# Expression Study and Clinical Correlations of MYC and CCAT2 in Breast Cancer Patients

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Received 7 December 2016; revised 1 January 2017; accepted 2 January 2017

#### **ABSTRACT**

Background: Colon cancer-associated transcript 2 (CCAT2) is a newly recognized IncRNA transcribed from the 8q24 genomic region. It functions as an oncogene in various types of cancers including breast cancer, in which it affects Wnt/β-catenin pathway. Previous studies have shown a putative interaction between this IncRNA and MYC proto-oncogene. Methods: In the current study, we evaluated the expression of CCAT2 in breast cancer tissues with regards to the expression of its target MYC. In addition, we assessed the relationship between CCAT2 and MYC expression levels in tumor tissues and the clinical prognostic characteristics of breast cancer patients. Results: MYC expression levels were significantly up-regulated in tumor tissues compared with adjacent noncancerous tissues (ANCTs), while such analysis showed no statistically significant difference between these two tissue types in CCAT2 expression. Starkly increased CCAT2 gene expression levels were found in 12/48 (25%) of cancer tissue samples compared with their corresponding ANCTs. Furthermore, significant inverse correlations were found between CCAT2 expression and stage, as well as lymph node involvement. Besides, a significant inverse correlation was found between the relative MYC expression in tumor tissues compared with their corresponding ANCTs and disease stage. Conclusions: These results highlight the significance of MYC and CCAT2 expressions in the early stages of breast cancer development and suggest a potentially significant role for CCAT2 in a subset of breast cancer patients, which could be applied as a potential therapeutic target in these patients. DOI: 10.18869/acadpub.ibj.21.5.303

Keywords: Long non-coding RNA, Breast cancer, c-MYC

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### INTRODUCTION

ccumulating evidence suggests that a group of non-coding RNAs ranging in size from 200 to 1000 nucleotides participates in tumorigenesis processes<sup>[1]</sup>. Such long non-coding RNAs (lncRNAs)

have been shown to control critical pathways for tumor initiation and progression. The lncRNAs have a tissue-specific function and tumor stage-specific expression, which potentiate them as important biomarkers and therapeutic targets<sup>[2]</sup>. Consequently, researchers have focused on the identification of their tumor-specific

DOR: 20.1001.1.1028852.2017.21.5.6.0 ]

DOI: 10.18869/acadpub.ibj.21.5.303

signature in several cancer types  $^{[3]}$  including breast cancer  $^{[4,5]}$ .

Breast tumors are among cancers with frequent gene copy number aberrations. Among cytogenetic abnormalities detected in breast tumors, amplification of oncogene(s) located on 8q24 has been suggested to participate in the development and/or progression of a great number of primary breast cancers, principally invasive ones<sup>[6,7]</sup>.

Colon cancer-associated transcript 2 (CCAT2) is a recently identified lncRNA transcribed from the 8q24 genomic region<sup>[8]</sup>. This lncRNA has been regarded as an oncogene in several kinds of cancers including breast and colon cancer, in which it exerts its function via affecting Wnt/β-catenin pathway<sup>[8,9]</sup>. The CCAT2 genomic locus contains certain polymorphisms that have been demonstrated to be associated with susceptibility to various cancers<sup>[8]</sup>, as well as with the risk of metastasis in inflammatory breast cancer<sup>[10]</sup>. In addition, among these polymorphisms, G-allele genotype of rs13281615 has been regarded as a risk factor for developing breast cancer, while the AA genotype has been shown to protect against its occurrence<sup>[11]</sup>.

Previously, it has been noted that each risk locus in 8q24 region has epigenetic marks consistent with enhancer elements and forms a chromatin loop with the MYC proto-oncogene, which localizes numerous 100 kb telomerics to this region. Consequently, the 8q24 risk loci has been suggested as tissue-specific enhancers of  $MYC^{[12]}$ . Previous independent reports have shown the overexpression of CCAT2 in breast cancer tissues<sup>[2]</sup>, as well as overexpression of MYC in certain types of breast cancer<sup>[13]</sup>. Although the role of CCAT2 in up-regulation of MYC expression has been demonstrated in colon cancer in vitro<sup>[8]</sup>, there is no sufficient data regarding the possible interactions of these two genes in the tumorigenesis process in breast cancer samples. As a result, in the current study, we assessed the expression of CCAT2 in breast cancer tissues with regard to the expression of its target MYC. Subsequently, we evaluated the relationships between CCAT2 and MYC expression levels in tumor tissues and the clinical prognostic characteristics of breast cancer patients.

## MATERIALS AND METHODS

#### Patients' samples

The Ethical Committee of Shahid Beheshti University of Medical Sciences (Tehran, Iran) approved this study. All the samples were acquired with the patients' informed consent. Paired breast cancer tissue and adjacent normal breast tissue were obtained from 48 patients who had undergone surgical breast cancer resection at the Departments of Surgery in the Tehran University of Medical Sciences affiliated hospitals between 2014 and 2016. All the participants were female with the average age of 51.35±14.721. Early onset cases were excluded from the study. Breast cancer was diagnosed based on American Society of Clinical Oncology breast cancer guidelines. The nontumorous tissue samples were at least two cm from the edge of the tumor, comprised no apparent tumor cells, and were assessed by the pathologists. The tissue samples were frozen in liquid nitrogen immediately after surgical removal and stored at -80°C.

# RNA extraction and quantitative RT-PCR

Total RNA was extracted from tissue samples using the AccuZol<sup>TM</sup> total RNA extraction solution (Bioneer, Korea) according to the manufacturer's instructions. RNA purity and concentration were quantified by Scientific NanoDrop<sup>TM</sup> Thermo Spectrophotometer (Waltham, MA, USA). RNA (1 µg) was used in cDNA synthesis by using PrimeScript RT reagent kit (Takara Bio, Ohtsu, Japan). Quantitative RT-PCR reaction was performed on a Rotor-Gene 6000 Corbett detection system using SYBR Premix Ex Taq (Takara Bio, Ohtsu, Japan). Thermal cycling conditions were an initial activation step at 95° C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds, specific annealing temperature for 20 seconds and an extension step at 72°C for 20 seconds, followed by curve acquisition. Specific temperatures for MYC, CCAT2, and B2M were 56.5°C, 58.5°C, and 60°C, respectively. We also included no template control consisting of H<sub>2</sub>O in each run. B2M gene was used for normalization of RT-PCR data as indicated in a previous study<sup>[4]</sup>. The sequences of forward and reverse primers sequences are listed in Table 1.

Table 1. The nucleotide sequences for forward and reverse primers

Gene	Forward primer	Reverse primer
CCAT2	5'-AAGAGGGAGGTATCAACAGAGAC-3'	5'-TTTGGACGACGCCTTCATTTC-3'
MYC	5'-CACATCAGCACAACTACG-3'	5'-GTTCGCCTCTTGACATTC-3'
B2M	5'-AGATGAGTATGCCTGCCGTG-3'	5'-GCGGCATCTTCAAACCTCCA-3'

# Estrogen receptor (ER)/progesterone receptor (PR)

The ER/PR status of each patient was confirmed by immunohistochemical (IHC) staining and obtained from patients' medical records. Staining of >20% of tumor cell nuclei was regarded as positive and that of 5% to 19% as borderline. When <5% of tumor cell nuclei was stained, a negative result was reported. Both borderline and obviously positive results were considered as positive.

# Human epidermal growth factor receptor 2 (HER2/neu)

HER2/neu results were obtained from the medical record and accomplished by IHC assay. For the present study, a test result of 0-2+ was considered as negative and 3+ as positive.

### Ki-67

Ki-67 status was evaluated using IHC assay. Ki-67 values were reported as the percentage of positively marking malignant cells among the total number of malignant cells assessed using the anti-human Ki-67 monoclonal antibody MIB1 or alternatively as positive vs. negative.

#### Statistical analysis

Fold changes in gene expression were quantified by LinRegPCR (version 2) and Relative Expression Software Tool-RG©-version 3 (QIAGEN, Korea) by means of the amplification efficiencies and cycle thresholds from comparative quantification analysis. By the means of LinRegPCR, the baseline fluorescence was determined and subtracted and PCR efficiencies were then computed for each sample. Afterwards, Cq value and the starting concentration per sample (reported in an arbitrary unit) were measured. The calculated Cq and efficiency values were used for quantification analysis. The quantities of mRNAs in the tissues were standardized to the B2M mRNA and compared between tumor and non-cancerous tissues. The pairwise fixed reallocation randomization test with 2000 iterations in the REST 2009 software was used to express the significances. The level of statistical significance was set at P<0.05. The associations of demographic and clinical data with gene expression levels were assessed using SPSS v.18.0.1 (SPSS Inc., Chicago, IL, USA). The data were presented as the mean±SD. The McNemar's test was used to compare paired tumor and adjacent non-cancerous tissues (ANCTs). Chi-square and independent t-tests were applied to assess the significance of CCAT2 expression as correlated with clinicopathologic features in breast cancer. Significance was delineated as *P*<0.05.

### **RESULTS**

#### General statistical information

Data were analyzed according to the evidence obtained from questionnaires, interviews, as well as clinical and laboratory tests. Table 2 demonstrates the demographic and clinical data of patients, including age, histological features, tumor size, tumor grade, and stage.

# Expression of CCAT2 and MYC in patients' samples

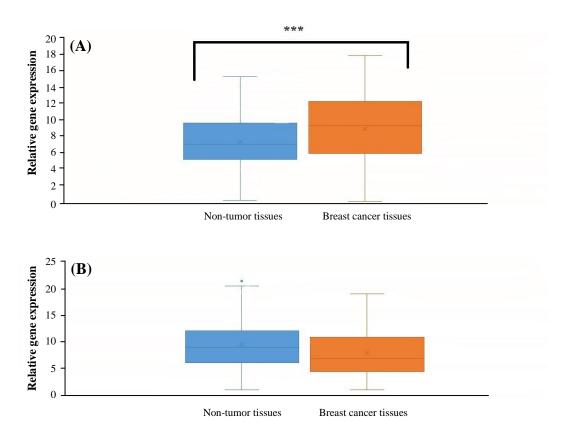
Comparison of *MYC* expression levels between total tumor and ANCT tissues showed a significant upregulation in tumor tissues (*P*=0.02) (Fig. 1A). However, such analysis showed no statistically significant difference between these two tissue types in *CCAT2* expression (*P*>0.05) (Fig. 1B). Starkly increased *CCAT2* gene expression levels were observed in 12/48 (25%) of cancer tissue samples, as compared with their corresponding ANCTs. Figure 2 shows the frequency and cumulative percentage of samples in each subgroup based on relative expression of *CCAT2* and *MYC* in tumor tissues compared with adjacent non-cancer tissues.

# Correlations between *CCAT2* expression and clinical characteristics

To further discover the role of CCAT2 in breast cancer, we subsequently assessed the associations transcript levels and between several A clinicopathological characteristics. significant inverse relationship was found between CCAT2 expression and stage, as well as lymph node involvement. The relationship between CCAT2 expression and clinicopathological variables is shown in Table 3. The 48 patients were then divided into two groups by the median value of relative CCAT2 transcript levels in tumor tissues compared with the corresponding ANCT: high (n=12) and low (n=36) expression groups. Statistical analyses between these two groups showed a significant inverse association between lymph node involvement and CCAT2 transcript levels (P=0.02). No significant correlation was found between transcript levels and other clinicopathological variables. Further analyses were performed in patients with low CCAT2 transcript levels in tumor tissues, as compared with the corresponding ANCT, by dividing into two subgroups based on the relative transcript levels (>0.5 and <0.5). No other significant relationship was found between CCAT2 transcript levels and clinicopathological variables.

Table 2. Demographic and clinical data of patients

Characteristics	Values 51.25 . 14.721 . (25.94)
ge (mean±SD)	51.35±14.721 (35-84)
lenarche age (mean±SD)	13.41±1.117 (12-16)
Ienopause age (mean±SD) irst pregnancy age (mean±SD)	49.84±4.413 (38-59) 21.41±5.056 (14-34)
reast feeding duration (months) (mean±SD)	50.20±52.206 (0-240)
ositive family history for cancer (%)	25.6
ody mass index (%)	
<18.5	7.1
18.5-24.9	57.1
>25	35.7
listory of oral contraceptive use (%)	
Yes	67.4
No	32.6
listory of hormone replacement therapy after m	
Yes No	18.6 81.4
	01.4
Cancer stage (%) I	7.0
II	58.1
III	30.2
IV	4.7
verall grade (%)	
I	11.9
II	54.8
III	33.3
uclear grade (%)	
I	7.1
II III	54.8 38.1
Subule formation (%)	30.1
I	7.1
II	38.1
III	54.8
litotic rate (%)	
I	33.3
II	50.0
III	16.7
umor size (%)	
<2 cm	16.3
≥2 cm, <5 cm ≥5 cm	76.7 7.0
	7.0
strogen receptor (%) Positive	70.7
Negative	29.3
rogesterone receptor (%)	
Positive	63.4
Negative	36.6
ler2/neu expression (%)	
0	26.8
1	22.0
2	26.8
3	24.4
i67 expression (%)	0.4.5
Positive	94.7
Negative	5.3



**Fig. 1.** Comparison of genes expression levels between total tumor and adjacent non-cancer tissues. (A) *MYC* relative expression levels; (B) CCAT2 relative expression levels. \*P>0.05; \*\*\*P<0.05

# Correlations between MYC expression and clinical characteristics

The relationship between MYC expression and clinicopathological variables is shown in Table 4. A significant inverse correlation was found between the relative MYC expression in tumor tissues in comparison with their corresponding ANCTs and disease stage. We further divided the 48 patients into two groups by the median value of relative MYC transcript levels in tumor tissues compared with the corresponding ANCT: high (n=14) and low (n=34) expression groups. Statistical analyses between these two groups showed a significant inverse association between MYC transcript levels and disease stage (P=0.030), as well as the tumor size (P=0.011). No significant association was found between transcript levels and other clinicopathological variables. Further analyses were performed in patients with low MYC transcript levels in tumor tissues compared with the corresponding ANCT by division into two subgroups based on the relative transcript level (>0.5 and <0.5). Such analyses showed a significant inverse correlation between histological grade and MYC relative expression level (P=0.007).

# Relative expression of CCAT2 and MYC in individual samples

In order to determine any correlation between the expressions of these two genes, the relative expression of these genes was compared in each set of samples. No significant association was observed between the levels of transcripts in tumor tissues ( $R^2$ =0.065, P>0.05) or ANCTs ( $R^2$ =0.17, P>0.05).

#### DISCUSSION

Identification of tumor-specific signature of non-coding RNAs, which are correlated with cancer detection, patient prognosis, and response to therapy, is the aim of many recent studies. In the present investigation, we evaluated the expression of *CCAT2* in breast cancer tissues in comparison with ANCTs and showed a significant *CCAT2* overexpression in tumor samples in a subset of patients. However, in other patients, the level of *CCAT2* transcripts was lower in tumor tissues compared with the corresponding ANCTs. This reduction can be explained by the high level of heterogeneity among breast cancer patients,

Table 3. CCAT2 expression and its associations with patients' clinical and demographic data

Characteristics	Down-	Up-	N	P
	regulation	regulation		
Age				0.205
< 50	12	12	24	
>=50	14	7	21	
Stage				0.001
Early stages (0, I, II)	12	18	30	
Advance stages (III, IV)	14	1	15	
Histological Grade				0.74
I	3	2	5	
II	14	10	24	
III	8	7	15	
Tumor Size				0.705
<2	5	2	7	
$\geq 2$ cm, $< 5$ cm	19	14	33	
≥5 cm	2	1	3	
Node Status				0.015
Negative	11	15	26	
Positive	15	4	19	
ER Status				0.525
Negative	6	5	11	
Positive	19	13	32	
PR Status				0.409
Negative	9	5	14	
Positive	16	13	31	
HER2/neu Status				0.475
Negative	18	14	32	
Positive	7	4	11	
Ki67 Status				0.348
Negative	1	23	24	
Positive	2	14	16	

N, number; ER, estrogen receptor; PR, progesterone receptor; HER2/neu, human epidermal growth factor receptor 2

which has been also highlighted in the Redis et al. [2] study. They showed CCAT2 overexpression in two out of three sets of patients and the correlation between transcript levels and clinical factors only for a subgroup of breast cancer patients. They also indicated CCAT2 expression in epithelial cells of both tumor and unmatched non-tumor breast tissues, by means of in situ hybridization with a higher expression in the former. Redis et al.'s<sup>[2]</sup> study demonstrated that the higher levels of MYC expression in breast tumor samples were positively associated with CCAT2, and the CCAT2 expression level was suggested as a predictor of metastasis and poor survival for a particular subgroup of breast cancer patients. In addition, in this subgroup of patients, a significant inverse correlation was detected between CCAT2 levels and ER and PR levels unlike in our study that no association was found. Another study has shown CCAT2 up-regulation in ovarian cancer samples compared with normal ovarian tissues and suggested a possible association with tumor progression and development<sup>[14]</sup>.

In the present study, we demonstrated significant inverse relationship between CCAT2 expression and disease stage, as well as lymph node status. Of note, the higher expression of CCAT2 was associated with early stages and negative lymph node status. Considering the role of CCAT2 as an oncogene, these data suggest that it might participate in the early stages of tumor development rather than late stages. The same association was found between MYC expression and disease stage, which is in accordance with the results of a previous study in breast cancer patients. Based on the mentioned study, MYC expression and MYC amplification were more commonly detected in earlyonset compared to late-onset tumors<sup>[15]</sup>. The inverse correlations observed between transcript levels of both studied genes in the present study and disease stage are in accordance with the parallel expression of CCAT2 and MYC expressions in breast tumors, which has been observed in a previous study<sup>[2]</sup>. The role of MYC in breast cancer has been highlighted by the participation of its target genes in cell growth, transformation, angiogenesis, and cell-cycle control, and also the fact

Table 4. MYC expression and its associations with patients' clinical and

Characteristics	Down-	Up-	N	P
	regulation	regulation		
Age				0.424
< 50	12	13	25	
>=50	9	13	22	
Stage				0.039
Early stages (0, I, II)	11	21	32	
Advance stages (III, IV)	10	5	15	
Histological Grade				0.621
I	2	4	6	
II	11	14	25	
III	7	8	15	
Tumor Size				0.152
<2	2	6	8	
≥2 cm, <5 cm	17	17	34	
≥5 cm	2	1	3	
Node Status				0.273
Negative	11	17	28	
Positive	10	9	19	
ER Status				0.547
Negative	5	15	20	
Positive	7	18	25	
PR Status				0.460
Negative	6	9	15	
Positive	14	16	30	
HER2/neu Status				0.167
Negative	17	17	34	
Positive	3	8	13	
Ki67 Status				0.391
Negative	2	1	3	
Positive	16	23	39	

N, number; ER, estrogen receptor; PR, progesterone receptor; HER2/neu, human epidermal growth factor receptor 2

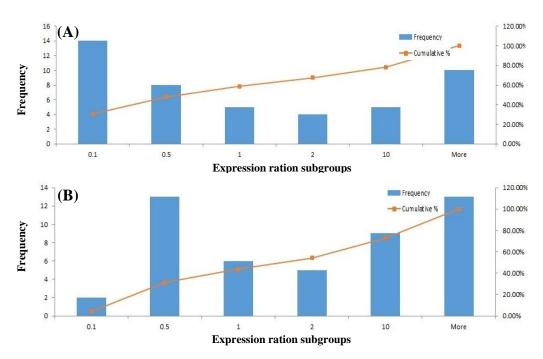


Fig. 2. Frequency and cumulative percentage of samples in each subgroup based on relative expression of CCAT2 (A) and MYC (B) in tumor tissues compared with adjacent non-cancer tissues.

that breast cancer 1 protein is involved in transcriptional regulation via interaction with  $MYC^{[16]}$ . MYC amplification has been shown to be considerably associated with aggressive tumor phenotypes and poor patients' survival. However, the relationship between amplification and overexpression is not obviously described<sup>[16]</sup>. In the present study, we assessed the relative expression level of MYC in tumor tissues regardless of its genomic amplification. Although a previous report has evaluated the associations between 8q24 region amplification and CCAT2 expression in breast cancer patients<sup>[2]</sup>, to our knowledge, there is no data regarding the association between CCAT2 expression and precise MYC copy number, which should be evaluated in future studies.

CCAT2 knock-down has resulted in the inhibition of cell proliferation and invasion *in vitro* and tumor formation *in vivo*<sup>[9]</sup>. In addition, its knock-down has impeded the Wnt/ $\beta$ -catenin signaling pathway transcriptional activity<sup>[9]</sup>. Considering the fundamental role of this pathway in the development of various cancers and its critical role in breast cancer<sup>[17]</sup>, the expression analysis of CCAT2 in breast cancer would pave the way for identification of the mechanism of aberrant signaling in breast cancer and designing more effective therapeutic strategies. The significant difference between CCAT2 expression levels in the studied patients indicates that treatment strategies should be designed based on information obtained from each patient. This observation is in accordance with the differences found in therapeutic responses, even between patients suffered from a certain subtype of breast cancer<sup>[18]</sup>. Regarding the advent of signal transduction therapies in breast cancer<sup>[19]</sup>, identification of biomarkers in each signaling pathway, which could predict the response to a specific treatment modality, is of practical significance. As different cancer therapies are effective in distinct subgroups of patients, it is necessary to find new predictive and prognostic markers to enhance the outcomes of treatments<sup>[20]</sup>. Taken together, these data suggest a potentially significant role for CCAT2 in a subset of breast cancer patients, which could applied as a potential therapeutic target in these patients.

#### **ACKNOWLEDGEMENTS**

The present study has been supported by a grant from Research Deputy, Medical School of Shahid Beheshti University of Medical Sciences (Tehran, Iran) and is a part of M.Sc. thesis of the first author.

### CONFLICT OF INTEREST. None declared.

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