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Expression analysis of long non-coding RNAs GSTT1-AS1 and IFNG-AS1 and their target genes in lung cancer

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ABSTRACT. Long non-coding RNAs (lncRNAs) have recently gained attention because of their regulatory roles on expression of genes involved in the immune response and cancer. In the present study, we evaluated expression of two lncRNAs (GSTT1-AS1 and IFNG-AS1) and their target genes (TNFA and IFNG) in human lung cancer samples and their adjacent non-cancerous tissues (ANCTs) to find their putative role in the pathogenesis of lung cancer. No significant difference was found in the expression of TNFA, IFNG, GSTT1-AS1 and IFNG-AS1 between tumoral and non-tumoral tissues. Expression levels of these genes were not associated with tumor stage after adjusting the effects of age. Moreover, expression levels of none of these genes were correlated with patients' age.

However, significant pairwise correlations were detected between expressions of genes in both tumoral and non-tumoral tissues. TNFA, IFNG, GSTT1-AS1 and IFNG-AS1 are not possibly involved in the pathogenesis of lung cancer.

Keywords: Lung cancer; TNFA; IFNG; GSTT1-AS1; IFNG-AS1

INTRODUCTION

Long non coding RNAs (lncRNAs) have recently gained attention of researchers because of their diverse functions in almost all aspects of cell life and aberrant expression in tumoral tissues of versatile origins (Nikpayam E, Tasharrofi B, Sarrafzadeh S, Ghafouri-Fard S, 2017; Dianatpour A and Ghafouri-Fard S, 2017). This proportion of human transcripts also regulates the expression of several genes and pathways involved in the immune response regulation (Eftekharian MM et al., 2017) and cancer pathogenesis (Nikpayam E, Tasharrofi B, Sarrafzadeh S, Ghafouri-Fard S, 2017). Dys-regulation of expression of lncRNAs has been reported in lung cancer samples by independent groups (Ghafouri-Fard S et al., 2018; Nie W et al., 2016; Seiler J et al., 2017). Of note, a number of lncRNAs participate in epigenetic regulation of IFN-γ and TNF-α production in CD8+ T cells (Wang Y et al., 2015). Meanwhile, decreased expression of these cytokines from CD8+ T cells in lung cancer tissues compared with non-cancer tissues has been suggested as an essential immune evasion mechanism of cancerous cells (Hodge G et al., 2014). Two lncRNAs have been shown to regulate expression of IFN-γ and TNF-α cytokines. First, the lncRNA GSTT1-AS1 whose sequence overlaps with the 5′ UTR of GSTT1 cooperates with a chromatin-modification enzyme to construct a more repressive chromatin configuration in IFNG and TNFA loci. Knock down of this lncRNA has resulted in over-production of these cytokines and enhancement of CD8+ T-mediated immunity (Wang Y et al., 2015). The second lncRNA namely IFNG-AS1 resides near to IFNG gene and acts as a crucial checkpoint for expression of IFNG in Th1 cells (Collier SP et al., 2014). The role of IFNG in the pathogenesis of lung cancer has been highlighted by the observed over-growth of lung tumors in the Ifng-/- mice (Redente EF et al., 2009). Moreover, plasma and intra-cellular IFNG levels were decreased in lung cancer patients in correlation with hypermethylation of the IFNG promoter in CD4+ T cells (Wang F et al., 2013). TNF has a dual role in the pathogenesis of lung cancer. While this cytokine is involved in the enhancement of epithelial-mesenchymal transition and successive stimulation of metastasis in lung cancer (Shang G-S, 2017), the expression of TNF by macrophages and mast cells in the tumor islets of patients with NSCLC has been associated with better patients' outcome (Ohri CM et al., 2010). Considering the role of IFNG and TNF in the pathogenesis of lung cancer on one hand and the reported dys-regulation of several lncRNAs in lung cancer samples on the other hand, we hypothesized that the expression of GSTT1-AS1 and IFNG-AS1 lncRNAs might be changed in lung cancer samples compared with adjacent non-cancerous tissues (ANCTs). Consequently, we conducted the current study to evaluate expressions of these lncRNAs in lung cancer samples and ANCTs.

MATERIAL AND METHODS

Patients

The current study was conducted on the tumoral samples and ANCTs obtained from 40 patients with definite diagnosis of non-small cell lung cancer (NSCLC). All patients were admitted at Labbafinejad Hospital during 2016-2017. Samples were excised before any radiotherapy or chemotherapy. Informed consent forms were obtained from all study participants. The study protocol was approved by the ethical committee of Shahid Beheshti University of Medical Sciences.

Expression analysis

Tissue samples were stored at -80°C until RNA extraction. Total RNA was extracted from all tissues using the TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA) based on company instructions. After verification of quantity and quality of the extracted RNA using Nanodrop equipment (Thermo Scientific), cDNA was

produced using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The relative expression levels of genes were compared between tumoral tissues and ANCTs using the rotor gene 6000 Corbett Real-Time PCR System. HPRT1 was used as normalizer. Primers and probes used for expression analysis were designed using the Allele ID 7 for 64x windows software (Premier Biosoft, Palo Alto, USA). The nucleotide sequences of primers are shown in Table 1. PCR program comprised a denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 60 sec and a final extension step in 72°C for 5 minutes. The Real Q Plus 2x. Master Mix (Ampliqon, Denmark) was used for expression analysis.

Table 1. The primers and PCR product length.

Gene name	Primer sequence	Primer length	Product length
B2M	F: AGATGAGTATGCCTGCCGTG	20	104
	R: CGGCATCTTCAAACCTCCA	19	
IFNG	F: GGCAAGGCTATGTGATTACAAGG	23	96
	R:CATCAAGTGAAATAAACACACAACCC	26	
IFNG-AS1	F: AGGAAGCTGGGTAATTGAATGC	22	94
	R: CTTAGGAGGAGAATTTTGGGAGAG	24	
TNFA	F: TCCACCCATGTGCTCCTCAC	20	97
	R: TCTGGCAGGGGCTCTTGATG	20	
GSTT1-AS1	F: CTTTTGCATAGAGACCATGACCAG	24	105
	R: TGGATAATAAACCTGGGCTCAGC	23	

Statistical analysis

Statistical analyses were performed in R 3.5.1 software. The spearman correlation and Bayesian multilevel model were used. The significance of difference in mean values of gene expression between two dependent groups was assessed using Kruschke's Bayesian estimation to fit two-sample Bayesian paired t-test. A student prior family was assumed for parameters with 200000 iteration and 5000 burn-outs. The 95% Highest Density Interval (HDI) was described as the 95% credible interval based on Bayesian approach.

RESULTS

Relative expression of genes in tumoral tissues compared with non-tumoral tissues

No significant difference was found in expression of mentioned genes between tumoral and non-tumoral tissues (Table 2). Moreover, Bayesian Multilevel analysis showed no association between genes expression and tumor stage after adjustment of the effects of age (Table 3). Spearmen correlation analysis showed no significant correlation between transcript levels of genes either in tumoral tissues or in ANCTs and patients' age (Table 4). Significant correlations were found between expression levels of genes in both tumoral and non-tumoral tissues (Table 5).

Table 2. The results of Bayesian test for comparison the relative expression between two paired groups (a: Expression $_{Tumoral}$ – Expression $_{Non-Tumoral}$; b: computed from frequentist method; c: 95% Highest Density Interval).

Gene	Posterior Mean		Relative Expression Difference [a]	Standard deviation	Effect Size	P-value [b]	95% HDI [c]
	Tumoral	Non-Tumoral					
IFNG	-1.215± 3.1	0.07± 3.17	-1.172	4.59	-0.239	0.145	[-2.68, 0.29]
IFNG-AS1	-1.54± 3.49	-1.108± 2.7	-0.639	3.75	-0.177	0.371	[-1.86, 0.58]
TNFA	-1.011± 3.55	-0.809± 3.45	-0.214	4.6	-0.165	0.817	[-1.68, 1.23]
GSST1-AS1	0.664± 3.68	0.38± 3.49	-0.059	6.2	-0.014	0.995	[-2.09, 1.93]

Table 3. Bayesian Multilevel results of association between genes expression and stage with adjusting the effects of age ([a] Stage 1= Reference group).

	IFNG				*IFNG-AS1*				*TNFA*				*GSST1-AS1*			
	Estimate	SE	P-value	95% Crl	Estimate	SE	P-value	95% Crl	Estimate	SE	P-value	95% Crl	Estimate	SE	P-value	95% Crl
Stage2	2.8	2.03	0.677	[-1.17, 6.79]	2.6	1.81	0.257	[-1.04, 6.16]	1.39	2.17	0.689	[-2.85, 5.64]	2.69	3.02	0.587	[-3.31, 8.55]
Stage 3	0.23	1.93	0.642	[-3.52, 4.04]	1.47	1.75	0.249	[-1.94, 4.91]	-1.34	2.08	0.422	[-5.42, 2.74]	-0.31	2.88	0.784	[-6, 5.31]
Age	-0.06	0.09	0.38	[-0.23, 0.12]	-0.02	0.07	0.719	[-0.17, 0.13]	-0.11	0.09	0.344	[-0.28, 0.07]	-0.11	0.12	0.19	[-0.36, 0.12]
Gender (Male/Female)	3.74	1.67	0.237	[0.47, 7.01]	1.87	1.46	0.72	[-0.95, 4.76]	2.45	1.62	0.511	[-0.73, 5.63]	1.33	2.42	0.439	[-3.4, 6.17]

Table 4. Spearman correlation between genes expressions and age.

	IFNG	*IFNG-AS1*	*TNFA*	*GSST1-AS1*
Tumor	-0.098	-0.228	-.086	-0.085
Non-Tumor	0.154	-0.163	0.259	0.269
Relative Expression difference	-0.198	-0.102	-0.222	0.082

Table 5. Pairwise correlation between expression levels of genes in each set of samples.

		IFNG-AS1	*TNFA*	*GSST1-AS1*
IFNG	Tumor	0.682**	0.451**	0.378*
	Non-Tumor	0.469**	0.537**	0.636**
	Expression Difference	0.745**	0.571**	0.515**
GSST1-AS1	Tumor	0.546**	0.578**	
	Non-Tumor	0.556**	0.695**	
	Expression Difference	0.721**	0.764**	
TNFA	Tumor	0.484**		
	Non-Tumor	0.440**		
	Expression Difference	0.661**		

**Correlation is significant at the 0.01 level.
*Correlation is significant at the 0.05 level.

DISCUSSION

In the current study, we evaluated expression of GSTT1-AS1 and IFNG-AS1 and their target genes in lung cancer samples and their paired non-tumoral tissues to find whether their expressions are altered in the context of cancer. LncRNAs contribute in the pathogenesis of several human cancers including lung cancer. Aberrant expressions of several lncRNAs have been associated with tumor initiation or poor survival of lung cancer patients (Xu EW et al., 2016; Tao H et al., 2016). LncRNAs that regulate immune responses might also been involved in the pathogenesis of lung cancer. Previous studies have mostly evaluated expression of cytokine coding genes from immune cells. However, based on the reported low frequency of lymphocyte infiltration in lung tumor tissues (Reynders K and De Ruysscher D, 2016), in the present study we evaluated their expression in lung tissue. Several mechanisms might explain the observed similar expression levels of these genes in tumoral and non-tumoral tissues. First, cytokines have crucial roles in the construction of tumor microenvironment (Alshaker HA and Matalka KZ, 2011). ANCTs might share the tumor microenvironment due to their proximity to tumoral tissues. Such effect may result in similar expression of these genes between tumoral tissues and ANCTs. Moreover, tumor microenvironment has several components including blood vessels, immune cells, fibroblasts, signaling molecules, and the extracellular matrix (Genova C, Rijavec E, Grossi F, 2017). Assessment of expression of genes in certain compartments of this environment might provide clues for their role in the pathogenesis of cancer. Another reason for the observed similar expression levels of genes between the mentioned sample sets is the relative small sample size of the study. Consequently, we suggest further assessment of genes expression in larger samples sizes with specific attention to cell type and inclusion of control samples from normal tissues.

We observed significant pairwise correlations between expression levels of IFNG, IFNG-AS1, TNFA, and GSST1-AS1. Assessment of IFNG and IFNG-AS1 in the Jurkat T cell model has previously indicated the role of IFNG-AS1 as a positive regulator of IFNG expression (Padua D et al., 2016). However, the lncRNA GSTT1-AS1 has been shown to induce a more repressive chromatin configuration in IFNG and TNFA loci leading to suppression of their expression in CD8(+) T cells in the context of tuberculosis infection (Wang Y et al., 2015). The observed positive correlation between expressions of IFNG-AS1, TNFA, and GSST1-AS1 in our current study might show a distinct role for GSST1-AS1 in lung tissues compared with T cells. Such hypothesis should be assessed in future studies.

CONCLUSION

In brief, in the current study we evaluated expression of two lncRNAs with putative role in regulation of immune response in lung tissues and found no difference either in their expression or in the expression of their target genes between tumoral and non-tumoral tissues. Based on the limitations of our study regarding sample size and source of control samples, we suggest future evaluations of their expression in larger sample sizes and with selection of appropriate controls.

DECLARATIONS

Ethics approval and consent to Participant

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent of publication

Not applicable.

Availability of data and materials

The analysed data sets generated during the study are available from the corresponding author on reasonable request.

Competing interest

The authors declare they have no conflict of interest.

Funding

Not applicable.

Authors' contributions

Mohammad Taheri and Soudeh Ghafouri-Fard supervised the study. Farbod Esfandi and Zabih Mir Hassani performed the laboratory assessment. Shahram Arsang-Jang analyzed the data.

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