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Expression of Treg-associated lncRNAs in breast cancer

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Abstract

Regulatory T cells (Tregs) have important functions in tumor microenvironment, particularly for induction of immune evasion. In order to find the underlying mechanism of dysregulation of Tregs in breast cancer tissues, we designed the current study to appraise expression of five Treg-related long non-coding RNAs (lncRNAs), namely FLICR (FOXP3 Regulating Long Intergenic Non-Coding RNA), NEST (IFNG-AS1), RMRP (RNA Component of Mitochondrial RNA Processing Endoribonuclease), MAFTRR (MAF Transcriptional Regulator RNA) and TH2-LCR (Th2 Cytokine Locus Control Region) in paired breast cancer and nearby noncancerous tissues. Expression levels of RMRP, TH2-LCR, MAFTRR and GATA3-AS1 were significantly higher in breast cancer samples compared with non-tumoral tissues. The calculated AUC values for GATA3-AS1, TH2-LCR, RMRP and MAFTRR were 0.66, 0.63, 0.63 and 0.60, respectively. There were significant positive associations between expression level of RMRP gene in tumor tissues and nuclear grade, tubule formation and tumor sizes. In addition, there was a significant positive association between expression levels of MAFTRR genes in tumor tissues and nuclear grade. Besides, expression levels of FLICR were different among tumors with different levels of HER2/neu receptor. Taken together, Treg-associated lncRNAs might contribute to the pathogenesis of breast cancer.

Key words: Breast cancer, regulatory T cell, lncRNA

Introduction

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Breast cancer is a malignancy with high frequency among women [16]. This type of cancer is associated with extensive cellular and molecular heterogeneity and vast numbers of molecular procedures contributing to cell growth, differentiation, proliferation, invasion, and metastasis [3]. A bulk of evidence has highlighted the importance of a subpopulation of T cells, named T regulatory cells (Tregs) in the progression of breast cancer [13, 18]. These cells contribute to the maintenance of tolerance to self-antigens [7]. In fact, Forkhead box P3 (FoxP3)-expressing Tregs have a crucial role in suppression of unwanted immune responses. In comparison to other subpopulations of T cells, Tregs have high reactivity to the selecting ligands in the thymus even following negative selection by the ligands [7]. It has become evident that the host immune responses contribute to the immune surveillance and demolition of cancer cells [14]. The presence of Tregs in the tumor microenvironment influences the immune responses to breast cancer cells and is implicated in the subsequent immunopathogenesis. Tumor-residing Treg cells have been shown to exert potent suppressive effects and their transcript signature resembles that of normal breast tissues, but differs from activated circulatory Tregs [13]. However, expression profile of some cytokine and chemokine receptor genes has been found to be different between tumor-resident and normal tissue residing Tregs [13].

It has also been evident that non-coding RNAs can modulate function and differentiation of Tregs [12]. Aberrant expression of non-coding RNAs is also implicated in the etiology of conditions that are linked with the activation of Tregs. In order to find the underlying mechanism of dysregulation of Tregs in breast cancer tissues, we intended to appraise expression of five Treg-related long non-coding RNAs (lncRNAs), namely FLICR (FOXP3 Regulating Long Intergenic Non-Coding RNA), NEST (IFNG-AS1), RMRP (RNA Component of Mitochondrial RNA Processing Endoribonuclease), MAFTRR (MAF

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Transcriptional Regulator RNA) and TH2-LCR (Th2 Cytokine Locus Control Region) in paired breast cancer and nearby noncancerous tissues.

Materials and methods

Selection of lncRNAs

Treg-related lncRNAs were selected through a literature-based approach as described previously [1, 4].

Patients

A total of 40 patients with approved diagnosis of breast cancer were enlisted in the present study. Expressions of Treg-related lncRNAs were estimated in breast tumors and nearby non-tumoral specimens. Tissue specimens were obtained from Farmanieh and Sina hospitals, Tehran, Iran during 2017-2020. All samples were quickly frozen in liquid nitrogen and transported to Medical Genetic Lab. Then, they were stored in -70º C. The study protocol was approved by the ethical committee of Shahid Beheshti University of Medical Science (IR.SBMU.CRC.REC.1400.045). All participants signed informed consent forms.

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Experiments

Total RNA was retrieved from tissues using the RNJia Kit (ROJE Technologies, Tehran, Iran). Then, 70 ng of total RNA was used for production of cDNA using AddScript cDNA synthesis kit (AddBio, Korea). Expressions of FLICR, MAFTRR, NEST, RMRP and TH2-LCR were enumerated in all samples using Ampliqon master mix (Denmark). Reactions were performed in StepOnePlus Real-Time PCR System. B2M was used as normalizer. Primers sequences are demonstrated in Table 1.

Table 1. Primer sequences and corresponding amplified regions.

Gene	Sequence 5→3	Primer Length (bp)
B2M	F- AGATGAGTATGCCTGCCGTG	20
	R- GCGGCATCTTCAAACCTCCA	20
FLICR	F- GGG CTT TTC CAG AAG GGT CT	20
	R- AGC CCA GGG TTC TAG TCG	18

MAFTRR	F- CTG AAG GGA CAG GAC GGA CAA C	22
	R- GGG GAA AAC CTG GAA AGA GGG A	22
NEST	F- AGC TGA TGA TGG TGG CAA TCT	21
	R- TGA CTT CTC CTC CAG CGT TTT	21
RMRP	F- GTA GAC ATT CCC CGC TTC CCA	21
	R- GAG AAT GAG CCC CGT GTG GTT	21
TH2-LCR	F- GCT CCC CAG GCT TTT GAG ATA	21
	R- TGG TGA TGC TGA AGG GAG AC	20

Statistical methods

GraphPad Prism version 9.0 (La Jolla, CA, USA) was used for this purpose. Expression levels of FLICR, MAFTRR, NEST, RMRP and TH2-LCR were measured in tumor samples and their matching control tissues. Expression of each gene was calculated using the Efficiency adjusted method.

Shapiro-Wilk test was used for appraisal of normal/gaussian distribution of data. Wilcoxon matched-pairs signed rank test was used to identify differentially expressed lncRNAs between tumoral and adjacent normal tissues. Correlations between gene expression levels in both study groups were measured using Spearman's rank correlation coefficient. Mann-Whitney test and Kruskal–Wallis one-way ANOVA were used for comparison of expression levels between different groups of patients. Chi-square test was used to find out the association between clinicopathological factors and genes expression levels. Receiver operating characteristic (ROC) curves were used to evaluate the diagnostic power of transcript levels of differentially expressed genes. P value < 0.05 was considered as significant.

Results

Table 2 shows the information about studied genes.

Table 2. Characteristic features of genes studied in this article.

Name/Gene ID	Accession number	Location	Official Full Name	Gene type
RMRP	NR_003051.3	9p13.3	RNA component of mitochondrial RNA processing endoribonuclease	ncRNA

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TH2-LCR	NR_132124.1, NR_132125.1 , NR_132126.1	5q31.1	T helper type 2 locus control region associated RNA	ncRNA
MAFTRR (LINCMAF4)	NR_104663.1	16q23.2	MAF transcriptional regulator RNA	ncRNA
IFNG-AS1 (NEST)	NR_104124.1, NR_104125.1	12q15	IFNG antisense RNA 1	ncRNA
GATA3-AS1	NR_024256.1, NR_104327.1, NR_104328.1, NR_104329.1, NR_104330.1, NR_104336.1	10p14	GATA3 antisense RNA 1	ncRNA
FLICR	NR_147988.1	Xp11.23	FOXP3 regulating long intergenic non-coding RNA	ncRNA

The study included 40 patients with breast cancer. Clinicopathological features of cases are shown in Table 3.

Table 3. Clinicopathological features of included cases.

case No	Age	stage	Grade	Nuclear Grade	Tubule Formation	Mitotic Rate	Tumor Size (cm)	Menarche Age (year)	Menopause Age (year)	Breastfeeding Duration (month)	First Pregnancy Age (year)	OCP	HRT	Obesity	ER	PR	Her2
1	**38**	ND	ND	2	ND	ND	ND	14	No	24	25	+	-	ND	ND	ND	ND
2	**35**	**1**	ND	ND	ND	ND	ND	12	No	No	No	-	-	2	ND	ND	ND
3	**81**	**1**	ND	1	ND	ND	1.5	13	50	120	16	-	-	3	ND	ND	ND
4	**39**	**2**	2	2	3	1	2.5	11	No	No	No	+	-	2	I	I	-
5	**59**	**1**	1	2	1	1	1.5	14	51	14	31	-	-	2	S	S	+
6	**62**	**3**	3	3	3	2	ND	13	50	No	No	-	-	ND	ND	ND	ND
7	**41**	**4**	2	2	2	2	3	12	No	24	17	-	-	2	W	W	+++
8	**48**	**2**	3	3	3	2	2.5	14	No	72	16	-	+	3	S	S	-
9	**51**	**3**	1	2	ND	ND	3	12	46	48	17	-	-	ND	S	S	-
10	**38**	**2**	ND	ND	ND	ND	7	14	No	72	23	+	-	2	W	W	+++
11	**57**	**0**	ND	ND	ND	ND	2	12	50	52	16	+	-	2	I	ND	ND
12	**46**	**1**	2	ND	ND	ND	ND	14	No	No	No	-	-	ND	I	I	-
13	**57**	**1**	2	2	2	1	2	14	45	48	24	-	+	2	S	S	-
14	**52**	**1**	1	1	2	2	0.5	12	49	No	No	-	+	ND	S	S	+
15	**81**	**2**	1	2	2	1	2.5	ND	ND	60	ND	-	-	ND	ND	ND	ND
16	**49**	**1**	ND	1	ND	ND	2	11	No	No	No	-	-	2	S	S	+

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17	**29**	**1**	2	2	3	2	1.5	13	No	No	No	-	-	2	S	S	-
18	**42**	**1**	2	1	2	2	1.8	18	No	114	22	+	+	1	W	W	-
19	**50**	**3**	3	3	3	3	1	12	48	60	19	+	-	3	I	I	+
20	**43**	**2**	1	2	1	1	2	13	No	50	19	+	+	2	I	I	-
21	**35**	**4**	3	3	3	3	2	14	No	18	24	-	-	2	I	W	+++
22	**48**	**3**	2	2	3	1	2	ND	No	ND	ND	ND	ND	ND	I	I	-
23	**55**	**3**	3	3	3	2	1	12	50	42	15	-	-	3	I	W	++
24	**34**	**2**	2	2	2	2	2	15	No	No	ND	+	+	3	W	W	+++
25	**75**	**2**	3	3	3	2	2	13	55	96	24	+	-	2	I	I	++
26	**77**	**3**	1	2	2	1	1.5	17	60	120	18	-	-	2	S	S	-
27	**48**	**2**	1	1	ND	ND	3	13	No	6	16	-	-	2	S	S	-
28	**36**	**3**	2	2	2	1	1.5	10	No	72	23	-	-	2	S	S	-
29	**34**	**1**	2	2	2	2	2	13	No	24	15	+	-	2	S	S	-
30	**47**	**3**	3	3	ND	ND	2.8	13	No	28	17	+	-	ND	I	W	-
31	**66**	**1**	ND	ND	ND	ND	1.5	12	45	30	24	+	-	2	S	S	-
32	**66**	**3**	3	3	3	2	3	10	55	33	22	+	-	ND	W	W	-
33	**60**	**2**	3	3	3	2	3	12	58	73	24	-	-	2	I	I	++
34	**81**	**3**	2	2	2	2	2	13	47	168	15	-	-	2	I	W	+++
35	**36**	**2**	2	2	3	2	2	14	No	35	23	+	-	3	W	W	+
36	**58**	**0**	2	2	2	2	ND	14	38	84	15	+	-	3	I	I	+++
37	**49**	**3**	1	1	1	1	2	14	No	No	ND	+	+	3	I	I	+
38	**67**	**3**	2	2	3	2	2	14	49	127	17	+	-	2	I	I	-
39	**64**	**2**	2	3	3	1	2	13	53	No	ND	-	+	3	I	I	+
40	**47**	**0**	3	3	3	3	ND	14	No	25	37	+	-	2	W	ND	+++

OCP: Oral contraceptives; HRT: Hormone replacement therapy; ER: Estrogen receptor; PR: Progesterone receptor; ND: Not determined; I: Intermediate; W: Weak; S: Strong.

There was significant difference in expression of all lncRNAs between tumoral and non-tumoral tissues, except for FLICR and NEST whose expression were not different between these two types of tissues (Figure 1).

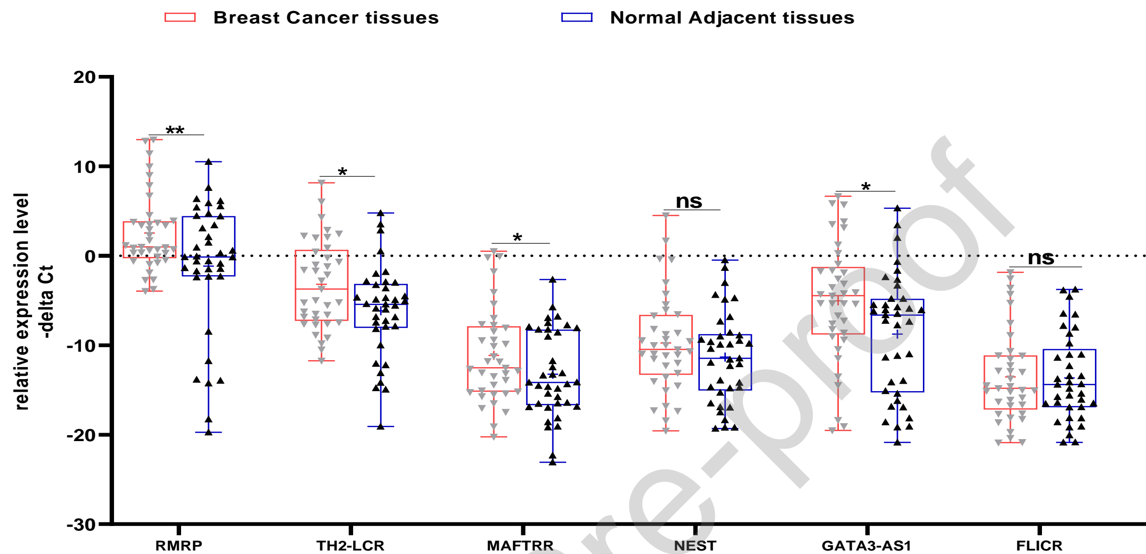

Figure 1. Relative expression levels of six lncRNA genes in breast cancer samples as compared to adjacent normal tissues as described by –delta Ct values (Ct Housekeeping gene- Ct Target gene). Median [line], mean [cross], interquartile range [box], and minimum and maximum values are shown. Data was assessed using the Wilcoxon rank-sum test, and P < 0.05 was considered significant. Asterisks show significant difference between two groups (*P value < 0.05, ** P value < 0.01).

Expression levels of RMRP, TH2-LCR, MAFTRR and GATA3-AS1 were significantly higher in breast cancer samples compared with non-tumoral tissues (Table 4). Expression ratio (95% CI) values for RMRP, TH2-LCR, MAFTRR and GATA3-AS1 are as follow: 12.9 (2.47-67.64), 7.84 (1.8-34), 4.34 (1.26-14.93) and 13.13 (1.85-93), respectively.

Table 4. The results of expression study of six lncRNA genes in tumoral samples compared with control tissues. Expression ratios are shown as mean and 95% confidence interval and SEM.

Studied genes	Expression ratio (95% CI)	SEM	P Value
RMRP	12.9 (2.47-67.64)	1.17	0.0065
TH2-LCR	7.84 (1.8-34)	1.045	0.0073
MAFTRR	4.34 (1.26-14.93)	0.8778	0.0209
NEST	2.98 (0.6-14.68)	1.134	0.1731
GATA3-AS1	13.13 (1.85-93)	1.394	0.0114
FLICR	1.07 (0.25-4.55)	1.025	0.8462

The calculated AUC values for GATA3-AS1, TH2-LCR, RMRP and MAFTRR were 0.66, 0.63, 0.63 and 0.60, respectively (Figure 2 and Table 5).

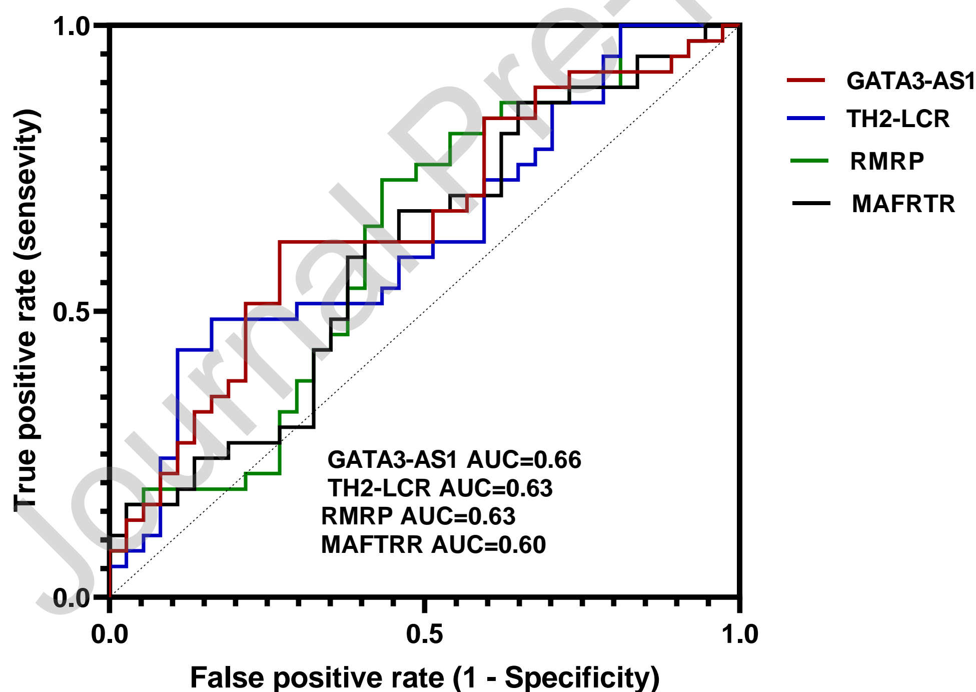

Figure 2. The receiver operating characteristic (ROC) curve of four differentially expressed lncRNAs genes for discrimination of breast tumors from adjacent normal tissues. AUC indicates area under the ROC curve.

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Table 5. The results of ROC curve analysis for six differentially expressed lncRNA genes for discrimination of breast tumors from adjacent normal tissues.

GATA3-AS1				TH2-LCR				RMRP				MAFTRR			
AUC ±SD	Sensitivity	Specificity	P Value	AUC ±SD	Sensitivity	Specificity	P Value	AUC ±SD	Sensitivity	Specificity	P Value	AUC± SD	Sensitivity	Specificity	P Value
0.65± 0.06	0.62	0.73	0.0204	0.63± 0.06	0.48	0.83	0.0461	0.63± 0.06	0.72	0.56	0.06	0.6 ±0.06	0.86	0.35	0.12

Significant correlations were found between almost all supposed pairs of lncRNAs in breast cancer tissues as well as non-cancerous tissues (Table 6). The strongest correlation was between GATA3-AS1 and TH2-LCR in tumor tissues (correlation coefficient= 0.89).

Table 6. Spearman's correlations between lncRNA expression levels among the breast tissue tumors (*N*= 38) and adjacent normal tissues (*N*= 38).

	TH2-LCR		MAFTRR		NEST		GATA3-AS1		FLICR	
	adjacent	Tumor	adjacent	Tumor	adjacent	Tumor	adjacent	Tumor	adjacent	Tumor
RMRP	0.52**	0.74**	0.67**	0.72**	0.24	0.59**	0.60**	0.77**	0.44*	0.59**
TH2-LCR			0.45*	0.52**	0.33*	0.44*	0.78**	0.89**	0.31	0.42*
MAFTRR					0.38*	0.53**	0.47*	0.50*	0.50*	0.75**
NEST							0.26	0.53**	0.38*	0.63**
GATA3-AS1									0.39*	0.46*

* $p < 0.05$
** $p < 0.001$

There were significant positive associations between expression level of RMRP gene in tumor tissues and nuclear grade, tubule formation and tumor sizes. In addition, there was a significant positive association between expression levels of MAFTRR genes in tumor tissues and nuclear grade. Besides, expression levels of FLICR were different among tumors with different levels of HER2/neu receptor (Table 7).

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Additionally, there were significant positive associations between mitotic rates and histological grades (χ^2 =11.25, p value= 0.004), nuclear grades (χ^2 =7.2, p value= 0.027) and tubule formation (χ^2 =4.74, p value= 0.029). Also, there was a negative association between mitotic rates and progesterone receptor status (χ^2 =7.1, p value= 0.029). OCP taking was negatively associated with estrogen receptor status (χ^2 =10.21, p value= 0.006) and progesterone receptor status (χ^2 =8.58, p value= 0.014) (Table 7).

Table 7. Expression levels of lncRNAs in tumor tissues based on clinicopathological factors. Mann-Whitney and Kruskal–Wallis one-way ANOVA tests were used for comparison of gene expression levels between different groups.

Parameters	Subclasses	Number of patients (%)	Relative expression level of RMRP (mean±SD)	P-value	Relative expression level of TH2-LCR (mean±SD)	P-value	Relative expression level of MAFTRR (mean±SD)	P-value	Relative expression level of NEST (mean±SD)	P-value	Relative expression level of GATA3-AS1 (mean±SD)	P-value	Relative expression level of FLICR (mean±SD)	P-value
Age	29-42	12 (32.43%)	1.4±1.12	0.34	-4.8±1.28	0.39	-12.42±1.6	0.47	-10.33±1.8	0.98	-8.6±2.2	0.16	-14.6±1.44	0.71
	43-60	17 (45.94%)	2.44±1.12		-2.28±1.25		-11.18±1.18		-9.6±1.21		-3.62±1.41		-13.28±1.16	
	61-84	8 (21.62%)	4.32±1.57		-2.64±1.81		-9.05±2.05		-9.18±2.15		-2.73±1.91		-12.4±2.2	
Clinical stage	I	14 (37.83%)	1.7±1.36	0.32	-3.46±1.38	0.59	-11.2±1.6	0.91	-8.98±1.69	0.74	-6.15±1.77	0.38	-13.12±1.5	0.98
	II	10 (27.02%)	2.8±1.3		-3.88±1.38		-10.9±1.57		-9.55±1.6		-4.98±1.98		-13.3±1.9	
	III & IV	12 (32.43%)	3.37±1.16		-1.91±1.54		-10.91±1.54		-10.17±1.44		-2.64±1.73		-13.63±1.07	

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Histological tumor grade	Low grade	8 (21.62%)	1.15 ±1.5	0.105	-3.39 ±2.06	0.409	-11.6 ±1.3	0.086	-12.26±0.89	0.25	-5.62 ±2.23	0.31	-15.7 ±1.22	0.18
	Moderate	13 (35.13%)	1.26 ±1.07		-4.54 ±1.24		-13.06±1.4		-9.36 ±1.52		-6.52 ±1.91		-14.2 ±1.18	
	High grade	9 (24.32%)	4.64 ±1.41		-2.1± 1.64		-8.21 ±1.73		-9.3± 1.71		-2.49 ±1.96		-11.2 ±1.74	
Nuclear Grade	Low grade	6 (16.21%)	-0.75 ±0.69	**0.019**	-4.92 ±1.23	0.33	-14.4 ±1.35	**0.04**	-13.06±1.48	0.56	-8.29 ±1.74	0.18	-15.4 ±1.2	0.11
	Moderate	17 (45.94%)	1.75 ±0.98		-4.03 ±1.31		-12.36±1.14		-10.6 ±1.24		-6.24 ±1.85		-15.3 ±1.07	
	High grade	9 (24.32%)	4.6± 1.41		-2.1± 1.64		-8.21 ±1.73		-9.3± 1.7		-2.49 ±1.96		-11.2 ±1.74	
Tubule Formation	10-75% in tubule form	13 (35.13%)	0.54 ±1.1	**0.023**	-4.81 ±1.18	0.207	-11.9 ±1.4	0.59	-10.8 ±1.48	0.46	-6.91 ±1.64	0.17	-14.65±1.32	0.409
	<10% in tubule form	14 (37.83%)	3.47 ±1.06		-2.8± 1.3		-10.58±1.46		-9.65 ±1.28		-3.83 ±1.78		-13.04±1.32	
Tumor Size (cm)	<2 cm	22 (59.45%)	1.86 ±1.07	**0.045**	-4.06 ±1.03	0.408	-11.76±1.3	0.117	-10.06±1.37	0.4	-6±1.51	0.25	-13.78±1.23	0.29
	=>2 cm	9 (24.32%)	3.53 ±0.93		-2.16 ±1.78		-10.37±0.91		-9.01 ±1.15		-3.01 ±1.46		-12.8 ±1.15	
Mitotic Rate	Slowest	9 (24.32%)	2.05 ±1.41	0.99	-3.71 ±1.54	0.87	-11.65±1.75	0.57	-9.49 ±1.71	0.57	-4.04 ±1.65	0.64	-15.47±1.63	0.08
	Moderate & quickest	18 (48.64%)	2.06 ±1		-3.79 ±1.12		-11.02±1.25		-10.56±1.19		-5.95 ±1.66		-12.99±1.11	
ER (Estrogen receptor)	Negative or weak	7 (18.91%)	2.57 ±1.28	0.61	-3.87 ±1.63	0.77	-10.7 ±2.11	0.9	-9.21 ±2.04	0.95	-6.03 ±2.42	0.61	-12.09±1.73	0.73
	intermediate	13 (35.13%)	3.26 ±1.29		-2.77 ±1.17		-10.8 ±1.51		-8.65 ±1.65		-3.22 ±1.54		-12.7 ±1.58	
	strong	12 (32.43%)	1.98 ±1.55		-3.49		-11.0		-9.58		-6.05		-13.3	

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					±1.82		8±1.65		±1.61		±2.2		8±1.47	
PR (Progesterone receptor)	Negative or weak intermediate strong	8 (21.62%) 10 (27.02%) 12 (32.43%)	1.5±0.62 2.63±1.3 1.98±1.5	0.76	-5.1±1.12 -3.06±1.13 -3.49±1.82	0.61	-11.84±1.38 -11.91±1.57 -11.07±1.65	0.77	-11.01±1.6 -9.04±1.51 -9.58±1.61	0.63	-7.78±2 -2.91±1.4 -6±2.2	0.3	-13.38±1.15 -13.6±1.75 -13.38±1.47	0.78
HER-2 neu receptor	Negative 1+ 2 & 3+	16 (43.24%) 6 (16.21%) 9 (24.32%)	2.26±1.11 -0.38±0.98 4.13±1.4	0.068	-3.8±1.39 -4.68±1.59 -2.46±1.26	0.54	-11.8±1.26 -14.12±1.81 -8.29±1.53	0.059	-8.9±1.19 -14.12±1.43 -7.68±1.62	0.054	-4.98±1.62 -8.93±2.77 -3.27±1.77	0.38	-13.94±1.17 -16.59±1.19 -9.59±1.38	**0.005**
KI67 percentage score	Low Moderate High	15 (24.32%) 6 (16.21%) 6 (16.21%)	2.38±1.2 0.35±1.27 1.87±1.08	0.61	-3.96±0.95 -5.2±2.33 -2.77±1.88	0.63	-10.63±1.52 -14.85±1.43 -11.5±0.8	0.15	-9.95±1.6 -11.43±1.65 -7.32±1	0.21	-5.71±1.69 -7.23±3.1 -4.47±1.56	0.76	-12.37±1.53 -16.19±1.4 -13.7±1.1	0.39

Discussion

Tregs have immunosuppressive properties and act in favor of progression of breast cancer [2, 21]. Thus, identification of the mechanism of dysregulation of this subpopulation of T cells has practical significance in the management of this kind of cancer. The present study aimed at evaluation of expression of five Treg-related lncRNAs in breast cancer tissues. Expression levels of RMRP, TH2-LCR, MAFTRR and GATA3-AS1 were significantly higher in breast tumors compared with non-tumoral tissues.

The impact of RMRP in the regulation of Th17 cell effector function has been verified previously [8]. Since Th17 and Treg cells have equivalent developmental requirements, this

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lncRNA has a putative effect in the regulation of Treg functions. Besides, RMRP has a sponging effect on miR-206 [17], a miRNA that modulates ratio of T17 cells to Tregs [19] and its expression in T cells is a putative marker for Th17-type immune response [6]. More importantly, RMRP has been shown to promote AKT-dependent growth and migratory aptitude of breast cancer through sponging miR-206 [9]. Moreover, miR-206 has been found to be under-expressed in estrogen receptor-positive breast cancer samples compared with their paired non-cancerous tissues [15]. TH2-LCR is involved in the modulation of production of Th2 cytokines [10].

There was no significant difference in expression levels of FLICR and NEST between two sets of samples. FLICR can modulate expression of Foxp3 and assist in development of a subpopulation of Tregs with low expression of FoxP3 [20]. NEST can affect methylation of the *IFN-G* locus and modulate expression of IFN-γ [5]. Besides, NEST can decrease Th1-stimulated proliferation of Treg cells [11]. Thus, in spite of functional relation between these two lncRNAs and Tregs, they are not related with the pathogenesis of breast cancer.

The calculated AUC values for GATA3-AS1, TH2-LCR, RMRP and MAFTRR were 0.66, 0.63, 0.63 and 0.60, respectively. Therefore, none of them is an ideal marker for separation of breast cancer tissues from non-cancerous tissues. However, it is possible that combination of expression levels of these transcripts with other markers increases the AUC values and diagnostic power of putative diagnostic panels.

There were significant positive associations between expression level of RMRP gene in tumor tissues and nuclear grade, tubule formation and tumor sizes. In addition, there was a significant positive association between expression levels of MAFTRR genes in tumor tissues and nuclear grade. Besides, expression levels of FLICR were different among tumors with different levels of HER2/neu receptor. Therefore, these three lncRNAs are functionally

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related with tumor characteristics. Taken together, Treg-associated lncRNAs might contribute to the pathogenesis of breast cancer. However, our study has a limitation, since we did not perform functional studies.

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. 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 (IR.SBMU.RETECH.REC.1400.045). All methods were performed in accordance with the relevant guidelines and regulations.

Consent of publication

Not applicable

Availability of Data and Materials

The analyzed 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.

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Authors' contributions

SGF wrote the draft and revised it. MT and EJ designed and supervised the study. BMH, LMR, MD, MFR and AG collected the data and designed the figures and tables. SE analyzed the data. All the authors read the submitted version and approved it.

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References

[1] M. Akbari, R. Eghtedarian, B.M. Hussen, S. Eslami, M. Taheri, S.M. Neishabouri, S. Ghafouri-Fard, Assessment of Expression of Regulatory T Cell Differentiation Genes in Autism Spectrum Disorder. Front Mol Neurosci 15 (2022) 939224.

[2] R. Allaoui, C. Hagerling, E. Desmond, C.-F. Warfvinge, K. Jirström, K. Leandersson, Infiltration of γ δ T cells, IL-17+ T cells and FoxP3+ T cells in human breast cancer. Cancer Biomarkers 20 (2017) 395-409.

[3] M. Beckmann, D. Niederacher, H.-G. Schnürch, B.A. Gusterson, H.G. Bender, Multistep carcinogenesis of breast cancer and tumour heterogeneity. Journal of molecular medicine 75 (1997) 429-439.

[4] M. Dadyar, B.M. Hussen, S. Eslami, M. Taheri, F. Emadi, S. Ghafouri-Fard, A. Sayad, Expression of T cell-related lncRNAs in multiple sclerosis. Front Genet 13 (2022) 967157.

[5] J.A. Gomez, O.L. Wapinski, Y.W. Yang, J.-F. Bureau, S. Gopinath, D.M. Monack, H.Y. Chang, M. Brahic, K. Kirkegaard, The NeST long ncRNA controls microbial susceptibility and epigenetic activation of the interferon-γ locus. Cell 152 (2013) 743-754.

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[6] J.D. Haas, K. Nistala, F. Petermann, N. Saran, V. Chennupati, S. Schmitz, T. Korn, L.R. Wedderburn, R. Förster, A. Krueger, I. Prinz, Expression of miRNAs miR-133b and miR-206 in the Il17a/f locus is co-regulated with IL-17 production in αβ and γδ T cells. PloS one 6 (2011) e20171.

[7] S. Hori, T. Nomura, S. Sakaguchi, Control of regulatory T cell development by the transcription factor Foxp3. Science 299 (2003) 1057-1061.

[8] W. Huang, B. Thomas, R.A. Flynn, S.J. Gavzy, L. Wu, S.V. Kim, J.A. Hall, E.R. Miraldi, C.P. Ng, F. Rigo, DDX5 and its associated lncRNA Rmrp modulate TH17 cell effector functions. Nature 528 (2015) 517-522.

[9] Y. Huang, B. Xie, M. Cao, H. Lu, X. Wu, Q. Hao, X. Zhou, LncRNA RNA component of mitochondrial RNA-processing endoribonuclease promotes AKT-dependent breast cancer growth and migration by trapping microRNA-206. Frontiers in cell and developmental biology (2021) 2591.

[10] B.H. Koh, S.S. Hwang, J.Y. Kim, W. Lee, M.-J. Kang, C.G. Lee, J.-W. Park, R.A. Flavell, G.R. Lee, Th2 LCR is essential for regulation of Th2 cytokine genes and for pathogenesis of allergic asthma. Proceedings of the National Academy of Sciences 107 (2010) 10614-10619.

[11] M. Luo, X. Liu, H. Meng, L. Xu, Y. Li, Z. Li, C. Liu, Y.-B. Luo, B. Hu, Y. Xue, IFNA-AS1 regulates CD4+ T cell activation in myasthenia gravis though HLA-DRB1. Clinical Immunology 183 (2017) 121-131.

[12] Y. Luo, H. Wang, Effects of Non-Coding RNA on Regulatory T Cells and Implications for Treatment of Immunological Diseases. Frontiers in immunology 11 (2020) 612060-612060.

Journal Pre-proof

[13] G. Plitas, C. Konopacki, K. Wu, P.D. Bos, M. Morrow, E.V. Putintseva, D.M. Chudakov, A.Y. Rudensky, Regulatory T Cells Exhibit Distinct Features in Human Breast Cancer. Immunity 45 (2016) 1122-1134.

[14] S.A. Rosenberg, Progress in human tumour immunology and immunotherapy. Nature 411 (2001) 380-384.

[15] M. Seifi-Alan, A. Dianatpour, L. Geranpayeh, R. Mirfakhraie, M.D. Omrani, S. Ghafouri-Fard, Expression analysis of selected miR-206 targets from the transforming growth factor-β signaling pathway in breast cancer. Journal of cellular biochemistry 120 (2019) 13545-13553.

[16] R.L. Siegel, K.D. Miller, H.E. Fuchs, A. Jemal, Cancer statistics, 2022. CA: a cancer journal for clinicians 72 (2022) 7-33.

[17] X. Wang, L. Peng, X. Gong, X. Zhang, R. Sun, J. Du, LncRNA-RMRP promotes nucleus pulposus cell proliferation through regulating miR-206 expression. Journal of cellular and molecular medicine 22 (2018) 5468-5476.

[18] M.A.E. Watanabe, J.M.M. Oda, M.K. Amarante, J. Cesar Voltarelli, Regulatory T cells and breast cancer: implications for immunopathogenesis. Cancer and Metastasis Reviews 29 (2010) 569-579.

[19] X. Ye, Q. Lu, A. Yang, J. Rao, W. Xie, C. He, W. Wang, H. Li, Z. Zhang, MiR-206 regulates the Th17/Treg ratio during osteoarthritis. Molecular Medicine 27 (2021) 1-9.

[20] D. Zemmour, A. Pratama, S.M. Loughhead, D. Mathis, C. Benoist, Flicr, a long noncoding RNA, modulates Foxp3 expression and autoimmunity. Proceedings of the National Academy of Sciences 114 (2017) E3472-E3480.

[21] Y. Zhou, N. Shao, N. Aierken, C. Xie, R. Ye, X. Qian, Z. Hu, J. Zhang, Y. Lin, Prognostic value of tumor-infiltrating Foxp3+ regulatory T cells in patients with breast cancer: a meta-analysis. Journal of Cancer 8 (2017) 4098.

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Authors' contributions

SGF wrote the draft and revised it. MT and EJ designed and supervised the study. BMH, LMR, MD, MFR and AG collected the data and designed the figures and tables. SE analyzed the data. All the authors read the submitted version and approved it.

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