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Increased Expression of CCAT2 LncRNA in Non-Melanoma Skin Cancer

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Abstract

Background: *The non-melanoma skin cancer is one of the most prevalent type of skin cancers, which at least involves 2-3 million people annually. In recent decades, we have witnessed a considerable rise in the incidence of NMSC in Iran. In this paper, we studied the expression of the new lncRNA colon cancer-associated transcript 2 (CCAT2) in cases of non-melanoma skin cancer (NMSC).*

Methods: *The sample included 36 patients and 30 healthy subjects, of whom, we extracted the total RNA from tissues. Using the cDNA synthase, we conducted the real time PCR. Using the SPSS software, we analyzed the data and drew the graphs by PRISM software. The index of P<0.05 was considered significant.*

Results: *The values of CCAT2, TCF7L2 and MYC indicated a considerable expression rise in the NMSCs in comparison with the controls. In addition, the expression of CCAT2 was found to be higher in high-grade tumors than low-grade tumors. According to results, there is a relationship between CCAT2 and NMSC initiation as well as the progression. The CCAT2 functions by its downstream genes, TCF7L2 and MYC, with an impact on the Wnt signaling pathway.*

Conclusions: *based on the results, the lncRNA CCAT2 acts as a potential biomarker for NMSC pathogenesis.*

Keywords: Skin cancer, CCAT2, TCF7L2, MYC, Squamous cell carcinoma.
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Introduction

Nearly the most frequent type of cancer throughout the world is the skin cancer, which involves higher than 35% of cases.[1]The non-melanoma skin (NMSC) is also the most prevalent type of skin cancer occurring minimally in 2-3 million people per year. The basal-cell cancers account for about 80% of the NMSCs, while squamous-cell carcinomas[2] involve the rest 20%. The epidermal keratinocytes[3] are the origin of non-melanoma skin cancers, including basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs). A dramatic rise has occurred in recent decades in the rate of NMSCs, which appears to be one of the most common malignancies. These malignancies cause major health problems (usually death) due to their high frequency of disabilities despite their low mortality rates. Studies have identified various genetic alterations as the etiology of NMSCs.[4]

A growing area of cancer research has focused on the noncoding RNA (ncRNA) in the last decade.[5,6] Current studies are more concentrated on two types of ncRNAs: microRNAs and long noncoding RNAs (lncRNAs). Through specifically pairing to the 5′UTR of mRNAs, the microRNAs do their regulatory function, and thus, mediate subsequent mRNA degradation or inhibition of translation.[7,8] Based on evidence, the lncRNAs play a role in all cellular biology aspects, indicating their involvement in more complex molecular mechanisms than the microRNAs.[9,10]The altered expression of lncRNAs has been seen in various types of cancer. Also, several deregulated lncRNAs are critical for cancer initiation, progression, and invasion.[11]

Colon cancer-associated transcript 2 (CCAT2) is an oncogenic lncRNA, which increases the tumor progression, metastasis, and chromosomal instability.[12]It was first discovered and identified as a highly expressed RNA in the microsatellite-stable colorectal cancer. The CCAT2 lies within the 8q24 genomic region with the role of producing a cancer-related single nucleotide polymorphism (SNP), rs6983276.[12]According to several genome-wide association studies (GWAS), the rs6983276 polymorphism is related to intestine cancers[13,14] and epithelial cancers.[15] Moreover, the WNT signaling pathway is activated by CCAT2 via TCF7L2 essential to the progression of multiple types of cancers.

As no studied have been done on CCAT2 in the NMSCs so far, we decided to assess the expression profile of CCAT2 in the NMSCs to distinguish its clinical correlations.

Materials and Methods

We gathered the information of 16 BCC cases, 15 differentiated SCC cases, and 5 undifferentiated SCC cases in this cross-sectional study from Cancer Center of Imam Khomeini Hospital, Tehran, Iran. The study sample included 36 cases and 36 controls (normal subjects). we reviewed all cases and the diagnosis was made independently for all instances. An informed consent form was signed and approved by all subjects. We graded the SCC cases based on Border`s criteria, while the basal cell carcinomas were categorized due to the Rippey's classification.

Employing the GRC's FFPE kit (Qiagen, Germany) and based on the manufacturer's instructions,[16] we extracted the total RNA from the tissues. Also, we treated the total RNAs with DNase I (Sigma, USA) for 30 min at 37 ºC to eliminate any possible genomic DNA contamination. We examined the integrity and concentration of extracted RNAs by Agilent RNA 6000 Nano Kit (Applied Bio Systems, USA). Using 3-5 µg of purified total RNA with the RevertAid™ reverse transcriptase (Applied Bio systems, USA) in a total 20 µl reaction mixture (according to the manufacturer's instructions), we conducted

the reverse transcription reaction for the first strand cDNA synthesis.

We estimated the mRNA expression levels of CCAT2, MYC, and TCF7L2 genes using proper primers. The relative expression of each gene was assessed compared to the housekeeping gene of Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) with specific primers. All primers were designed using the PRIMER EXPRESS software (Applied Biosystems, USA). Using the specific primers (Table 1), we made the amplifications. According to the manufacturer's instructions, the quantitative RT-PCR was done by the 7500ABI system (Applied Biosystems, Foster, CA, USA) in a final reaction using the volumes of 20 µl with 20 ngcDNA, 10 µl of SYBR Green I master mix (Takara, Shiga, Japan), and 200 nM of forward and reverse primers. The PCR reaction process was as follows: it started with denaturation of templates at 95 ºC for 3 min, which was followed by 40 cycles of denaturation at 95 ºC for 15 sec and annealing/extension at 60 ºC for 30 sec. The PCR products specificity was evaluated by running them on a 2% agarose gel to verify their size and dissociation curve analyses. A serial diluted cDNA was employed to achieve a standard curve and an amplification efficiency for each of the primers of gene transcript. The same procedure was used for all genes expression analyses with proper negative controls with no template controls to exclude or detect any possible contamination.

Table 1. Characteristics of primers of ccat2, tcf7l2 and myc genes used for real time pcr.

Tm	Product size	Sequence	Gene name
60	145	F-5-CCTCCTGCCTCGAGAAGGGC-3 R-5-CTCGCTCCCTCTGCCTCTCGC-3	MYC
59	142	F-ACGAGCACCTCCTGTATCTTC R-CTCGTTCCCAAGGATCCGATC	TCF7L2
59	182	F- TGGATGTTCTGGGTCTTGACC R-GGGAGTCTCTGTTGATACCTC	CCAT2
59	123	F- CCATGAGAAGTATGACAAC R-GAGTCCTTCCACGATACC	GAPDH

Using the comparative threshold cycle as described by Livak Briefly, we estimated the relative gene expression for each gene. Also, the mean threshold cycle (mCT) was obtained from triplicate amplification during the exponential phase of amplification. We then subtracted the mCT value of the reference gene of internal control gene (GAPDH) from mCT value of each Tubb3 and TopIIAgenes to achieve ΔCT for each gene. The relative expression of each gene was calculated by ratio formula (ratio= $2^{-\Delta\Delta Ct}$)[17] following the computation of ΔΔCT values of each sample. Three replications were at least made for all experiments.

We analyzed the real time quantitative reverse transcription of PCR with T-test using the Graphpad Prism 5.0 program and the SPSS software (SPSS, Chicago, IL, USA). A P-value ≤0.05 was considered significant and the data were indicated as mean±standard deviation (SD).

Results

The patient's age was from 47 to 85 years (a mean age of 68.72). The samples included 15 cases of differentiated SCC, 5 cases of undifferentiated SCC, and 16 cases of basal cell carcinomas (BCC). Based on the Broders` criteria, 5 out of SCC cases were grade I, while 11 were grade II and 4 of grade III. In BCC cases, 6 were nodular, 6 were Metatypical, and 4 cases were classified as mixed (Table 2).

Table 2. Summary of pathological data of the cases

	BCC	Undifferentiated SCC	Differentiated SCC
Biopsy	16	5	15
Grade I	-	1	4
GradeII	-	3	8
GradeIII	-	1	3
Nodular	6	-	-
Metatypical	6	-	-
Mixed	4	-	-

We used the Real Time PCR for cases and controls to analyze the expression levels of CCAT2, TCF7L2, and MYC genes. According to the Real Time PCR results, a significant rise was seen in the CCAT2 rates in the NMSC samples in comparison to the normal samples (P<0.05). The expression levels of TCF7L2 and MYC also showed a significant increase in the NMSCs compared to the controls (Figure 1).

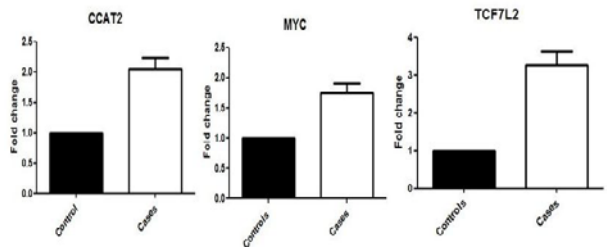

Figure 1. Expression profiles of CCAT2, MYC and TCF7L2 genes in cases and controls

The expression levels of CCAT2, TCF7L2, and MYC genes were also assessed between BCC and SCC samples. Indicated by the results, a significant increased expression of CCAT2 was seen in the SCC samples compared to the BCC samples. Moreover, the analysis demonstrated a statistical rise in the levels of TCF7L2 and MYC in the SCC samples (Figure 2).

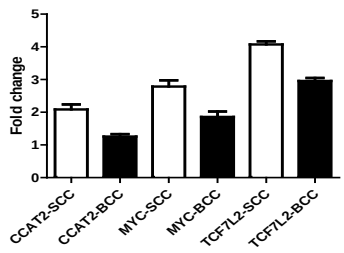

Figure 2. Expression profiles of CCAT2, MYC and TCF7L2 genes in SCC and BCC samples

We compared the expression levels in different grades of SCC and BCC samples to evaluate the expression of CCAT2 and its downstream genes (TCF7L2 and MYC in this study). The analysis results suggested no significant difference in the expression of CCAT2 between grades I and II. The expression

of CCAT2 in grade III was however considerably higher than the grade I and II samples. As for TCF7L2 and MYC genes, the expression level showed a significant rise in the grade III compared to grade I tumors (P<0.05). Nevertheless, no difference was found in the gene expression of TCF7L2 and MYC between grades III and II, and between grades II and I as well (P>0.05)(Figure 3).

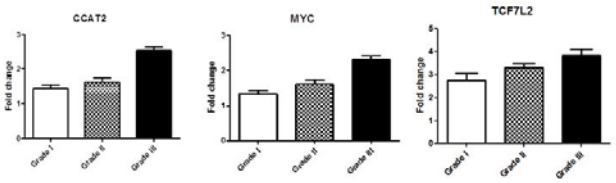

Figure 3. Expression profiles of CCAT2, MYC and TCF7L2 genes in different grades of SCCs

We also evaluated the expression level of these genes in different types of BCC. We observed that the CCAT2 expression was significantly higher in the nodular type compared to the metatypical samples. Compared with mixed and metatypical tumor samples, the TCF7L2 expression showed increase in the nodular samples (P<0.05). However, no significant difference was found in the expression level of MYC in the BCC samples (P>0.05).

Discussion

CCAT2 is a new LncRNA in the 8q24 genomic region, which harbors rs6983267 SNP. Based on reports by previous research, CCAT2 can function as an oncogenic gene involved in multiple cancers progression.[12]In this study, we examined the expression of CCAT2 and its downstream genes (TCF7L2 and MYC) in the non-melanoma skin cancer samples. Indicated by the results, the levels of CCAT2, TCF7L2, and MYC genes expression significantly increased in the skin cancer cases in comparison with the controls. This result is consistent with previous studies by Redis et al., Ling et al. and Wang et al.[12,18,19] The human genome is transcribed actively with just a small protein-coding genes fraction, while nearly 95 % of the transcripts are non-protein-coding genes or ncRNAs, once treated as junks.[20,21]Recent lncRNAs studies have demonstrated critical regulatory roles for ncRNAs along with involvement in all cancer biology aspects. Various cancer-associated lncRNAs have been well described, including MALAT1, HOTAIR, HULC, CCAT2, CCAT1, and BCAR4.[22-25]TheselncRNAs prove the human transcriptome complexity, introducing a novice paradigm for cancer research. For example, the microRNA-mediated cross talk between lncRNAs and mRNAs, BceRNA hypothesis has been validated in numerous types of cancer.[26,27]Moreover, GWAS have identified many cancer-associated SNPs and hot loci, most of which are located in the chromosome region of gene desert with no protein-coding gene.[28,29]

According to Redis et al. research, the CCAT2 is up-regulated in breast cancer samples, functioning as an oncogene with correlation with cancer progression.[18]Ling et al. also demonstrated the regulation of Wnt signaling pathway by CCAT2 through TCF7L2 and MYC, which promotes the progression and metastasis[12] of colon cancer. Based on the results, TCF7L2 and MYC indicated increased level of expression in the NMSCs, implying that CCAT2 may regulate the TCF7L2 and MYC in the skin cancer as well. Another outcome was the highly expressed CCAT2 is in the malignant skin tumors rather than the low grade tumors, suggesting the possible correlation of CCAT2 with increased cancer progression and metastasis.

In conclusion, one can say that the new lncRNA CCAT2 is highly expressed in the NMSCs and there is an association between CCAT2 expression levels with the skin cancer progression. This may indicate that the CCAT2 can be used as a potential biomarker for NMSCs.

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Conflict of Interest

The authors declared that they have no conflict of interest.

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