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The Expression of *CCAT2*, *UCA1*, *PANDA* and *GHET1* Long Non-coding RNAs in Lung Cancer

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Abstract

Background: Long non-coding RNAs (lncRNAs) have been considered to be prospective biomarkers for diagnosing lung cancer due to the fundamental roles they hold in the regulating several cancer-related pathways.
Methods: Using the quantitative real-time polymerase chain reaction method, we evaluated the expression of *CCAT2*, *UCA1*, *PANDA* and *GHET1* lncRNAs in 32 lung cancer tissue samples and their corresponding adjacent non-cancerous tissues (ANCTs) from lung cancer patients admitted to the Labbafi-Nejad Hospital from 2015 to 2016.
Results: No significant differences were found in the expression of lncRNAs within the tumoral and non-tumoral tissue samples. Bayesian Multilevel analysis showed no association between the expression of lncRNAs and the patient's tumor node metastasis (TNM) stage following adjustments for age. Spearman correlation analysis revealed an inverse correlation between the expression of *PANDA* in tumoral tissues and age. Additionally, the difference in *CCAT2* expression among the tumoral and non-tumoral tissues was inversely correlated with patients' age. Significant pairwise correlations were found between the expression of lncRNAs in both the tumoral and non-tumoral tissues.
Conclusions: Despite the findings supporting a role for the lncRNAs, *CCAT2*, *UCA1*, *PANDA* and *GHET1* in the pathogenesis of lung cancer, our data suggests no relationship for expression of these lncRNAs in lung cancer, questioning their potential as lung cancer biomarkers.

Keywords: *CCAT2*, *GHET1*, lncRNAs, Lung cancer, *PANDA*, *UCA1*.

Introduction

Globally, lung cancer is one of most prevalent causes of cancer-related deaths. Despite current medical interventions, the prognosis and survival rate of a diagnosed individual is very poor. This has created the necessity for understanding the biological pathways and genetic abnormalities involved in lung cancer pathogenesis in order to design more effective treatments. Several driver mutations have been characterized in lung cancer samples which have been implicated to hold a significant role in carcinogenesis. Additionally, genomic and transcriptomic sequencing methods have revealed long non-coding RNAs (lncRNAs) to be highly expressed in cancerous tissues and have a critical role in cancer pathogenesis. However, despite the fundamental roles of lncRNAs in the transcriptional, post-transcriptional, and epigenetic modification of genes, their patterns of gene expression are poorly characterized in lung cancer (1).

Although limited, previous research has examined the expression of various lncRNAs in lung cancer. Work by Qui *et al.* examined the expression profile of the lncRNA, *colon cancer-associated transcript 2 (CCAT2)*, in non-small cell lung cancer (NSCLC). Their findings revealed *CCAT2* to be over-expressed specifically within adenocarcinoma and not squamous

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cell cancer. Furthermore, the over-expression of *CCAT2* was associated with enhanced invasiveness of NSCLC (2). A separate study by Zhao *et al.* investigated the relationship between *CCAT2* and its associated regulatory genes in the context of NSCLC. Over-expression of *CCAT2* within NSCLC tissues was found to be accompanied by Pokemon over-expression. Furthermore, *CCAT2* knockdown experiments resulted in a decrease in Pokemon expression, cell invasion and viability. Their findings suggest that the overexpression of *CCAT2* promotes tumorigenesis via Pokemon over-expression (3). The lncRNA, *urothelial carcinoma-associated 1* (*UCA1*), has also been shown to be over-expressed in NSCLC tissues. Increased *UCA1* expression was found to result in significantly worse prognosis for the patient (4). The lncRNA, *P21-associated noncoding RNA DNA damage-activated* (*PANDA*), has been observed to be down-regulated in NSCLC tissues and be negatively associated with increased tumor size and advanced tumor node metastasis (TNM) stage. Furthermore, *PANDA* was shown to be a direct transcriptional target of p53 in NSCLCs *in vitro* and *in vivo* (5). Finally, the lncRNA, *gastric carcinoma high expressed transcript 1* (*GHET1*) has been regarded as oncogenic in lung cancer based on its high expression within NSCLC tissues and its association with lymph node metastasis, TNM stage and patient survival (6). Based on these findings and the potential role for lncRNAs in lung cancer, we examined the expression of the lncRNAs, *CCAT2*, *UCA1*, *PANDA* and *GHET1* in a cohort of Iranian patients with NSCLC to assess their potential application as diagnostic or prognostic biomarkers for lung cancer within the Iranian population.

Materials and methods

Patient Samples

Tumoral tissues and non-tumoral tissues of 32 patients (24 males and 8 females, mean age ± SD=57.9±7.7) diagnosed with NSCLC were collected. All patients were admitted to Labbafi-Nejad Hospital during 2015 and 2016. Samples were harvested during surgery prior to receiving any form of radiotherapy or chemotherapy. Tissue samples were immediately flash frozen in liquid nitrogen and transferred to the genetic laboratory. Written informed consent was obtained from all study participants. The study was approved by the ethical committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1395.525).

LncRNA Expression Study

All tissue samples were subjected to RNA extraction and cDNA synthesis using the TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA) and the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The transcript levels of lncRNAs (relative to *HPRT1* expression) were compared between the tumoral and non-tumoral tissues of each patient in the rotor gene 6000 corbett Real-Time PCR System using TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA). The primer and probe sequences and the PCR product lengths are provided in Table 1.

Table 1. The primer and probe sequences of each lncRNA and the PCR product length.

Gene name	Primer and probe sequences	Primer and Primer and probe length	Amplicon length
HPRT1	F: AGCCTAAGATGAGAGTTC	18	88
	R: CACAGAACTAGAACATTGATA	21	
	FAM -CATCTGGAGTCCTATTGACATCGC- TAMRA	24	
CCAT2	F: GCGCTGACAGAGATTGCTTAC	21	141
	R: CCAGAGTAGAACAGGGGAAGC	21	
	FAM- TGTGCTCCAAGTGCTTGCCAGGCT-TAMRA	24	
UCA1	F: TCTCCATTGGGTTCACCATTCC	22	100
	R: GCTCTCGGCCTAATCTTGTGG	21	
	FAM- AGCCATGCCCATCAGACAGCCAGC- TAMRA	24	
PANDA	F:GTTTTCCTGTTCGTCGATTCTGG	24	81
	R: GGAAAGCTGAGAGAGACTTTGAAC	23	
	FAM- CTGGACCACCTCTGAAGGCAGGCA -TAMRA	24	
GHET1	F: AGTCAGCTCCCTACAGAGGTG	21	94
	R: TCCTTAGGTGGTGGTTTCTGTTC	23	
	FAM- TCCCACTGCCCAAGATCCCTGCCT - TAMRA	24	

Statistical analysis

Relative expression of lncRNA levels in tumoral tissues compared with the ANCTs was calculated using the (Ln [Efficiency^ΔCT]) formula. The significance between the mean values of transcript levels between the paired tumoral and non-tumoral tissue samples were determined via the Kruschke's Bayesian estimation and was used to fit two-sample Bayesian paired t-test. A student prior family was assumed for parameters with 200000 iterations and 5000 burn-outs. Highest Density Interval was described as the 95% credible interval calculated based on Bayesian approach. The Spearman rank order correlation test was used to estimate the correlation between relative expression levels of lncRNAs and patients' age. Statistical analyses were performed using R software version 3.5. A *P* value of < 0.05 was considered statistically significant.

Results

Relative expression levels of lncRNAs in tumoral tissues compared to non-tumoral tissues

Expression analysis revealed no significant difference in the expression of lncRNAs among the tumoral tissues and the non-tumoral tissues (Table 2).

Table 2. Bayesian t test for the comparison of relative gene expression between two paired samples (tumoral and non-tumoral tissues) ([a]: computed from frequentist method).

Gene	Posterior mean		Relative Expression difference	Standard Deviation	Effect Size	P-value[a]	95% Highest Density Interval
	Tumoral	Non-Tumoral					
GHET1	2.94±3.73	3.05±4.27	0.016	5.43	0.004	0.885	[-1.74, 1.74]
PANDA	2.31±4.2	1.83±4.26	0.508	4.32	0.119	0.511	[-0.89, 1.86]
CCAT2	3.24±4.62	2.23±4.45	1.066	6.09	0.178	0.263	[-0.88, 3]
UCA1	4.09±4.46	2.82±4.68	1.147	7.23	0.161	0.351	[-1.13, 3.46]

The transcript levels of lncRNAs were also compared between samples with different TNM stages. Bayesian Multilevel analysis showed no association between the expression of lncRNAs and TNM stage after adjusting for patients' age (Table 3).

Table 3. The results of Bayesian Multilevel analysis for the association of lncRNA expression and TNM stage following adjustments for age (Stage 1 was considered as reference).

	CCAT2				*GHET1*				*UCA1*				*PANDA*			
	Estimate	SE	P-value	95% Crl	Estimate	SE	P-value	95% Crl	Estimate	SE	P-value	95% Crl	Estimate	SE	P-value	95% Crl
Stage 2	1.82	2.9	.928	3.8, 7.8]	4.3	2.55	0.29	[-0.75, 9.3]	3.55	3.38	.436	[-2.8, 10.3]	3.87	2.15	.14	[-.32, 7.9]
Stage 3	-.85	2.77	.409	6.4, 4.5]	1.89	2.16	0.84	[-2.56, 6.15]	0.24	3.69	.764	[-7.2, 6.9]	2.14	1.97	.294	[-1.7, 5.9]
Age	-.18	0.12	.036	-.4, .06]	-0.06	0.11	0.652	[-0.27, 0.15]	-0.09	.15	.994	[-.38, .19]	0.01	0.09	.918	[-.16, .17]

We assessed the correlation between the expression of lncRNAs and patients' age (Table 4). Spearman correlation analysis revealed an inverse correlation between *PANDA* expression in tumoral tissues and patients' age. Moreover, the expression of *CCAT2* in tumoral and non-tumoral tissues was inversely correlated with patients' age.

Table 4. Spearman correlation examining the expression of lncRNAs and age.

	CCAT2	*GHET1*	*UCA1*	*PANDA*
Tumor	-.177	-.151	-.12	-.361*
Non-Tumor	.25	0.105	.187	-.251
Expression difference	-.306*	-.199	-.184	-.145

*Correlation is significant at the 0.05 level.

Finally, we assessed pairwise correlations between the expression of lncRNAs in both tumoral and non-tumoral tissues. Our findings show a significant correlation among the different types of tissues (Table 5).

Table 5. Pairwise correlation between the expression levels of lncRNAs in tumor and non-tumor tissue samples.

		CCAT2	***GHET1***	***UCA1***
PANDA	Tumor	.591**	.644**	.556**
	Non-Tumor	.482**	.589**	.735**
	Expression Difference	0.71**	0.754**	0.788**
UCA1	Tumor	.57**	.326*	
	Non-Tumor	.752**	.744**	
	Expression Difference	.738**	0.512**	
GHET1	Tumor	.584**		
	Non-Tumor	.583**		
	Expression Difference	0.661**		

Discussion

In the present study, we assessed the expression of four previously identified lncRNAs in the cancerous lung tissue in a cohort of Iranian patients with NSCLC to evaluate their potential diagnostic power within the Iranian population. Despite the previous research indicating a role for these lncRNAs in lung cancer, our findings did not show any significant differences in their expression when comparing the tumoral and non-tumoral tissues of patients. Such findings call into question the validity of these lncRNAs as potential biomarkers for lung cancer. In line with our data, Yang *et al*. did not find any significant difference in *PANDA* expression between the tumoral and non-tumoral lung samples within a cohort of Chinese patients (7). The discrepancies between our results and the results of previous work may be explained by sample size, differences in the etiology of lung cancer and differences in the genetic background of patients. Among different populations, there may be a distinct lncRNA signature. Several lines of evidence point towards an influence for environmental pollutants in modulating the expression of lncRNAs in both *in vivo* animal models and in humans. For example, Martinez-Guitarte *et al*. observed an up-regulation of certain lncRNAs following 24-hour exposure to bisphenol A (BPA) (8). Bhan *et al*. has previously reported a dysregulation in the expression of the lncRNA, *HOX transcript antisense intergenic RNA* (*HOTAIR)*, due to BPA and diethylstilbestrol (DES) exposure (9). Furthermore, smoking has been demonstrated to alter the expression of several lncRNAs involved in the metabolic function and immune response within lungs (10). Additional studies have shown that a dysregulation of the lncRNAs, *HOTAIR* and *MALAT1 (Metastasis associated lung adenocarcinoma transcript 1)* in human bronchial epithelial cells occurs following exposure to cigarette smoke extract (11, 12). Therefore, it is possible that among different populations where the incidence of smoking is highly variable, the influence environmental influence on lncRNA expression within the lung tissues is also variable, which can lead to distinct patterns in lncRNA expression among different populations.

We also compared the expression of lncRNAs between distinct TMN stages using Bayesian Multilevel analysis. Our results show no association between the expression of lncRNAs and TNM stage after adjusting for age. These findings further question their potential application as prognostic markers. Consistent with our data, Qiu *et al.* found no significant association between *CCAT2* expression and TNM stage in lung cancer

patients (2). However, *GHET1*, *UCA1* and *PANDA* expression has been previously found to be associated with TNM stage (5, 6, 13). Such inconsistencies within the literature may be due to the application of different statistical methods and not accounting for the potential effects of confounding variables.

Spearman correlation analysis revealed an inverse correlation between *PANDA* expression in tumoral tissues and the patients' age. Moreover, the difference in *CCAT2* expression among tumoral and non-tumoral tissues was inversely correlated with patients' age. Age-related patterns of lncRNAs expression has been previously described in several cell types, including oocytes (14) and neurons (15). The observed age-related gene signature may reflect the differences in the length of exposure to environmental risk factors. This potential factor should be assessed in a large-scale epidemiological study.

Finally, we detected significant pairwise correlations between the expression of the examined lncRNAs in the tumoral and non-tumoral tissues suggesting the presence of regulatory mechanisms within the tissues. Such significant correlations indicate that the expression level of a certain lncRNA may be based on the expression of other lncRNAs.

In conclusion, similar patterns of lncRNAs expression between tumoral and non-tumoral tissues in this cohort of Iranian patients highlights the significance of population-based studies for biomarker discovery and targeted therapies.

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The authors declare they have no conflicts of interest.

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