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Research Paper

CT120: A New Potential Target for c-Myc in Head and Neck Cancers

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Abstract

Background: CT120 is a universally expressed protein with seven transmembrane domains. It functions in cell proliferation, survival and apoptosis by activating Raf/MEK/ERK and PI3K/Akt signaling pathways. Evidence suggests that CT120 plays important roles in lung carcinogenesis and oncogenic pathway activation. c-Myc is an important transcription factor modulating cell progression, apoptosis and cellular transformation. Previous studies have shown that *MYC* gene is amplified in many types of cancer including head and neck squamous cell carcinoma (HNSCC). Myc can regulate expression of many genes by binding to E-boxes. The aim of this study was to investigate the relationship between c-Myc protein and *CT120* gene.

Methods: Tumor and normal tissue samples from 50 patients with HNSCC were investigated with chromatin immunoprecipitation assay (ChIP), Illumina MiSeq, bisulphite sequencing and qRT-PCR.

Results: c-Myc binds to all E-boxes except E-box 5 on *CT120* promoter. The CpG dinucleotides were found to be partially methylated in all tumor and normal tissue samples. Bisulphite sequencing showed a 10% down-regulation in the methylation levels of the tumor tissues. *CT120* gene was hypomethylated and up-regulated in 56% of the tumor tissue samples. Expression of c-Myc was significantly higher in tumor tissues than in non-cancerous tissue samples. *MYC* was overexpressed in 68% of the tumor tissue samples compared to normal tissues. The mean *MYC* levels were 2.42-fold higher in the tumor tissue samples. In 48% of the tumor tissues, *MYC* and *CT120A* mRNA were up- or down-regulated simultaneously ($p<0.001$).

Conclusion: We show that *CT120* gene is a target of c-Myc and it contributes to cancer progression in HNSCC.

Key words: *CT120, FAM57A*, c-Myc, HNSCC, bisulphite, sequencing, methylation.

Introduction

The novel human plasma membrane-associated gene *CT120/FAM57A* (GenBank accession No: AF 477201) has been isolated from chromosome 17p13.3 by positional cloning and rapid amplification of cDNA ends (RACE) by He et al [1]. The *CT120* gene spans over 10.288 bp on chromosome 17p13.3 and is composed of 5 exons. Its main product is a 257 amino acid protein.

CT120 is universally expressed in different tissues except lung tissue and various human tumor cell lines [1]. In human two isoforms of *CT120* have been identified [2]. Functional predictions based on the amino acid sequence of the *CT120A* gene using the SMART and TMHMM software revealed that the protein has seven transmembrane domains [1]. Homology comparison studies also revealed that the gene has been conserved during evolution which indicates that it exerts essential physiological functions. A variant of *CT120, CT120B* has been isolated from large cell lung carcinoma [2]. In this isoform, the fourth exon of *CT120A* is spliced. It consists of four exons and encodes a protein

composed of 225 amino acids. By using a yeast two-hybrid system two partners of *CT120* have been identified. These are the SLC3A2 (CD98) and GGTL3B proteins which function in the amino acid transport and glutathione metabolism, respectively [1].

In 2004, He et al. [3] have shown that two major signaling pathways are activated as a result of ectopic CT120 expression. These are the Raf/Mek/ERK and PI3K/Akt signal pathways, both of which are involved in cell proliferation, survival and apoptosis. CT120 may also contribute to the overexpression of metastasis-associated genes such as cathepsin B, cathepsin D, cathepsin L, and MMP-2/TIMP2 [3]. Silencing of *CT120* enhances *p53* and *caspase 3* expression and reduces *cyclin D1* and *CDK4* expression which results in G1 arrest and apoptosis [4]. It has also been shown that knocking down *CT120* in the SPCA1 human lung adenocarcinoma cell line results in reduced *in vitro* cell growth rates and in decreased tumorigenicity in nude mice [5]. These results indicate that CT120 may be a key modulator in lung carcinogenesis and might contribute to the activation of oncogenic pathways in human lung cancer cells. In a previous study we have observed that the *CT120A* isoform is overexpressed also in head and neck squamous cell carcinoma (HNSCC) tumors [6]. However, no detailed information is available on the structural features of the *CT120* gene. Therefore, we first sequenced the promoter region of the *CT120* gene to investigate the presence of any regulatory sequences.

The c-Myc protein belongs to the myc family of transcription factors and plays a fundamental role in cell cycle progression, apoptosis and cellular transformation [7, 8]. Amplification of the *MYC* gene is observed in many different types of cancer including HNSCC [9, 10]. In the human genome, the *MYC* gene is located on chromosome 8 and its protein product regulates expression of 15% of all genes through binding to the Enhancer box (E-box) sequences. An E-box is a specialized region that functions as a protein binding site and regulates gene expression [11-13]. The CANNTG consensus sequences of the E-box are known as the canonical E-boxes. There are several other non-canonical E-box motifs such as the GATGTG, CATGCG, CACGCG, CACGAG, CGCGAG and CAACGTG sequences [14, 15].

It is also well known that the gene expression rates are under the control of epigenetic modifications [16]. Methylation of the CpG islands which are found in the promoter regions is one of the most frequently studied epigenetic modifications. In this study, to investigate the possible epigenetic regulation of *CT120* and the effect of the promoter methylation to the binding of c-Myc, we also investigated the methylation level of the *CT120* promoter.

Materials and methods

Human tissue

Tumors and the corresponding normal tissue samples were obtained from 50 patients with HNSCC undergoing surgery at the Istanbul University Cerrahpasa Medical Faculty, Department of Otorhinolaryngology. The study was approved by the Ethics Committee of Istanbul University, Cerrahpasa Medical Faculty (No: 83045809/604.01/02-41972). Written informed consent was obtained from all patients prior to the study and the study was performed in accordance with the 1964 Declaration of Helsinki. The experiments have been performed twice to confirm results.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assay was performed using the Tissue Acetyl-Histone H4 ChIP Kit (Abnova, Taipei City, Taiwan) according to the manufacturer's instructions. The strips were activated with the antibody buffer, then, Mouse monoclonal IgG (c-Myc antibody) (200 µg/0.1 ml) (SC-40X, SantaCruz Biotechnology, Dallas TX, USA) and normal Mouse IgG (0.5 mg/ml) were added into the strip wells and incubated for 90 min. The tissue samples were cut into small pieces on ice and the histones and transcript-related proteins were cross-linked to DNA by 1% formaldehyde at room temperature on an orbital shaker for 20 min. After washing with ice-cold PBS, the tissue pieces were homogenized for 5 min using the Bullet Blender homogenizer (Next Advance, NY, USA). The samples were incubated on ice for 10 min after adding 1% of protease inhibitor cocktail. The DNA was sheared by 4-5 cycles of 20 s strokes using a Sonoplus HD2070 sonicator (Bandelin electronic GmbH&Co, Berlin, Germany), resting the samples on ice between each cycle. Unused samples were kept at -80^{0}C until used. The supernatant was diluted and 5 µl of the supernatant was labeled as input DNA. The antibody solutions were removed and the supernatant containing the protein was immunoprecipitated in the strip wells for overnight at 4^{0}C on a Janke Kunkel KS 250 rocking platform (IKA Works GmbH& Co. KG, Staufen, Germany). After washing the wells with 1X TE Buffer, reversal of the cross-linked DNA was performed by adding the DNA-release buffer containing Proteinase K and incubating the samples at 65^{0}C for 90 min. Purified and eluted DNA samples were used for PCR. PCR was performed using 10 ng/µl of DNA and appropriate primers which are given in Table 1. PCR reactions were as follows: 95^{0}C for 10 min, then 35 cycles of 94^{0}C for 30 s, 57-63^{0}C for

30 s (depending on the amplicon) and 72^{0}C for 30 s, with a final extension for 10 min at 72^{0}C (the annealing temperatures for E-box PCR are shown in Table 1).

Analysis of Promoter Methylation Levels by Targeted DNA Methylation Analysis

Promoter methylation levels of the *CT120* gene were analyzed on the Illumina MiSeq system (Illumina Inc., CA, USA) and the methylation level of the 1st island was also confirmed by bisulphite sequencing. For this purpose genomic DNA was obtained from tumor and normal tissue samples using the Roche High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) and the samples were modified with sodium bisulphite using the EZ-DNA Methylation Gold Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions.

The PCR products were purified and eluted for Nextera XT library preparation with dual indexing. The libraries were multiplexed and sequenced on the Illumina MiSeq (Illumina, San Diego, CA, USA) following the manufacturer's protocol. The sequence data was demultiplexed and assessed for sample read coverage, mapping efficiency, unique CpG coverage and bisulfite conversion rate.

The coordinates, methylated CpG counts and total CpG counts were used to investigate the methylation status on the various positions of the promoter region. The methylated and unmethylated regions were investigated using USCS Genome Browser on Human Feb. 2009 (GRCh37/hg19). Assembly and the reports were generated on Excel files. The methylation ratio of the promoter region and the methylation status of each CpG island on each position on Chr 17:633812-635936 were determined.

Bisulphite Sequencing

To confirm the results of the 1st CpG island in the *CT120* promoter region targeted bisulphite sequencing was performed. For cycle sequencing modified DNA samples were amplified by PCR specific to the methylated promoter regions of *CT120* (primers are given in Table 1) and the products were analyzed on 2% agarose gels. After purification of the PCR samples with Ethanol/EDTA/Sodium Acetate, cycle sequencing was performed using the ABI 3100 Genetic Analyzer capillary sequencing system (Applied Biosystems, Waltham, MA, USA). The PCR conditions for cycle sequencing were as follows: Denaturation at 96^{o}C for 30 sec, hybridization at 55^{o}C for 30 sec and an extension step at 60^{o}C for 2 min for 44 cycles.

RT-PCR and Real-Time Quantitative RT-PCR

Total RNA from the tissues was isolated by using the PureLink RNA Mini Kit (Ambion, USA) according to the manufacturer's instructions. cDNAs were prepared from 200 ng of total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). Expression levels of the *MYC* gene in the tumors and non-cancerous tissue samples were analyzed by quantitative Real Time PCR (qRT-PCR) using the LightCycler 480 system (Roche Diagnostic, Mannheim, Germany). PCR reactions were performed in a final volume of 20 µl containing 1x Master Mix, 200 nM gene specific primers (forward: 5'- GCTGCTTAGACGCTG GATTT-3' and reverse: 5'- TAACGTTGAGG GGCATCG-3') and 200 nM hydrolysis probe (UPL Probe No.66) for *MYC* that was 5'-labeled with fluorescein (FAM) and 3'- labeled with a dark quencher dye. The Succinate Dehyrogenase *(SDHA)* gene was used as the reference to normalize the quantification of mRNA levels. Primers (forward: 5'-GGACCTGGTTGTCTTTGGTC-3' and reverse: 5'-CCAGCGTTTGGTTTAATTGG-3') and 200 nM hydrolysis probe (UPL Probe No: 80) that is 5'-labeled with fluorescein (FAM) and 3'-labeled with dark quencher dye. Relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method [17].

Table 1. Sequences of the primers for ChIP analysis and bisulphite sequencing.

Primer	Sequence	Annealing Temperature	Amplicon length	Location
E-box-1	F-5'- AGGAAGATACAGACCAAAGCAAA -3' R- 5'- GGACAGGCGTCTAAACTTGAAT -3'	61 °C	146 bp	-1405/-1259
E-box-2/3	F-5'- TAGACGCCTGTCCCGATAAC -3' R-5'- AGACACCATCCAGAGGCGTA -3'	62 °C	389 bp	-1272/ -883
E-box-4	F-5'- CAGAAGCCGGGAGCCGCG -3' R-5'- ATAGGGCGGGGCGGGGTC -3'	64 °C	207 bp	-488/-281
E-box-5	F-5'- GACCCCGCCCCGCCCTAT -3' R-5'- GCCGCGCGATTTCAACCCT -3'	58 °C	123 bp	-299/-176
E-box-6	F-5– AGGGTTGAAATCGCGCGG -3' R-5'- AGTCGGTGCGGCTCCAT -3'	57 °C	190 bp	-195/-5
1st CgP Island	F-5'- AGGTATTATTATATTTGGTTAATTTTGTAT-3' R-5'-AAAAACTACAAACTCTTACTACCTCTC -3'	58 °C	255 bp	-1093/-838

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6 for Windows, (GraphPad Software, La Jolla California USA). We used One-way ANOVA test to analyze the association between gene expression levels and promoter methylation. $p<0.05$ was considered statistically significant.

Results

Characterization of the Promoter Region of the CT120 Gene

Using sequence analysis we identified 6 E-boxes in the promoter region of the *CT120* gene (Fig. 1). To investigate binding of c-Myc to the *CT120* gene promoter we performed ChIP assays. Our data have shown that c-Myc binds to all E-boxes which are located in the *CT120* promoter except the E-box 5 (Fig. 2).

ACTGAAATTCCTGTCTTCATC**CATGTG**ATTGGTGCTTGGCACAAATGAAATGTAT
TCCAATTCATTCAATTTAGACGCCTGTCCCGATAACTGTTTTTTTGTTTGTTTGTTT
GTTTGTTTTTTGAGACAGAGTCCTGCTCTGTGGCCCAGGCTGGAGTGCAGTGGCG
TGATCTCGGCTCACTGCAACCTCTGCCTCCAGGTTCAAGCGATTCTCCTGCCTCAG
CCTCCAGAGTAGCTGGGACTACAGACAGGCACCACCACACCTGGCTAATTTTGTA
TTTTTAGTAGAGACGGGGTTTCACCGTGGTGGTCAGGCTGGTCTCAACTCCTGAC
GT**CAAGT**GATCTGCCCGCCTCGGCCTCCC**CAAGTGC**TGGGGTTACAGGCGCGCC
CGACCCCGATACTTTTAAATAACGACACGTTGTGAACAACACCACAGTCCCTACG
CCTCTGGAGGTGTCTCCGGATAGATCCAAAGCCGAGAGGCAGTAAGAGCCTGCA
GCCTCCACAGTCCGCCTCCTGGTGCAGGTGGAGAGGGGCTGCTGGAGGCCCCCGG
ACCTCACGCTTCCTGAGGGCCACCCCGAGCGGCTGCAGCCGCTGGCGGCACCGGG
AAAAGCATCTCGGCCGTGCGGGGAGAGGCCACATCCTGAGGCCTCCCACCGAGA
AGCACCAGGGCTTGGTGGACGGGGCCCGCGAACCCAGGGCCACAGCCGCCTCCC
TCCTTCCTTCCCCGCGGGCCTCGCCCCACCCCGGAGGGGGTTCCTACCTGGCTTCC
TCGGCCCCGCGCCCACCCCGCCCCTTCGCCCGGGGCCGGCGAGAAGAGCAGGTC
GGGGAGGGCGCGTCCGCTGACGGAACAGAAGCCGGGAGCCGCGGCCCTTCCCGC
CTCCGCCTGGGCCCCGCGGCCGCTCGGGAGGGAGCGGCGGACTCGGAACGCGCG
CGCGCCGCGGCCCCGGCCACAGCCGCGCCTCGGTCCCCGCCTCCGCCCCCGCCCA
CCCGCCCGCCGGGGACGCCGGGGAACCCTCCGGGCCCAGGGAC**CGCGAG**AGAC
CCCGCCCCGCCCTATCGCAGCCGCCGGGCCTGGT**CACGCG**CAGAGCGCGCCGCG
GGATCGGGGCCAGCGCCGGGCGGAGGCGGGACGGGGACGGGGGCGGAGGGTTG
AAATCGCGCGGCCGGGCCGGGGCGCGCCGAGCCGAACCCAGC**CACGCG**GCGCC
AGCGAGGCGGCCGACCCGCAGCCCCGATGCTGCTGACGCTGGCCGGGGGCGCGC
TCTTCTTCCCGGGGCTCTTCGCGCTCTGCACCTGGGCGCTGCGCCGCTCCCAGCCC
GGATGGAGCCGCACCGACTGCGTG*ATG*ATCAGCACCAG

Figure 1. Nucleotide sequence of the promoter region (-1334 to +14) of the *CT120* gene. The putative binding sites for c-Myc are highlighted in bold. The start codon (ATG) is italicized and underlined

Target sequences of the transcription factors contain CpG islands and methylation of the CpG dinucleotides which are located in these regions may affect the binding of the factors to DNA. Therefore, to predict CpG islands in the promoter region of *CT120* we analyzed the target sequences using the MethPrimer 3 software. We identified two putative CpG-rich regions in the *CT120* gene promoter (Fig. 3).

In order to reveal whether *CT120* expression or binding of c-Myc is attributable to epigenetic mechanisms, the methylation status of the *CT120* promoter was analyzed. In all tumors and non-cancerous tissue samples the CpG dinucleotides were partially methylated (Fig 4). However, there were no differences in the methylation levels of the CpG dinucleotides which are located in the E-boxes 4, 5 and 6. The CpG dinucleotides of the E-boxes 1, 2 and 3 were hypomethylated in tumor tissues compared to non-cancerous tissues (Fig. 5). Bisulphite sequencing also showed that there was a 10% down-regulation in the methylation levels of the tumor tissues with respect to their normal counterparts. In all of our study samples the *CT120* expression levels had been analyzed previously. Therefore, we also analyzed the methylation levels in association with *CT120* expression. Our results showed that 28 (56%) of the tumor tissue samples which displayed CT120 up-regulation were hypomethylated (Table 2 and Fig. 6).

MYC Expression

In order to determine the level of *MYC* expression qRT-PCR was performed using the tumor and adjacent non-cancerous tissue samples. Expression of *MYC* was significantly higher in tumor tissues than in non-cancerous tissue samples (Table 3). We observed *MYC* overexpression in 68% (34/50) of the tumor tissue samples when compared to non-cancerous tissues. The mean *MYC* expression levels were 2.42-fold higher in the tumor tissue samples (Table 3).

To understand the effect of c-Myc on the *CT120* expression levels we evaluated the correlation between these two genes. As a result of this analysis we observed a concordant change in the expression levels of both genes (Table 4). In 48% of the tumor tissues, *MYC* and *CT120A* mRNA were up- or down-regulated concurrently. This correlation was statistically significant ($p<0.001$). In 40% (20/50) and 8% (4/50) of the samples an increase or decrease in the c-Myc level resulted in a concurrent increase or decrease in the *CT120A* level, respectively.

Table 2. Correlation between *CT120* gene expression and promoter methylation

		CT120 Expression			
		Increased	Decreased	No change	
CT120 Methylation	Increased	13 (26%)	6 (12%)	3 (6%)	p<0.001
	Decreased	16 (32%)	11 (22%)	1 (2%)	

Table 3. *MYC* expression levels in tumors and non-cancerous tissue samples from HNSCC patients

	C_t Reference	C_t Target	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$	p
Tumor	27.91	26.46	-1.451	-1.27	2.42	0.001
Normal	28.22	28.04	-0.174	0	1	

Table 4. Correlation between *CT120* and *MYC* expression in HNSCC tumors

		CT120 Expression			
		Increased	Decreased	No change	
MYC Expression	Increased	20 (40%)	11 (22%)	3 (6%)	p<0.001
	Decreased	7 (14%)	4 (8%)	1 (2%)	
	No change	2 (4%)	2 (4%)	-	

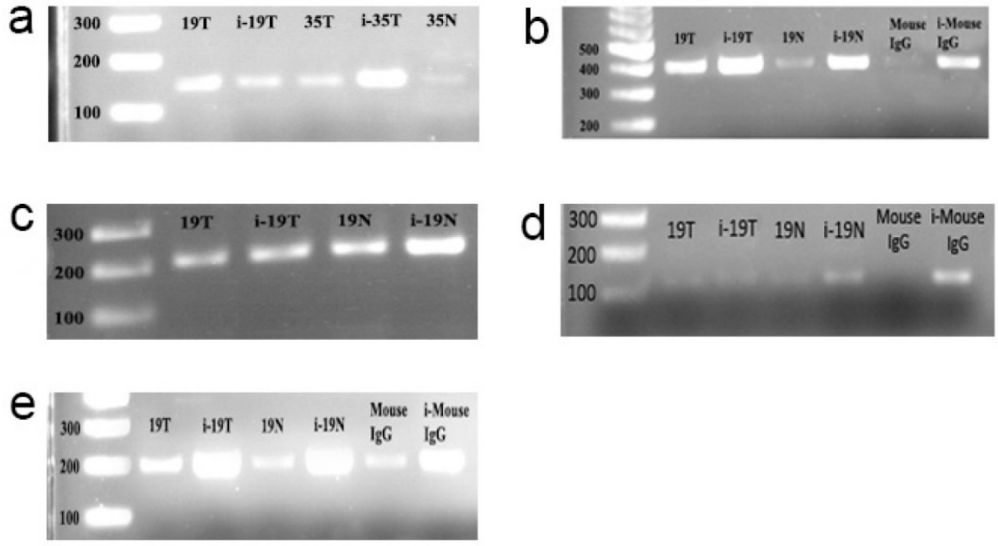

Figure 2. ChIP assay electrophoregrams to demonstrate that c-myc binds to the E-boxes which are found in the promoter of the *CT120* gene. i-19T: input DNA, T. Tumor, N: Normal, Mouse IgG: PCR product derived from DNA template immunoprecipitated by normal IgG. First lanes are 100 bp markers. a: PCR products which were amplified using E-box 1-specific primers; b: PCR products which were amplified using E-box 1-2-specific primers; c: PCR products which were amplified using E-box 4-specific primers; d: PCR products which were amplified using E-box 5-specific primers; e: PCR products which were amplified using E-box 6-specific primers.

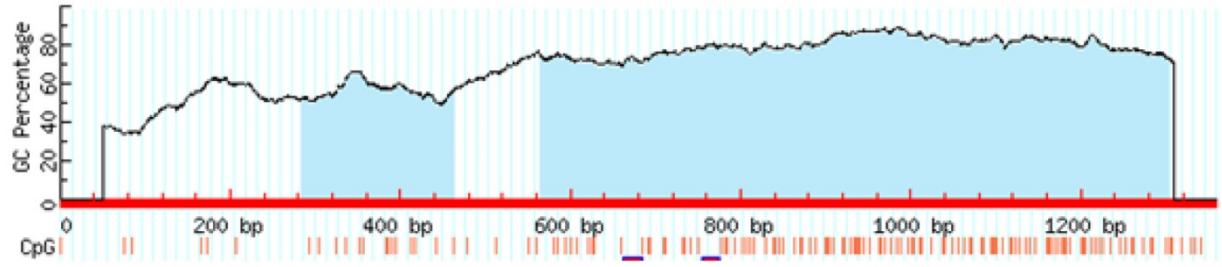

Figure 3. CpG islands of the *CT120* promoter

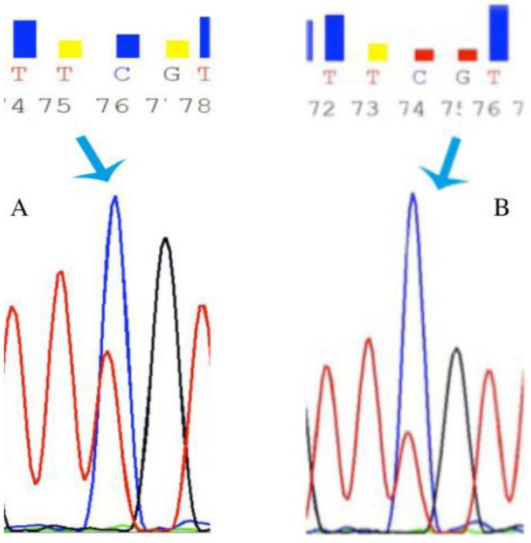

Figure 4. Representative chromatograms of bisulphide sequencing of the promoter region. a: 21 T partial methylation; b: 21 N partial methylation. Blue arrow shows hypomethylated region.

Discussion

The *CT120* gene is located on chromosome 17p13.3 region and codes for a human plasma membrane-associated protein [1]. Although it has been characterized and associated with tumor progression more than a decade ago its exact mechanism of action has not been investigated in detail. Depending on its partners SLC3A2 and GGTL3B a function in the amino acid transport and glutathione metabolism has been suggested [1]. On the other hand, some investigators reported that CT120 is involved in major signaling pathways such as PI3K/Akt and Ref/Mek/ERK which are de-regulated in carcinogenesis [3]. Until recently *CT120* had been associated only with lung cancer [3-5]. In our previous report we have shown that *CT120* acts as an oncogene in HNSCC [6]. Depending on our previous data we hypothesized that *CT120* may have important functions in the progression of HNSCC needing further investigation.

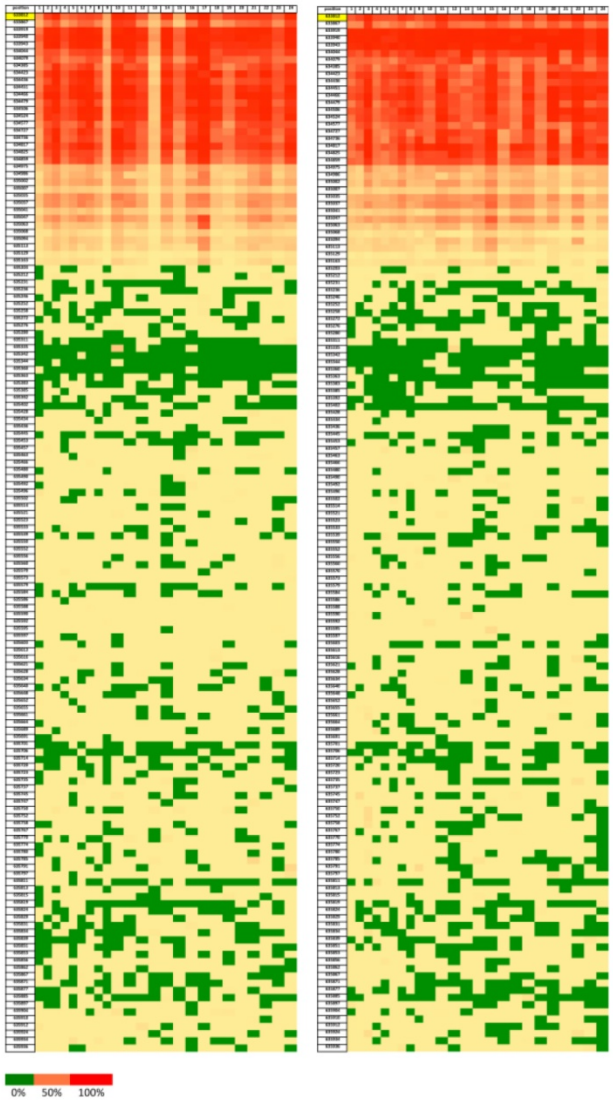

Figure 5. Heatmap showing the ratios of methylation on each position of the CpG dinucleotides (Chr 17:633812-635936). Green color represents lower methylation ratio while red color shows increased methylation up to 100%. Left for tumor samples and right for normal tissue samples.

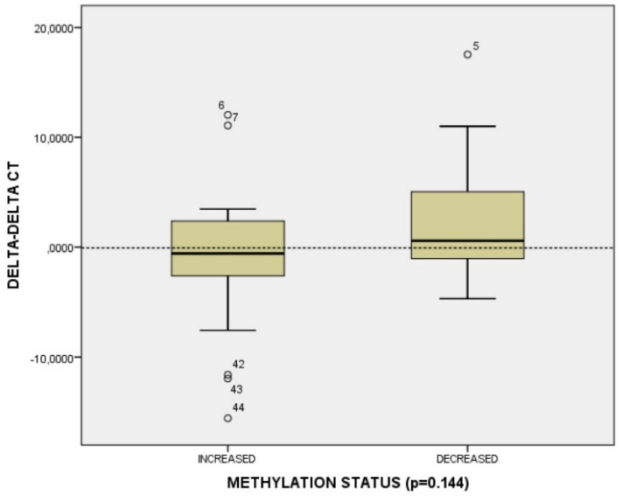

Figure 6. *CT120* methylation and gene expression distribution in HNSCC tumors.

The sequence analysis showed that 6 E-boxes were present in the promoter region of the *CT120* gene. To test the binding of c-Myc to the *CT120*'s promoter we performed ChIP assays. The result of this analysis confirmed binding of c-Myc to the E-boxes 1,2,3,4, and 6 which are located on *CT120*'s promoter. Our results show for the first time that *CT120* is a new target of c-Myc.

On the other hand, Myc-target sites are rich in CpG islands [18]. When we investigated the promoter region of the *CT120* gene we identified two CpG-rich regions. c-Myc was found to strongly bind to the E-boxes 2 and 3 which are in the first CpG-rich region. Aberrant DNA methylation within the CpG islands is among the earliest and most common alterations in human cancers, leading to abnormal expression of a broad spectrum of genes. Methylation of the CpG dinucleotides prevents binding of transcription factors. It is also known that CpG-dinucleotides within the E-boxes prevent the binding of c-Myc [18-20]. Therefore, a decrease in the methylation level of the promoter region in the tumor samples leads to an increase in the expression level by allowing binding of the c-Myc protein. Parallel changes in the expression of the *MYC* and *CT120* genes supports this correlation.

Dysregulated expression or function of c-Myc is one of the most common abnormalities in human malignancies including HNSCC. Several studies have reported that *MYC* amplification is a frequent event in HNSCC [9, 10, 21, 22]. However, reported amplification frequencies vary from 6 to 38% [23-28]. In our previous study we detected *MYC* amplification in 10% of the HNSCC tumor samples by MLPA analysis [29]. Therefore, in this study we investigated the expression of c-Myc to identify whether over-expression of c-Myc occurs as a result of amplification in HNSCC. However, in our study cohort overexpression of c-Myc was more frequent than amplification. Overexpression of *MYC* gene at the mRNA and protein level has been reported in HNSCC with different frequencies (21-68%) [21-29].

In our study overexpression of the *MYC* gene was higher than the previous reports [21-28]. This may be due to the low sensitivity of the methods which have been used in previous studies. In most of these studies the c-Myc protein levels were investigated using immunohistochemistry. As to our knowledge this is the first report in the literature investigating the *MYC* mRNA using a PCR-based method.

As a result, this is the first report indicating the *CT120* gene as the target of the c-Myc protein. Our data suggest that CT120 participates in HNSCC

progression under the control of c-Myc and epigenetic regulations.

Abbreviations

HNSCC: Head and neck squamous cell carcinoma; E-box: Enhancer box; ChIP: Chromatin Immunoprecipitation; qRT-PCR: quantitative Real Time PCR.

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Competing Interests

The authors have declared that no competing interest exists.

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