggested that as candidate c-Myc target genes, MYCT1-TV and MYCT1 really have some of the known phenotypic features associated with c-Myc, but whether they have the other properties of c-Myc and the precise mechanism between c-Myc and MYCT1-TV/MYCT1 need to be further studied.

As newly discovered genes, MYCT1-TV and MYCT1 showed no significant differences in the expression or biological function in LSCC or cells. Maybe both of them should be the main forms for MYCT1 to achieve its function efficiently. At present, the role of MYCT1-TV and MYCT1 is unceasingly studied. Our present study provides a fundamental clue for the further exploration on understanding the function and the molecular mechanism of MYCT1-TV/MYCT1 in LSCC.

Materials and Methods

Cell culture

The human laryngeal cancer cells Hep2, the human hepatoma cells Bel7402, the human epithelial carcinoma cells HeLa, the human gastric carcinoma cells BGC823, SGC7901 and MKN1, and the human gastric epithelial cells GES1 were grown in complete RPMI 1640 medium containing 10% fetal bovine serum. The human embryonic kidney cells HEK293 were maintained in Dulbecco's high glucose modified Eagle's medium (DMEM) containing 10% fetal bovine serum. All the cells above were purchased from Cell Biology Institute of Shanghai, Chinese Academy of Science.

Samples

Forty cases of LSCC tissues were obtained from patients treated at the Ear, Nose and Throat (E.N.T) department of the 463 Hospital of PLA of China after receiving their written informed consent and the approval of the hospital authorities. No patients had previously received any neoadjuvant treatment, e.g. chemotherapy, before the surgery. The specimens, including cancerous tissues and paired adjacent normal laryngeal tissues typically 4–15 mm in diameter were obtained during diagnostic biopsy or operation, and confirmed by pathologists. The clinical pathological characteristics of the patients were evaluated according to the International Union Against Cancer guidelines. All specimens were frozen immediately after surgery and stored at −80°C. Approval for the study was received from the Ethics Committee of China Medical University. Patient information is shown in Table 2.

Rapid Amplification of cDNA Ends (RACE)

The 5′ and 3′ end of the gene was elucidated by RACE-PCR using the SMART™ RACE cDNA amplification kit (Clontech, Heidelberg, Germany), according to the manufacturer’s protocol. Total RNA was extracted from normal human blood by an RNA extraction kit (Galen Biopharm, Beijing). The first round PCR of 5′ RACE was performed with the Universal Primer A Mix UPM obtained from the kit and the gene-specific primer GSP1 (5′-CAT AGG CAG GTG GGG GGT GGT TT-3′). PCR product was used in a secondary nested PCR with the Nested Universal Primer A NUP obtained from the kit and the nested gene-specific primer NGSP1 (5′-TGG ACC AGT CAG GAC GAG TGA GTC GA-3′). The 5′-cDNA ends were amplified using the first primer pair GSP2 (5′-TAT CCA TGG CAA TCG GGC TGG TAC T-3′) and UPM followed by a nested PCR with the second primer pair NGSP2 (5′-AGT GGG TCT TGT GAA CGT CGA AGC AAG C-3′) and NUP. The obtained new MYCT1 transcript variant was deposited in GenBank (MYCT1-TV, GenBank accession No. GU1997693).

Sequence prediction and analysis of MYCT1

A series of bioinformatics softwares were used as follows: BDGP (www.fruitfly.org/search_tools/promoter.html); Promoter 2.0 (www.cbs.dtu.dk/services/Promoter/); Promoter Scan (www.bimas.cit.nih.gov/molbio/proscan/); ExPASy proteomics server (http://www.expasy.org/tools/dna.html); NCBI blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Plasmid construction

For cloning of the MYCT1 promoter-driven reporter plasmid, genomic DNA was prepared from the human blood using the TIANamp Genomic DNA Kit (Tiangen, China). Plasmids used for functional analysis of the MYCT1 promoter activity were generated using the promoterless luciferase reporter plasmid pGL3-Basic (Promega, USA). Three 5′ deletion constructs of P667 (–852/+12), P799 (–799/+12) and P667 (–667/+12) were generated by PCR amplification method. Three different forward primers contained an internal site for SacI restriction enzyme each and their sequences are: primer 852: 5′-TTT GAG CTC GTG GCC GAG CGC AGT-3′; primer 799: 5′-TTT GAG CTC GGA GGT GGA TCA CGT CTT GAG G-3′; and primer 667: 5′-TTT GAG CTC GCT GAG GGA GAA TCG-3′. The sequence...
of the reverse primer containing site for HindIII enzyme is 5'-TTT AAG CTT GTT ATT AGC CAT AAT ATC CAC AAG A-3'.

Mutated reporter plasmids were generated by using the Gene Tailor site-directed mutagenesis system (Invitrogen, USA), according to the manufacturer's instructions using primers designed to replace the E-box A core sequence “CGCATG” with “TTCATG” (named P852-mutA), replace the E-box B core sequence “CAAGCCG” with “CAAGGG” (named P852-mutB) and replace both E-box core sequences above (named P852-mutAB). The mutated sequences were cloned into the SacI/HindIII site of pGL3-Basic vectors.

Luciferase and RNAi assays

Cells in 24-well plates were transfected in triplicate using Lipofectamine 2000 (Invitrogen, USA) with 0.8 μg, respectively, of specific plasmids. After 48 h of transfection, cells were harvested in 100 μl of Passive Lysis Buffer (Promega) and luciferase assay was performed using the Dual Luciferase Assay System (Promega, USA) according to the manufacturer’s instructions. Relative luciferase activity was calculated as the ratio of sample luciferase activity to renilla luciferase activity. Values are expressed as fold changes versus pGL3-Basic.

The c-Myc translation was silenced in Hep2 and HEK293 cells using the siRNA duplexes c-Myc siRNA (5'-UAU UUC UAC UTT-3') synthesized by Gene Pharma. A nonspecific siRNA (5'-UUC UCC GAA CGU GUC ACG UTT-3', Gene Pharma) was used as negative control (NC). Cells were transfected with 20 pmol of siRNA, 0.2 μg of P852, P799 or empty construct of the luciferase reporter gene, and 16 ng pRL-TK were cotransfected into cells using Lipofectamine 2000 (Invitrogen, USA) following the manufacturer’s instructions. The results of gene knockdown were determined at 48 h after transfection.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from Hep2 and HEK293 cells using a nuclear extract kit (Active Motif, USA) following the manufacturer’s instructions. The following double-stranded oligonucleotides were used (wild type and mutant binding sites are underlined): E-box A, 5'-CGA GCG CAG TGG CGC ATG GCT GTA ATC CGA-3'; E-box B, 5’-CGG GTG TG’G TGG CAC GGG CCT GTA ATC CGA-3'; E-box A mutant, 5’-CGA GCG CAG TGG CAT GGG GCT GTA ATC CGA-3'; E-box B mutant, 5’-CGG GTG TG’G TGG CGT TAG CCT GTA ATC CGA-3'. Oligonucleotide labeling was performed using the Biotin 3’ End Labeling Kit (Pierce, USA). EMSA used the Lightshift Chemiluminescent EMSA kit (Pierce, USA) according to the protocols provided. For competition assays, samples were preincubated with a 100-fold excess of the unlabeled wild type or mutated oligonucleotide duplex competitors. For the supershift reaction, 1 μg each of anti-c-Myc antibody (N-262X, Santa Cruz) was preincubated with the nuclear extracts in the absence of poly(dI·dC) for 1 hour at 4°C. Subsequently, poly(dI·dC) was added and incubated for 5 min, followed by the addition of a probe for an additional 20 min. Protein DNA complexes were separated by electrophoresis on a 6% non-denaturing acrylamide gel in 0.5×TBE, transferred to positively charged nylon membranes, and visualized by streptavidin-horseradish peroxidase followed by chemiluminescent detection (Pierce, USA).

Chromatin immunoprecipitation assay (ChIP)

ChIP assay were performed as previously described [40,41]. Cells were fixed with 1% formaldehyde in growth medium at 37°C for 10 min. After washed with ice-cold PBS, cells were lysed with lysis buffer containing protease inhibitors and sonicated to obtain DNA fragments. Small amount of DNA fragments was saved as input DNA. The rest was diluted, treated with anti-c-Myc antibody (N-262X, Santa Cruz) or rabbit IgG, and immunoprecipitated by protein A/G plus-agarose (Santa Cruz, USA). Each precipitated DNA was eluted and extracted with phenol-chloroform and subjected for PCR. PCR was performed with MYCT1 promoter-specific primers amplifying the c-Myc binding regions (c-Myc A, forward, 5’-GTT TTC CCT CCT TGA TTT-3’; and reverse, 5’-GTT TTC CCT CCT TGA TTT-3’), c-Myc B, forward, 5’-GAG ATG GGC ATG CCT GAT CCG TCA-3’, and reverse, 5’-GTT TTC CCT CCT TGA TTT-3’. 

Construction of MYCT1-TV-GFP and MYCT1-GFP expression vectors

The entire open reading frames of MYCT1-TV and MYCT1 complementary DNA (cDNA) were obtained by RT-PCR from normal human blood. The associated primer sequences are as follows: MYCT1-TV-GFP, forward, 5’-TTT GTC GAC ATG GCT AAT ACC ACA AGT TTA G-3’; and reverse, 5’-TTT GTC TAC TCA GGA GAA ATC TGA CCT GAG TCA-3’. MYCT1-GFP, forward, 5’-TTT GTC GAC ATG CAG ACA CAA GAT TAT GAG G-3’; and reverse, 5’-TTT GTC TAC TCA GGA GAA ATC TGA CCT GAG TCA-3’. Two different forward primers contains a Sall site and two different reverse primers contains a BamHI site. The PCR fragments were cloned into the Sall/BamHI site of pEGFP-C1 plasmids (BD, USA). The fidelity of the constructs was then confirmed by sequencing, and plasmids were prepared for transfection using TIANpure Mini Plasmid Kit (Tiangen, China).

Table 2. The characteristics of the patients (n = 40).

| Characteristic | Response | Cases (%) |
|---------------|----------|-----------|
| Age           | <62 y    | 19 (47.5) |
|               | ≥62 y    | 21 (52.5) |
| Sex           | Male     | 28 (70.0) |
|               | Female   | 12 (30.0) |
| Cancer stage  | I        | 7 (17.5)  |
|               | II       | 8 (20.0)  |
|               | III      | 10 (25.0) |
|               | IV       | 15 (37.5) |

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Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from Bel7402 cells, Hep2 cells, HeLa cells, BGC823 cells, SGC7901 cells, MKN1 cells, GES1 cells, HEK293 cells, human blood, LSCC tissues and paired adjacent normal laryngeal tissues using Trizol reagent (Invitrogen, USA) following the protocol of the manufacturer. cDNA was synthesized by reverse transcription using an AMV RNA PCR kit (TaKaRa, China) in keeping with the standard operating procedure. The MYCT1 primers were the same as those for construction of MYCT1-GFP, which were expected to produce a 726 bp DNA fragment. The primers for MYCT1-TV were 5′-CGT TCC AGT TT-3′ and 5′-AGT CAC TTG ATG GAG T-3′, which were expected to produce a 929 bp DNA fragment. β-actin served as an internal control in each sample. The primer sequences for β-actin were 5′-CAC CTT CCC CAG TCA TGT-3′ and 5′-AGT CAC TTG ATG GAG T-3′, which were expected to produce a 511 bp DNA fragment. The electrophoresis images were scanned by Fluor-S MultiImager (Bio-Rad, USA) and the original intensity of each specific band was quantified with the software Multi-Analyt (Bio-Rad, USA). Data were analyzed after being normalized by the intensity of β-actin.

Cell viability assay

Cell viability was assessed by the MTT colorimetric assay. 50 mg of MTT (Sigma, USA) was dissolved in 10 mL of PBS. After being seeded for 24 h in a 96-well plate, Hep2 and HEK293 cells (1×10⁴ cells/well) were transfected with GFP only, MYCT1-TV-GFP and MYCT1-GFP for 48 h, with untransfected cells serving as a control. At 48 h after transfection, 10 μL of MTT solution (5 mg/mL) was added to each well. After incubation for 4 h at 37°C, the medium was replaced with 200 μL of DMSO and the plate was allowed to shake on a plate shaker for 10 min. Absorbance at 490 nm was measured using a microplate reader, and the results were expressed as a percentage (%) of the control. All experiments were done with three parallel wells each and repeated three times [42].

Apoptosis assay

Apoptotic cells were measured by using an annexin V-PE/7-AAD Kit (KeyGEN, China) according to the manufacturer’s protocol. The apoptotic rates were analysed by flow cytometry on a FACs Calibur (Becton Dickinson, USA). Cells that were annexin V-PE positive and 7-AAD negative indicated early apoptotic cells [42].

Transwell chamber invasion assay

Twenty-four-well invasion chambers were obtained from Costar (USA). After being transfected with negative control GFP, MYCT1-TV-GFP, and MYCT1-GFP, Hep2 (2×10⁴ cells) and HEK293 (4×10⁵ cells) were detached and resuspended in serum-free medium, and seeded to the upper chambers. 500 μl of medium containing 10% FBS was added to the lower chambers. After incubation at 37°C for 24 h, cells remaining attached to the upper surface of the filters were carefully removed with cotton swabs. The filters were fixed with methanol and stained with haematoxylin and eosin. The cells that invaded to the underside of the filter were counted [43].

Statistics

Data were subjected to statistical analysis and shown as mean ± standard error of the mean (SEM). Differences in mean values were analyzed using one-way analysis of variance (ANOVA). Comparisons related to age or sex in clinical characteristics were made by the Mann-Whiney U test. The comparisons of mRNA levels between MYCT1-TV and MYCT1 in LSCC and paired adjacent normal laryngeal tissues were made by Levene’s test. In the figures, the statistically significant values were marked with asterisks (*, p<0.05; **, p<0.01).

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Author Contributions

Conceived and designed the experiments: SF W-NF. Performed the experiments: SF YG W-NF. Contributed reagents/materials/analysis tools: Z-MX G-BQ MZ K-LS W-NF. Wrote the paper: SF W-NF.
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