

Down-regulation of MEG3, PANDA and CASC2 as p53-related lncRNAs in breast cancer

Soudeh Ghafouri-Fard^a, Behnoush Sohrabi^b, Bashdar Mahmud Hussien^c, Elham Mehravaran^d, Elena Jamali^e, Shahram Arsang-Jang^f, Mohadeseh Fathi^g, Mohammad Taheri^{h,*} and Majid Samsami^{i,*}

^aDepartment of Medical Genetics, Shahid Beheshti University of Medical Sciences, Tehran, Iran

^bDepartment of Biology, Faculty of Sciences, Arak University, Arak, Iran

^cDepartment of Pharmacognosy, College of Pharmacy, Hawler Medical University, Kurdistan Region, Iraq

^dMotamed Cancer Institute, Tehran University of Medical Sciences, Tehran, Iran

^eDepartment of Pathology, Loghman Hakim Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran

^fCancer Gene Therapy Research Center, Zanjan University of Medical Science, Zanjan, Iran

^gMen's Health and Reproductive Health Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

^hSkull Base Research Center, Loghman Hakim Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran

ⁱCancer Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Abstract. *TP53* encodes a major tumor suppressor protein which blocks carcinogenesis process in a variety of tissues including breast tissue. Expression and function of this gene is regulated by a number of long non-coding RNAs (lncRNAs) among them are *PANDA*, *MEG3* and *CASC2*. We measured expression of *TP53* and these transcripts in a cohort of Iranian breast cancer patients. Expression levels of *TP53*, *MEG3*, *CASC2* and *PANDA* were significantly lower in tumoral samples compared with non-tumoral samples (Posterior mean differences = -4.26, -1.66, -5.98 and -3.13, respectively; *P* values < 0.0001). Expression of *CASC2* was higher in Her2 1+ cases compared with Her2 negative cases (Beta = 1.85, *P* value = 0.037). Expression levels of *MEG3* and *TP53* were lower in grade 2 samples compared with grade 1 (Beta = -1.86, *P* value = 0.006 and Beta = -2.24, *P* value = 0.003, respectively). There was no other significant association between expression of genes and clinical variables. *CASC2* had the best performance among these genes with area under curve value of 0.78 and sensitivity and specificity values of 56.33% and 88.73%, respectively (*P* value < 0.0001). The current investigation supports the role of *TP53*-related lncRNAs in the pathogenesis of breast cancer.

Keywords: *TP53*, lncRNA, *PANDA*, *MEG3*, *CASC2*

1. Introduction

Being located on chromosome 17p, *TP53* gene encodes a nuclear protein that hinders proliferation

of genetically altered cells [1]. This tumor suppressor protein regulates genomic stability by governing cell cycle progression and prompting apoptosis when DNA damage is beyond the repair capacity of the cell [2]. Although *TP53* mutations have been detected in minority of breast cancers, changes in levels of interacting molecules or target genes have been shown to decrease the capacity of this protein in management of stress events [2]. Notably, epigenetic alterations in p53 transcriptional targets of p53 are additional mechanisms for modulation of p53 function

*Corresponding authors: Mohammad Taheri, Skull Base Research Center, Loghman Hakim Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran. E-mail: mohammad_823@yahoo.com. Majid Samsami, Cancer Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran. E-mail: samsamimd@gmail.com.

in breast cancers that do not harbor *TP53* mutations [3]. Moreover, mutations in this gene predict poor survival of breast cancer patients [3]. Recent studies have shown interaction between this tumor suppressor gene and a number of long non-coding RNAs (lncRNAs). For instance, *P21-associated non-coding RNA DNA damage-activated (PANDA)* is an lncRNA whose expression is activated following DNA damage [4]. This lncRNA suppresses apoptosis by inhibiting the function of nuclear transcription factor Y. Moreover, *PANDA* has been shown to stabilize p53 protein in response to DNA damage [5]. *Maternally expressed 3 (MEG3)* has been shown to enhance p53 protein levels and induce p53-dependent transcription from a p53-responsive promoter. Moreover, *MEG3* down-regulates MDM2 levels to enhance p53 stability [6]. Finally, lncRNA *cancer susceptibility candidate 2 (CASC2)* is a tumor suppressor lncRNA that down-regulates CDK1 and BCL2 [7,8]. Both CDK1 and BCL2 have suppressive effects on p53 function [9,10]. Therefore, *CASC2* indirectly affects p53-related pathways. Forced up-regulation of *CASC2* in thyroid cancer tissues has suppressed proliferation of these cells and attenuated their migratory potential and invasiveness. Besides, this lncRNA can suppress epithelial-mesenchymal transition through enhancing E-cadherin levels and decreasing expressions of ZEB1 and N-cadherin [11]. In the current study, we compared transcript levels of *TP53*, *MEG3*, *CASC2* and *PANDA* in breast cancer tissues versus non-tumoral tissues to identify their relevance with breast carcinogenesis.

2. Material and methods

2.1. Patients

Expressions of *TP53* and related lncRNAs were quantified in 69 pairs of tissues including both tumoral and non-tumoral tissues. Patients recruited for this study were hospitalized in Farmanieh and Sina hospitals, Tehran, Iran during 2017–2020. The study protocol was approved by the ethical committee of Shahid Beheshti University of Medical Science and all methods were performed in accordance with the relevant guidelines and regulations (IR.SBMU.RETECH.REC.1400.087). All enrolled patients signed informed consent forms. Specimens were gathered during surgery and prior to any chemotherapy or radiotherapy. Medical records were collected to obtain pathological and clinical data.

Table 1
Primer sequences

Gene		Primer sequence
<i>B2M</i>	Forward	5'-AGATGAGTATGCCTGCCGTG-3'
	Reverse	5'-CGGCATCTTCAAACCTCCA-3'
<i>CASC2</i>	Forward	5'-CAAGAAACTTCCCAAGGTATC-3'
	Reverse	5'-CATGCAACACACTTCTTACTCG-3'
<i>MEG3</i>	Forward	5'-TGGCATAGAGGAGGTGAT-3'
	Reverse	5'-GGAGTGCTGTTGGAGAATA-3'
<i>PANDA</i>	Forward	5'-GTTTTCTGTTCGTCGATTCTGG-3'
	Reverse	5'-GGAAAGCTGAGAGAGACTTTGAAC-3'
<i>TP53</i>	Forward	5'-CTGTCATCTTCTGTCCCTTC-3'
	Reverse	5'-TGAATCAACCCACAGCTGCA-3'

2.2. Experiments

All obtained tissues were subjected to RNA extraction using the RiboEx kit (GeneAll, Seoul, South Korea). After that, 75–100 ng of RNA was converted to cDNA using the ExcelRT™ Reverse Transcription Kit II (SMOBIO, Taiwan). Expressions of *TP53* and related lncRNAs were measured in all paired samples in the ABI step one plus PCR machine. Expression levels were normalized to transcripts of *B2M*. Reactions were prepared using RealQ Plus 2x PCR Master Mix (Ampliqon, Odense, Denmark). Forward and reverse primers were designed using Primer3 software (Table 1).

2.3. Statistical methods

Bayesian estimation supersedes the *t*-test (BEST) was used to test the significance of difference in means of genes expression levels between tumoral and non-tumoral samples. A *t* student prior family was assumed for parameters with 5000 iterations and 2000 burn-outs. *P* values were estimated using frequentist methods including median test. The Spearman correlation coefficients were calculated to assess the association between expression levels of genes. These analyses were performed using BEST, RJags, and ggplot2 packages in the R 4.0.3 software.

3. Results

Expression assays have been conducted on the same cohort of breast cancer patients as our previous study [12]. Detailed demographic and clinical data of

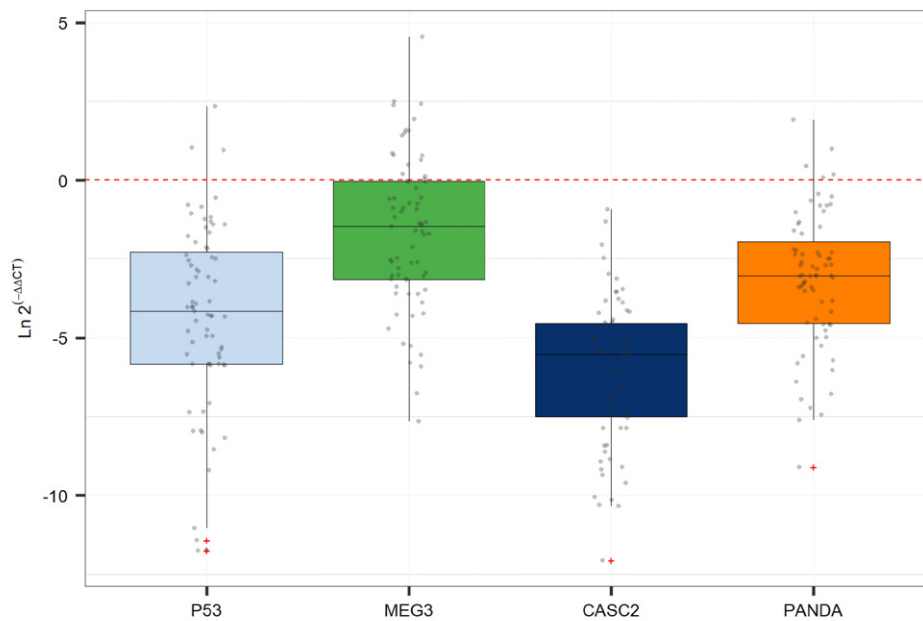


Fig. 1. Expression levels *TP53*, *MEG3*, *CASC2* and *PANDA* in tumoral tissues versus non-tumoral tissues as calculated by $\text{Ln } 2^{(-\Delta\Delta\text{Ct})}$. The data is expressed relative to the matched healthy tissue.

Table 2

The comparison of relative expression of genes between tumoral and non-tumoral samples (Results of Bayesian estimation supersedes *t*-test)

Gene	Posterior mean difference	SD	Effect size	<i>P</i> -value	95% HDI
<i>TP53</i>	-4.26	2.46	-1.789	<0.0001	[-5.02, -3.5]
<i>MEG3</i>	-1.666	2.36	-0.711	<0.0001	[-2.24, -1.1]
<i>CASC2</i>	-5.893	2.1	-2.856	<0.0001	[-6.47, -5.31]
<i>PANDA</i>	-3.13	2.08	-1.519	<0.0001	[-3.64, -2.62]

Abbreviations; SD: Standard deviation. HDI: highest density interval.

these patients have been reported formerly [12]. Figure 1 shows expression levels *TP53* and related lncRNAs in breast cancer tissues versus non-tumoral tissues as calculated by $\text{Ln } 2^{(-\Delta\Delta\text{Ct})}$.

Expression levels of *TP53*, *MEG3*, *CASC2* and *PANDA* were significantly lower in tumoral samples compared with non-tumoral samples (Posterior mean differences = -4.26, -1.66, -5.98 and -3.13, respectively; *P* values < 0.0001) (Table 2).

Expression of *CASC2* was higher in Her2 1+ cases compared with Her2 negative cases (Beta = 1.85, *P* value = 0.037). Yet, expression of this gene was similar between Her2 2+ or Her2 3+ cases compared with Her2 negative ones. Expression levels of *MEG3* and *TP53* were lower in grade 2 samples compared with grade 1 (Beta = -1.86, *P* value = 0.006 and Beta = -2.24, *P* value = 0.003, respectively). There was no other

significant association between expression of genes and clinical variables (Table 3).

While expression of none of mentioned genes was correlated with patients' age, there were significant correlations between all pairs of genes with correlation coefficients ranging from 0.66 (*MEG3/PANDA*) to 0.37 (*MEG3/CASC2*) (Fig. 2).

Finally, the diagnostic value of *TP53*, *PANDA*, *MEG3* and *CASC2* in differentiation between tumoral and non-tumoral tissues was evaluated through plotting receiver operating characteristic (ROC) curves (Fig. 3). *CASC2* had the best performance among these genes with area under curve (AUC) value of 0.78 and sensitivity and specificity values of 56.33% and 88.73%, respectively (*P* value < 0.0001). *TP53* with AUC value of 0.62 ranked second in this regard.

Table 3
Association between expression levels of TP53, PANDA, MEG3 and CASC2 genes and clinical data

Variable	CASC2			MEG3			PANDA			TP53		
	Beta	CI	P-value	Beta	CI	P-value	Beta	CI	P-value	Beta	CI	P-value
Abortion (yes/no)	-1.937	[-4.51, 0.63]	0.137	-0.142	[-2.88, 2.6]	0.918	-1.4	[-3.78, 0.98]	0.245	-0.434	[-4.89, 4.02]	0.846
Age (year)	0.041	[-0.02, 0.1]	0.18	0.032	[-0.04, 0.1]	0.361	0.007	[-0.06, 0.07]	0.834	-0.017	[-0.09, 0.06]	0.649
Breast feeding duration	0.001	[-0.01, 0.02]	0.859	-0.003	[-0.02, 0.02]	0.793	-0.003	[-0.02, 0.01]	0.703	-0.006	[-0.02, 0.01]	0.484
ER (Positive/negative)	-0.018	[-0.04, 0.01]	0.184	0.004	[-0.02, 0.02]	0.726	-0.004	[-0.02, 0.02]	0.724	0.018	[-0.03, 0.07]	0.477
PR (positive/negative)	-0.014	[-0.04, 0.02]	0.365	0.003	[-0.02, 0.02]	0.794	0.001	[-0.02, 0.03]	0.956	0.023	[-0.02, 0.07]	0.3
First pregnancy age	-0.002	[-0.21, 0.21]	0.984	-0.034	[-0.28, 0.21]	0.783	-0.04	[-0.19, 0.11]	0.586	0.017	[-0.3, 0.33]	0.914
Her2 (1+/negative)	1.845	[0.11, 3.58]	0.037	0.164	[-2.19, 2.52]	0.889	0.784	[-0.8, 2.36]	0.325	0.802	[-2.69, 4.29]	0.647
Her2 (2+/negative)	-0.847	[-2.91, 1.21]	0.413	0.263	[-1.71, 2.23]	0.79	-0.369	[-2.09, 1.35]	0.669	-0.428	[-2.98, 2.13]	0.738
Her2 (3+/negative)	-0.151	[-2.48, 2.18]	0.897	-1.019	[-5.46, 3.42]	0.647	0.539	[-1.74, 2.82]	0.638	-1.079	[-5.34, 3.18]	0.613
Histological Grade (2/1)	-0.62	[-2.16, 0.93]	0.426	-1.862	[-3.17, -0.55]	0.006	-0.62	[-2.16, 0.93]	0.426	-2.244	[-3.68, -0.81]	0.003
Histological Grade (3/1)	-0.365	[-2.07, 1.34]	0.669	0.172	[-1.28, 1.62]	0.813	-0.365	[-2.07, 1.34]	0.669	-0.36	[-3.29, 2.58]	0.807
HRT (yes/no)	-1.837	[-4.21, 0.54]	0.128	0.164	[-2.34, 2.67]	0.896	-0.555	[-2.47, 1.36]	0.563	1.192	[-2.05, 4.44]	0.465
KI67 (positive/negative)	-1.156	[-3.55, 1.23]	0.336	-1.135	[-2.64, 0.37]	0.137	-0.505	[-1.71, 0.7]	0.403	1.806	[-0.88, 4.49]	0.183
Laterality (Bilateral/unilateral)	0.644	[-1.04, 2.32]	0.447	-0.267	[-1.87, 1.33]	0.74	-0.067	[-1.08, 0.94]	0.894	-1.017	[-3.15, 1.12]	0.345
Lymph node involvement (yes/no)	-1.672	[-3.9, 0.56]	0.136	-0.263	[-3.82, 3.29]	0.881	0.074	[-1.94, 2.08]	0.941	-0.831	[-5.65, 3.99]	0.727
Menopause Age	0.067	[-0.18, 0.32]	0.587	0.05	[-0.24, 0.34]	0.724	0.046	[-0.21, 0.3]	0.715	0.098	[-0.31, 0.51]	0.627
Obesity (yes/no)	0.448	[-1.65, 2.54]	0.67	1.216	[-0.36, 2.79]	0.128	1.098	[-0.4, 2.6]	0.147	-0.888	[-2.68, 0.9]	0.323
OCP (yes/no)	0.155	[-1.21, 1.52]	0.821	-0.164	[-1.79, 1.46]	0.84	0.02	[-1.25, 1.29]	0.975	0.224	[-1.98, 2.42]	0.84
Parity 1/0	0.94	[-3.27, 5.15]	0.656	2.534	[-1.07, 6.14]	0.164	2.717	[-1.47, 6.91]	0.199	5.291	[-11.18, 21.76]	0.523
Parity 2/0	2.513	[-0.62, 5.64]	0.113	0.107	[-3.29, 3.51]	0.95	2.52	[-1.45, 6.49]	0.209	0.976	[-4, 5.95]	0.696
Parity 3/0	0.702	[-2.39, 3.79]	0.651	-0.858	[-3.82, 2.11]	0.565	1.75	[-2.1, 5.6]	0.367	-0.058	[-4.91, 4.79]	0.981
Parity 4/0	3.023	[-0.5, 6.55]	0.092	0.842	[-2.26, 3.95]	0.59	2.255	[-1.65, 6.16]	0.252	1.195	[-9.21, 11.6]	0.819
Parity 5/0	2.035	[-1.53, 5.6]	0.258	0.135	[-3.51, 3.78]	0.941	1.947	[-1.81, 5.7]	0.304	-0.498	[-5.09, 4.09]	0.829
Stage (2 versus 1)	0.305	[-2.5, 3.11]	0.828	0.033	[-1.89, 1.96]	0.973	0.473	[-1.48, 2.43]	0.63	-1.178	[-3.23, 0.87]	0.256
Stage (3 versus 1)	1.442	[-0.8, 3.68]	0.203	1.13	[-0.83, 3.09]	0.254	1.5	[-0.43, 3.43]	0.126	-0.565	[-3.19, 2.06]	0.669
Tumor size (between 2-5 cm/less than 2 cm)	0.024	[-2.43, 2.48]	0.985	0.17	[-2.08, 2.42]	0.88	1.164	[-0.82, 3.15]	0.245	-0.008	[-3.34, 3.32]	0.996
Tumor size (Larger than 5 cm/less than 2 cm)	-1.215	[-3.85, 1.42]	0.36	-0.074	[-1.99, 1.84]	0.939	0.801	[-1.22, 2.82]	0.431	0.96	[-2.11, 4.03]	0.534

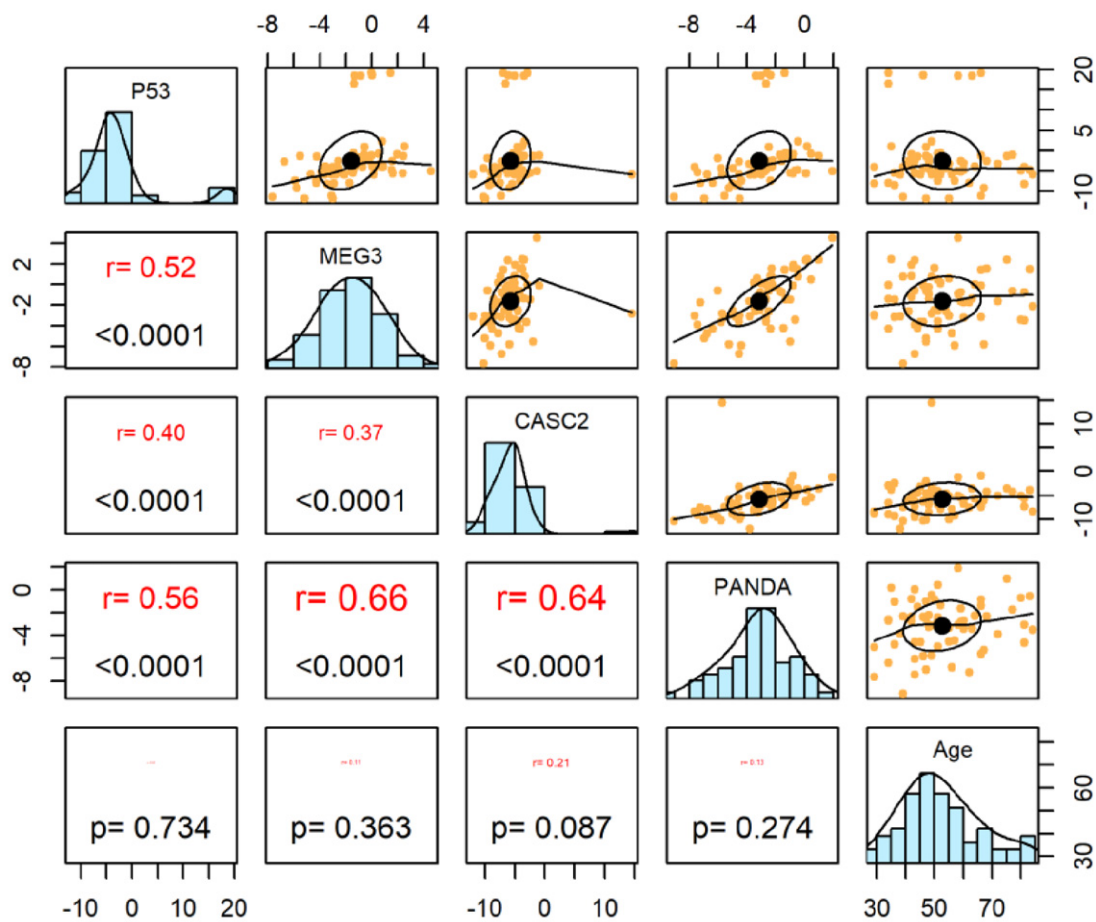


Fig. 2. Correlation between expression levels of *TP53*, *PANDA*, *MEG3* and *CASC2*. The distribution of expression levels of *TP53*, *PANDA*, *MEG3* and *CASC2* genes is shown on the diagonal. On the bottom of the diagonal, *P* values and correlation coefficients are shown. The bivariate scatter plots are displayed in the upper part.

4. Discussion

As the first identified tumor suppressor gene, *TP53* has been shown to eradicate and suppress the proliferation of genetically abnormal cells, thus averting neoplastic transformation [3]. However, the frequency of mutations in this gene in breast cancer is lower than what has been expected [3], suggesting an alternative route for inactivation of the encoded protein in this kind of cancer. LncRNAs have critical roles in the pathoetiology of breast cancer [13]. Dysregulation of lncRNAs not only affects evolution of breast cancer, but also influences the clinical progression and outcomes of this type of malignancy [14]. Various studies have shown dysregulation of lncRNAs in breast cancer specimens or sera of affected females signifying these transcripts as diagnostic molecules for breast cancer [14].

LncRNAs have been demonstrated to influence breast cancer development through several mechanisms among them is modulation of activity of tumor suppressor genes [15]. In order to find putative mechanism of p53 inactivation in breast cancer, we evaluated expression of three lncRNAs which have been formerly recognized as modulators of p53 stability or function [4,6,7]. Most notably, expression levels of *TP53*, *MEG3*, *CASC2* and *PANDA* were significantly lower in tumoral samples compared with non-tumoral samples. *PANDA* and *MEG3* have been shown to directly enhance p53 transcription or stability [5,6]. Meanwhile, *CASC2* influences functions of two downstream targets of p53 i.e. CDK1 and BCL2 [7,8]. Therefore, down-regulation of *MEG3*, *CASC2* and *PANDA* in breast cancer samples not only attenuates p53

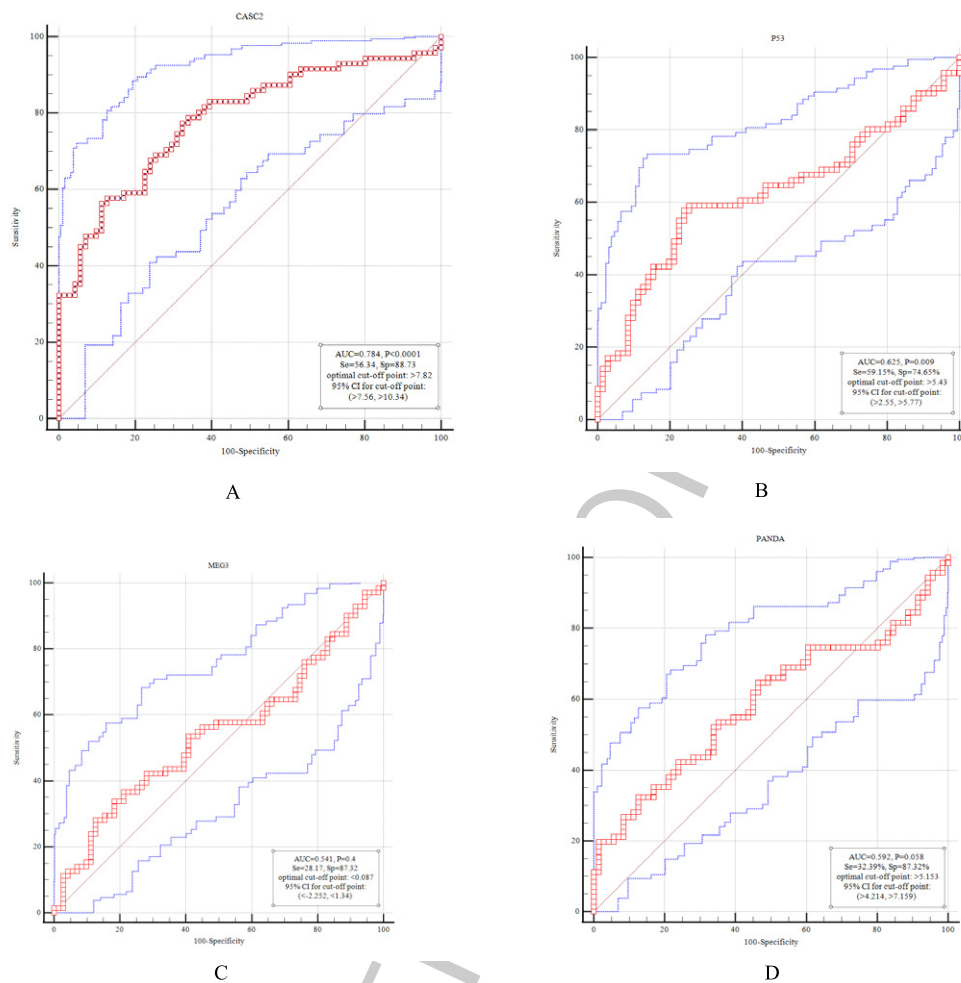


Fig. 3. Diagnostic value of *CASC2* (A), *TP53* (B), *MEG3* (C) and *PANDA* (D) in differentiation between tumoral and non-tumoral tissues. Area under curve (AUC), sensitivity, specificity, optimal cut-off point and *P* values are shown.

stability and function, but also over-activates oncogenes which are normally suppressed by p53. Taken together, the current study suggests abnormality in the function of p53-related molecular cascades in breast cancer which might be an explanation for low frequency of *TP53* mutations in this neoplasm. *CASC2* has been shown to inhibit breast cancer cell growth and metastasis via modulation of miR-96-5p/SYVN1 cascade [16]. Expression of *CASC2* has been remarkably reduced in breast cancer tissues and cell lines compared with neighboring non-affected tissues and non-malignant breast epithelial cells. Most notably, *CASC2* up-regulation could inhibit viability, migratory potential and invasive properties of breast cancer cells, while elevating their apoptosis [16]. In addition, *MEG3* has

been demonstrated to suppress breast carcinogenesis through influencing expression of miR-21 and activity of PI3K/Akt, p53 and NF- κ B [17,18]. However, another study in a small-sized cohort of breast cancer patients has demonstrated up-regulation of *PANDA* in breast cancer samples compared to breast cysts tissues [19]. Low sample size of the mentioned study and the source of control tissues might explain the discrepancy between our results and the results of this study.

Expression of *CASC2* was higher in Her2 1+ cases compared with Her2 negative cases. Her 2 has been shown to be up-regulated in 15–30% of invasive breast cancer samples. Moreover, over-expression of this factor has both prognostic and predictive implications [20,21]. Besides, expression levels of *MEG3*

and *TP53* were lower in grade 2 samples compared with grade 1. Although these findings might indicate changes in the expression of mentioned lncRNAs throughout the course of breast carcinogenesis, the exact underlying mechanisms for these observations should be uncovered.

Consistent with the role of these lncRNAs in the regulation of activity of p53, significant pairwise correlations were detected between expression levels of *TP53* and these lncRNAs. Moreover, *CASC2* had the best performance for differentiation between tumoral and non-tumoral tissues, potentiating this lncRNA as a probable marker for breast cancer.

In brief, we demonstrated abnormal down-regulation of *TP53* and its related lncRNAs in breast cancer tissues, which is in line with the critical role of this tumor suppressor gene in prevention of cancer. However, we state lack of functional studies as a limitation of our work.

Conflict of interest

The authors declare they have no conflict of interest.

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