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Moonlight human ribosomal protein L13a downregulation is associated with p53 and HER2/neu expression in breast cancer

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Abstract
Breast cancer is the most common malignancy among females worldwide. Recent studies have shown extra-ribosomal roles of the moonlight ribosomal proteins in the development of human cancers. Accurate quantification of the gene expression level is based on the selection of the reference genes whose expression is independent of cancer properties and patient's characteristics. The aim of this study was the evaluation of the expression level of a previously proposed ribosomal protein as moonlight, L13a (RPL13A), in breast cancer samples and their adjacent tissues. Its association with genes of known roles in developing cancers was also investigated. Traditionally used housekeeping genes were selected and their expression was analyzed in 80 surgically excised breast tissue specimens (40 tumors and 40 tumor-adjacent tissues) by applying three software tools including GeNorm, NormFinder, and BestKeeper to select the most stable reference genes. Then, mRNA expression levels of *RPL13A* and *p53* were evaluated. Additionally, protein expression levels of RPL13A were measured. It was demonstrated that *PUM1* and *ACTB* are the most reliable reference genes and *RPL13A* is the least stable gene. There was a positive correlation between *RPL13A* and *p53* mRNA expression levels in all the tumor samples. Moreover, significant downregulation of *RPL13A* expression levels was revealed in HER2+ tumor samples compared to HER2- ones. There was also a marked decrease in *p53* mRNA expression levels in HER2+ tumor subtypes. Our results suggest that there is a probable relationship between RPL13A decreased expression with p53 and HER2/neu expression in the breast cancer.

Keywords: Breast neoplasms; ErbB receptors; Ribosomal proteins; Tumor suppressor protein p53

Highlights:
- *RPL13A* was least stable gene reference gene in breast cancer samples.
- Positive correlation between *RPL13A* and *p53* mRNA expression levels was detected in all studied tumor samples.
- Significant downregulation of *RPL13A* expression levels was revealed in HER2+ tumor samples compared to HER2- tumor samples.
- *p53* mRNA expression levels showed considerable decrease in HER2+ tumor subtypes.

Abbreviations:
rRNAs, ribosomal ribonucleic acids; RPs, ribosomal proteins; RPL13A, human ribosomal; protein L13a; GAIT, interferon-gamma-activated inhibitor of translation; cDNA, complementary DNA; qRT-PCR, quantitative reverse transcription PCR; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ACTB, actin β; B2M, beta-2-microglobulin; PUM1, pumilio RNA binding family member 1; Ct, threshold cycle; IHC, Immunohistochemical; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; DAB, 3, 3-diaminobenzidine tetrahydrochloride; HER2+, human epidermal growth factor receptor 2 positive; TCGA, The Cancer Genome Atlas; IRESs, internal ribosome entry sites; oxLDL, oxidized low-density lipoprotein

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Introduction

Breast cancer has a high prevalence among females (Bray et al., 2018). The complexity of the prognosis, diagnosis, and treatment of this disease originates from the biological heterogeneity of neoplastic breast epithelial cells, which in turn is caused by genomics, transcriptomics, proteomics and epigenetic diversities (Cava et al., 2015). However, early-stage diagnosis of breast cancer results in a more effective response to treatment, improved survival prospects, and reduced risk of metastasis and mortality (Cheng and Ueno, 2012).

The eukaryotic complex structure of the ribosome is essential for protein biosynthesis in the cell. This organelle is made up of ribosomal ribonucleic acids (rRNAs) and ribosomal proteins (RPs). The integrated 80S ribosome consists of two large (60S) and small (40S) subunits (Bhavsar et al., 2010). Due to large variations in the type of proteins and the rate of protein synthesis in different cells, tissue-specific expression of ribosomal components has been reported in normal human tissues (Buszczak et al., 2014). Moreover, it was shown that specific ribosomal protein genes are expressed differently in some pathological conditions such as cancer and cardiovascular diseases (Wang et al., 2015). Contrary to the general assumption, RPs independent of their main involvement in the protein biosynthesis and ribosome biogenesis can also participate in important cellular functions including DNA replication and repair, cell proliferation and differentiation, and apoptosis (Xu et al., 2016). These multifunctional RPs are called moonlight ribosomal proteins. By revealing the function of RPs in multiple extra-ribosomal activities, the role of these proteins as diagnostic and/or prognostic markers has been demonstrated in cancer patients (Molavi et al., 2019). Notably, human ribosomal protein L13a (RPL13A) is a component of the 60S ribosomal subunit. However, it does not have a proven role in the protein synthesis, assembly of mature 80S ribosomes, and rRNA processing. This molecule is a moonlight ribosomal protein in the mature ribosome compartment with the release capacity from the ribosomal subunit in the phosphorylated state. The phosphorylated RPL13A blocks the translation of interferon-gamma-activated inhibitor of translation (GAIT) element-containing target mRNAs following the IFN-γ-induced signaling (Mukhopadhyay et al., 2008; 2009). To understand how molecular alterations drive cancer, recent studies have focused on the detection of expression differences between cancerous and normal tissues (Pierouli et al., 2019).

This study aimed to evaluate the expression of RPL13A in both mRNA and protein levels in tumors and related adjacent tissues of breast cancer patients. We also measured P53 mRNA expression and its association with RPL13A in these patients. Furthermore, stability of this gene was compared to the stability of other common housekeeping genes by applying three different software tools.

Material and methods

Collection of human breast cancer specimens

A total of 40 fresh breast tumors and the paired adjacent tissues were obtained from newly diagnosed breast cancer patients and were confirmed with an oncologist. These specimens were recruited from Valiasr Hospitals, Tabriz, Iran between 2018 and 2019. Table 1 shows the clinicopathological information of the participants. Notably, the patients were not treated with chemotherapeutic agents at the time of sampling. Fresh samples were divided into two segments, one of which was used for pathology analysis, whereas the second was collected in liquid nitrogen and stored frozen at –80 °C.

Table 1. Characteristics of transitional breast cancer samples

Characteristic	Patients, *n*
Total number of patients	40
Age	
Median (range)	45 (21– 65)
Histological grade	
1	10
2	22
3	8
Tumor type	
ER+	16
ER–	24
HER2+	12
HER2–	28

Evaluation of the mRNA expression level of RPL13A and p53

Total RNA isolation and cDNA synthesis

The preserved tumor and tumor-adjacent samples were cut into the smallest possible pieces and total RNA was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) by mechanical homogenizing and according to the manufacturer's protocol. Then, the isolated RNA was purified using the Phenol/Chloroform extraction method (Toni et al., 2018). For complementary DNA (cDNA) synthesis, PrimeScript RT Master Mix (Takara Bio, Inc.) was applied in the retro-transcription reaction mixture.

Real-time PCR

Gene expression is modulated by different intra- and extracellular factors. Therefore, identification of gene expression modification via common methods such as real-time quantitative reverse transcription PCR (qRT-PCR) requires normalization of the target-genes expression levels to the expression of the housekeeping gene with minimum variation in a wide variety of conditions. To date, some genes such as glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and actin β (*ACTB*) have been used as the general-purpose reference genes (Kasai et al., 2003). Despite their wide-spread nature, the variability of known reference genes in human cancers was shown (Jo et al., 2019). Since *RPL13A* is a conventionally used reference gene for the normalization of the gene expression in different cells and tissues, its reliability as a reference gene was validated in this study. Then, mRNA expression levels of *RPL13A* and *p53* were evaluated relative to the most stable reference genes. For this purpose, one microliter of RT products was used as a template for the real-time PCR reaction with the SYBR Premix Ex Taq™ II (TaKaRa, #RR820A/B, Japan) reagent on the LightCycler 96 System (Roche Diagnostics, Mannheim, Germany). Table 2 presents sequences of forward and reverse primers used in PCR reactions to amplify *p53* and five candidate reference genes, including *ACTB*, beta-2-microglobulin (*B2M*), *GAPDH*, *RPL13A*, and pumilio RNA binding family member 1 (*PUM1*). Each reaction was carried out with three technical replicates.

Table 2. Characteristics of the selected genes and their primers

Gene	Primers
GAPDH	F 5'-TCTCCTCTGACTTCAACAGCGA-3' R 5'-CCCTGTTGCTGTAGCCAAATTC-3'
ACTB	F 5'-TCACAATGTGGCCGAGGACTTT-3' R 5'-AGAAGTGGGGTGGCTTTTAGGATG-3'
RPL13A	F 5'-GCAAAGATCCATTACCGGAAG-3' R 5'-ACAGTCTTTATTGGGTTCACAC-3'
B2M	F 5'-CTTATGCACGCTTAACTATCTTAACAA-3' R 5'-TAGGAGGGCTGGCAACTTAG-3'
PUM1	F 5'-AGTGGGGGACTAGGCGTTAG-3' R 5'-GTTTTCATCACTGTCTGCATCC-3'
p53	F 5'-TCAACAAGATGTTTTGCCAACTG-3' R 5'-ATGTGCTGTGACTGCTTGTAGATG-3'

Data analysis

To determine the most stable reference genes, qRT-PCR data related to the housekeeping genes were analyzed using three Excel-based widely applied software tools, GeNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), and BestKeeper (Pfaffl et al., 2004). GeNorm sorts the tested reference genes according to M value. M value or gene expression normalization factor is an average pair-wise variation of a single candidate with all others. The higher the M value revealed the less stability of the selected gene expression (Vandesompele et al., 2002). NormFinder identifies optimal reference genes by the combination of the estimated intra- and inter-group variations for each candidate reference gene, called stability value (Andersen et al., 2004). BestKeeper helps users to determine the best reference genes using pair-wise correlation analysis of candidate reference genes (Pfaffl et al., 2004). Finally, the two most stable genes were used as the internal controls to normalize the expression of RPL13A and p53 using the 2-ΔΔCt method (Livak and Schmittgen, 2001).

Evaluation of the protein expression level of RPL13A

Immunohistochemical staining

All tissue samples were subjected to the Immunohistochemical (IHC) process to determine the expression levels of RPL13A. Briefly, tissues were fixed in 20% buffered formalin and then embedded in paraffin to prepare the blocks of tissues. Then, the blocks were sectioned (4 μm thickness) and placed on the glass slides. Afterward, the slides were put aside at 60 °C overnight, de-paraffinized in xylene, and subsequently rehydrated by ethanol series. Besides, 3% H_2O_2 in methanol was used to quench endogenous peroxidase activity of samples. The tissue sections were subjected to heating in the sodium citrate buffer at pH 6.0 for 30 min to retrieve antigen. After a triple round of washing with phosphate-buffered saline (PBS, pH 7.4), the tissue sections were incubated with rabbit polyclonal anti-RPL13A antibody (Abcam Inc.) at 4 °C overnight followed by incubation with anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP) (Dako, EnVision system, Denmark) at room temperature for 45 min. After washing with PBS solution, 3, 3-diaminobenzidine tetrahydrochloride (DAB)/H_2O_2 solution was applied for about 5 min to visualize the peroxidase activity. For the next stage, the sections were washed with water, and then 0.1% hematoxylin was used for counterstaining. Finally, after cover slipping the slides with a rapid mounting medium (Entellan, Merck, Germany), they were analyzed under a light microscope. Negative controls were obtained by omitting the primary antibodies from the staining procedure (Jeddi et al., 2018).

Immunohistochemical analysis

The expression level of RPL13A was evaluated based on a scoring system, in which the staining intensity was scored from 0 to 3 (no staining = 0, weak = 1, moderate = 2, and strong = 3). Also, according to the percentage of the stained cells relative to all epithelial cells, the expression was scored as (0, <5%; 1, 6–10%; 2, 11–50%; and 3, >51%). The expression index was calculated by adding the two individual scores (Kasai et al., 2003). Then, the specimens were graded from 0 to 3 based on the total scores, i.e. the score of 0 as negative staining (–), 1–4 as weakly positive (1+), 5–8 as moderately positive (2+), and 9–12 as strongly positive (3+). For statistical analysis, the negative and weakly positive groups (– and 1+) were recorded as negative, while the moderately and strongly positive groups (2+ and 3+) were defined as positive.

Results

Expression levels of candidate reference genes

To assess the Ct values of all five candidate reference genes in the samples, qPCR method was used. mRNA expression levels of candidate genes displaying median Ct values ranging from 16.99 (GAPDH) to 28.04 (B2M) are shown in Fig. 1.

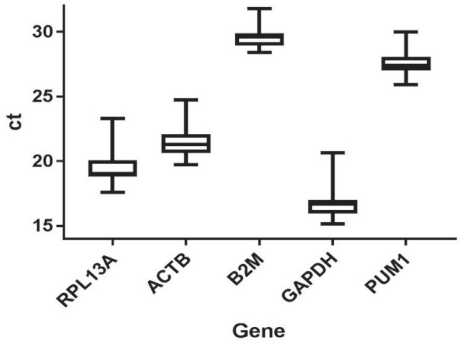

Fig. 1. Ct values of the candidate reference genes in 80 breast tissue specimens

The selected reference genes were further analyzed in tumor and tumor-adjacent with the software programs of GeNorm, NormFinder, and BestKeeper (Tables 3 and 4). GeNorm calculates the stability of the expression according to the M value. As shown in Table 3, the genes with the smallest M value were *PUM1* and *ACTB* (1.07), which were the most stable genes in all samples in the present study. However, the least stable gene was *RPL13A* with a high M value of 1.34. The results of the NormFinder analysis, which evaluates the expression stability of each candidate independently, are shown in Table 3. Consistent with the results of GeNorm, *PUM1* was ranked the first in the list of the most stable reference genes, and *RPL13A* was the most variable reference gene compared to the other candidates. The analysis results of BestKeeper, which evaluates the stability of the reference gene expression using raw Cq values, are given in Table 4. The results showed that covariance and standard deviation values of *B2M* and *RPL13A* were the lowest and the highest of the reference genes, respectively.

Table 3. Ranking of housekeeping genes by GeNorm and NormFinder

Candidate genes	GeNorm		NormFinder		
	Rank	M value[a]	Rank	SD[b]	Acc. SD[c]
PUM1	1, 2	1.07	1	0.7117	0.7117
ACTB	1, 2	1.07	2	0.8157	0.5413
GAPDH	3	1.20	3	0.9092	0.4712
B2M	4	1.22	4	1.017	0.435
RPL13A	5	1.34	5	1.263	0.4304

[a] stability factor of expression; [b] standard deviation (stability value); [c] accumulated standard deviation.

Table 4. Results of five housekeeping genes by BestKeeper

Gene	Ct					x-fold		
	GM[a]	Min	Max	SD[b]	%CV	Min	Max	SD[c]
B2M	29.54	28.41	31.78	0.65	2.19	–2.19	4.73	1.57
PUM1	27.49	25.92	29.99	0.71	2.58	–2.97	5.66	1.64
ACTB	21.44	19.74	24.75	0.92	4.28	–3.25	9.92	1.89
GAPDH	16.8	15.16	20.66	0.92	5.46	–3.12	14.51	1.89
RPL13A	19.52	17.59	23.31	1.08	5.51	–3.80	13.87	2.11

[a] stability factor of expression; [b] standard deviation (stability value); [c] accumulated standard deviation.

Considering the differences among the ranking results of the three used algorithms, a method was used to calculate the final ranking of the reference genes (Chen et al., 2011). The ranking of each reference gene in all the algorithms was listed and then the geometric means of the three ranking numbers were calculated. The smallest geometric mean shows the most stable genes. As shown in Table 5, *PUM1* and *ACTB* were determined as the most reliable reference genes.

Table 5. Final ranking of five candidate reference genes in all samples

Ranking	geNorm	Norm Finder	BestKeeper	Overall
1	PUM1-ACTB	PUM1	B2M	PUM1
2	PUM1-ACTB	ACTB	PUM1	ACTB
3	GAPDH	GAPDH	ACTB-GAPDH	B2M
4	B2M	B2M	ACTB-GAPDH	GAPDH
5	RPL13A	RPL13A	RPL13A	RPL13A

mRNA expression levels of RPL13A and p53

According to the research design, quantitative-PCR was performed to evaluate the *RPL13A* and *p53* mRNA expression in all tissue samples. As can be seen in Fig. 2 and 3, *RPL13A* and *p53* expression levels were significantly down-regulated in the tumor tissues compared to the related tumor-adjacent tissues ($p < 0.05$) in human epidermal growth factor receptor 2 positive (HER2+) subtype samples. Moreover, a decrease of the *RPL13A* expression in the tumor of breast cancer subtypes of luminal-like and basal-like was observed in comparison to their adjacent samples, but their reduction *p*-values were not significant ($p > 0.05$). In addition, the decrease of *p53* expression levels in the luminal-like subtype tumor samples and its increase in the basal-like subtype samples were observed, but the *p*-values of their expression changes were not significant ($p > 0.05$). Finally, it was found that a positive correlation exists between expression levels of *RPL13A* and *p53* in the breast cancer tissue samples (Fig. 4).

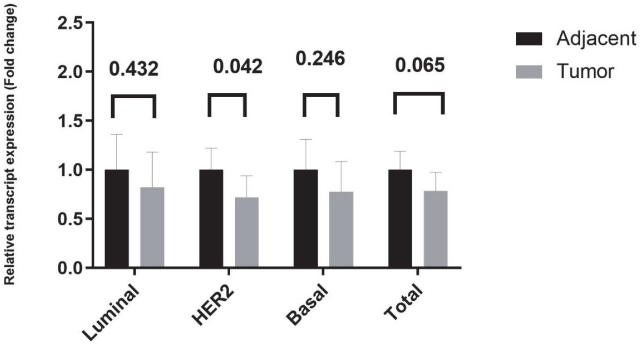

Fig. 2. Quantitative PCR analysis of mRNA expression levels of *RPL13A* in different subtypes of samples

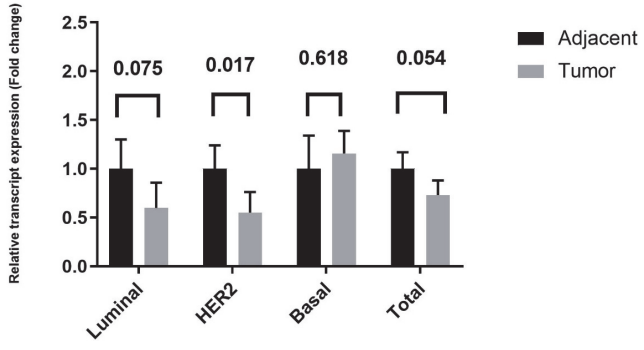

Fig. 3. Quantitative PCR analysis of mRNA expression levels of *p53* expression in different subtypes of samples

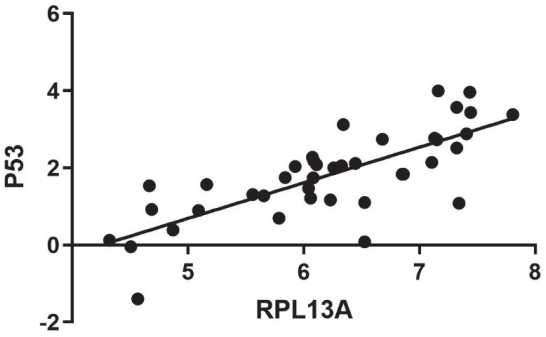

Fig. 4. Pearson correlation analyses of the relationship between mRNA expression levels of *RPL13A* and *p53* in breast cancer tissue samples

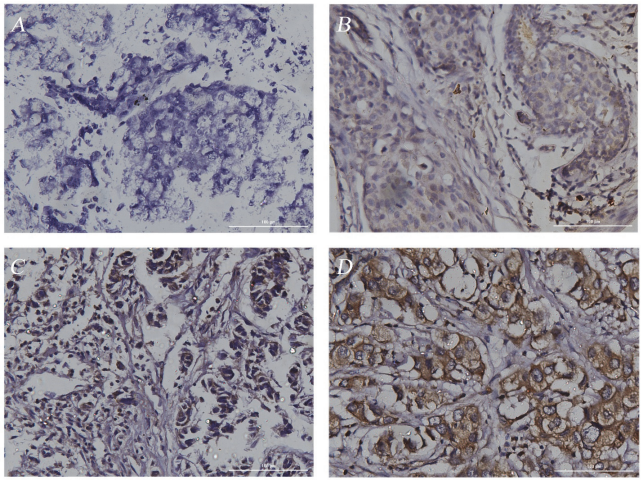

Fig. 5. Immunohistochemical staining of RPL13A expression. (**A**) Negative staining; (**B**) Weakly positive and (**C**) Moderately positive; and (**D**) Strongly positive staining of RPL13A in breast cancer tissues. Scale bars = 100 μm.

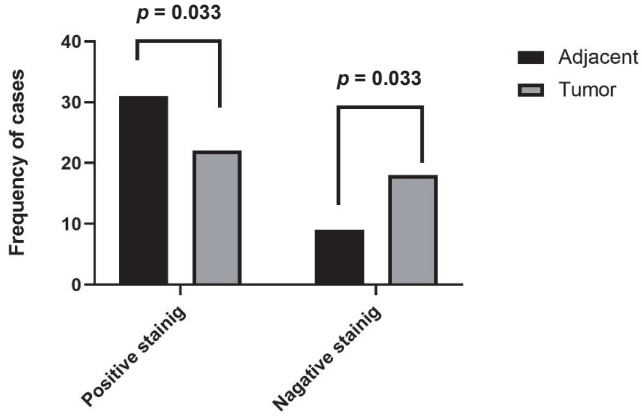

Fig. 6. Immunohistochemical analysis of expression – RPL13A protein expression was significantly down-regulated in the tumor tissues compared to the adjacent tissues ($p < 0.05$)

Protein expression levels of RPL13A

We determined the protein level of RPL13A by immunohistochemical staining in the collected tumor and tumor-adjacent tissues. Our results showed that RPL13A is predominantly expressed in the cytoplasm of the breast carcinoma cells (Fig. 5). It was also found that (Fig. 6) the positive staining rate of RPL13A expression was 27.5% (22/80) in tumor tissues, which was significantly ($p < 0.05$) lower than that in the tumor-adjacent tissues 38.5% (31/80).

Association between RPL13A expression and clinicopathological criteria

Table 6 summarizes the correlation analyses between the clinicopathological criteria and the mRNA expression levels of *RPL13A*. According to the table, no significant association was observed with age, tumor size, and histological grade. The decreased expression level of *RPL13A* was markedly associated with HER2 expression status of tumors ($p = 0.023$). Notably, the same results were obtained when correlation analysis was performed between clinicopathological criteria and protein expression levels of RPL13A (Table 7).

Table 6. Association between clinicopathological criteria and mRNA expression of *RPL13A*

Clinicopathological criteria	*RPL13A* expression (Mean ± SEM)	*P* value (*T*-test)
Age		
≥45 years	–6.180 ± 0.204	0.425
<45 years	–5.945 ± 0.206	
Tumor size		
≤5 cm	–6.160 ± 0.177	0.574
>5 cm	–5.994 ± 0.240	
Histological grade		
1	–6.016 ± 0.160	0.437
2	–5.956 ± 0. 240	
3	–6.443 ± 0.277	
Tumor type		
ER+	–5.899 ± 0.245	
ER–	–6.181 ± 0.177	0.346
HER+	–5.859 ± 0.176	
HER–	–6.557 ± 0.193	**0.025**

The bold characters denote that the *p* value is less than 0.05, which has the statistical significance.

Discussion

It is commonly agreed that breast cancer is the second cause of death in women throughout the world. Besides, genetic and epigenetic diversities of individuals have been considered as the main causes of clinical and morphological heterogeneity of breast cancer tumors. Notably, one of the main properties of cancer are the complex signaling pathways. Therefore, identification and control of the genes, whose dysregulation is involved in the tumorigenesis, is a main challenge of investigators worldwide. Therefore, the aim of the present study was the evaluation of the expression of one of the ribosomal proteins, RPL13A, whose extra-ribosomal functions in translational control of the genes involved in cancer have been verified recently. The schematic representation of the methodology and obtained results in this research are given in Fig. 7.

Table 7. Association between clinicopathological criteria and protein expression of *RPL13A*

Clinicopathological criteria	No. of patients (%)	Positive *n* (%) = 18 (45)	Negative *n* (%) = 22 (55)	*P* value (*T*-test)
Age				
≥45 years	21	10 (47.6)	11 (52.4)	0.726
<45 years	19	8 (42.1)	11 (57.9)	
Tumor size				
≤5 cm	18	9 (50)	9 (50)	0.565
>5 cm	22	9 (40.9)	13(59.1)	
Histological grade				
1	10	5 (50)	5 (50)	0.848
2	22	9 (40.9)	13 (59.1)	
3	8	4 (50)	4 (50)	
Tumor type				
ER+	16	6 (37.5)	10 (62.5)	
ER–	24	12 (50)	12 (50)	0.436
HER+	12	9 (75)	3 (25)	
HER–	28	9 (32.1)	19 (67.9)	**0.013**

The bold characters denote that the *p* value is less than 0.05, which has the statistical significance.

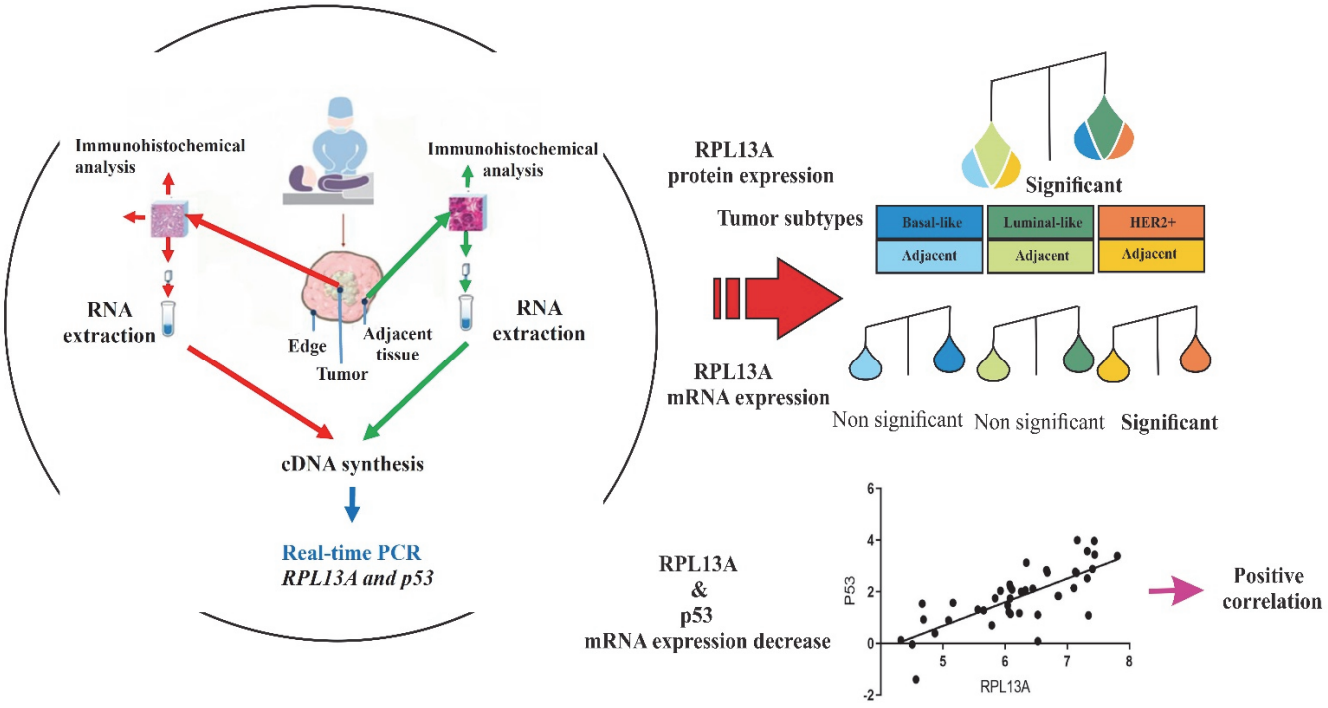

Fig. 7. Schematic representation of the methodology and obtained results in this research

RT-qPCR is one of the most frequently used methods for gene expression profiling. However, for the following reasons, the selection of reliable reference genes is the prerequisite of this study: (1) Data obtained from qRT-PCR should be normalized with appropriate reference genes. (2) The traditionally used reference genes are not necessarily suitable for normalization of the gene expression in human cancer research. (3) *RPL13A* is conventionally considered as a housekeeping gene in many studies. Two of the ideal reference gene criteria are: (1) its abundance in the studied tissues and (2) a low expression variation in the investigated samples. In this study, according to the analyses of three software tools (geNorm, NormFinder, and BestKeeper), the final ranking of the most reliable reference genes was indicated as follows: *PUM1* > *ACTB* > *B2M* > *GAPDH* > *RPL13A*.

Thus, *PUM1* and *ACTB* were the most reliable reference genes. This result was in parallel with the results obtained from the analysis of large-scale cancer gene expression datasets from the Cancer Genome Atlas (TCGA) database, which validated *PUM1* and *ACTB* genes among the top stable reference genes (Jo et al., 2019). Also, the pan-cancer analysis of TCGA Data revealed *PUM1* as the most suitable reference gene for qPCR normalization of expression data related to breast cancer (Krasnov et al., 2019). Moreover, the suitability of *PUM1* as a reference gene was demonstrated in another large-scale screening of the potential candidate reference genes from RNA-seq data. Notably, the large expression variation of *RPL13A* was also shown in the same study (Tilli et al., 2016). Contrary to the results of this study, the results of another study about the selection of the housekeeping genes as the

references for the normalization of quantitative PCR data in breast cancer indicated the combination of *PUM1/RPL13A* as suitable reference genes (Kılıç et al., 2014). Such a condition may be due to the difference in the tumor subtypes samples used in these studies.

Fig. 2 shows that the mRNA expression level of *RPL13A* in tumor samples is weaker than the level in the tumor-adjacent samples, which is significant ($p < 0.05$) only in the comparison of HER2+ tumor samples with their adjacent samples. *RPL13A* mRNA expression decrease was reported in breast cancer through an investigation of the expression of ~10,000 human tumors and over 700 normal tissues from TCGA (Dolezal et al., 2018). Additionally, *RPL13A* expression decrease was reported in pancreatic and cervical cancer tumors compared to normal tissues (Crnogorac-Jurcevic et al., 2001; Rajkumar et al., 2011). Interestingly, the increased level of the expression of *RPL13A* was shown in breast cancer and ovarian cancer patients following chemotherapy (Clarke et al., 2004; Modlich et al., 2004). Moreover, the decrease of *RPL13A* mRNA expression in H605 breast cancer cells, which were derived from HER2/neu transgenic mice, was previously reported (Chen et al., 2010).

According to the results presented in Fig. 4, there is a positive correlation between the *RPL13A* and *p53* expression levels. It was demonstrated that some mRNAs, including p27, and p53 contain internal ribosome entry sites (IRESs) in their 5'untranslated region (5'-UTR), which mediate cap-independent translation initiation of these mRNAs. It was found that methylation posttranslational modification of rRNA increases the translation via IRES. Moreover, RPL13A causes methylation of rRNA and efficient translation via IRES elements of these mRNAs (Chaudhuri et al., 2007; King et al., 2010). However, as shown by the results of Fig. 3, there is a significant reduction in *p53* expression levels in HER2+ tumor subtypes. Here, it is emphasized that previous studies verified the inverse relationship of p53 and p27 expressions with HER2 expression (Le et al., 2003; Payandeh et al., 2016). Therefore, the inverse relationship of RPL13A with HER2 expression may be mediated by p53 and p27. As can be seen in Tables 6 and 7, there is a significant reduction in *RPL13A* expression levels in the HER2+ tumor samples compared to the HER2- samples with a *p*-value lower than that of the HER2+ tumor samples with their adjacent samples (Fig. 2). Therefore, it can be expected that HER2+ tumor-adjacent cells may express fewer levels of HER2 compared to tumor cells (Margan et al., 2016; Zubor et al., 2015).

According to gene-expression profiling, the major subtypes of breast cancer are HER2+, luminal-like, and basal-like (Perou et al., 2000). The luminal-like subtype is $ER^{low\ or\ high}$ and $HER2^{low}$. The basal-like subtype is ER- and HER2- (Malhotra et al., 2010). The results of Fig. 3 showed insignificantly decreased expression of p53 in this subtype. The inverse expression of p53 with the increased ERα expression in luminal tumors has been shown previously (Bado et al., 2018). As seen in Fig. 5, the expression level of RPL13A protein was significantly decreased in total tumor tissues compared to their adjacent tissues. It should be mentioned that the weaker expression of this ribosomal protein was shown in the results of a study, which investigated the expression differences of ribosomal proteins in the human normal and neoplastic colorectum (Kasai et al., 2003). However, the difference in the significance of RPL13A reduction in mRNA and protein levels in total samples (Fig. 2 and 6) may be derived from the posttranslational expression changes of RPL13A. GAPDH could function as a chaperone, protecting RPL13A from proteasomal degradation. Moreover, it has been shown that this shielding activity is lost following cell treatment with oxidized low-density lipoprotein (oxLDL) (Jia et al., 2012). According to the findings, breast cancer patients exhibited higher levels of oxLDL (Cedó et al., 2019).

Conclusions

The selection of suitable reference genes is important for seeking novel markers in studies of breast cancer in the future. The results of the present study demonstrated that moonlight ribosomal protein RPL13A is not always the outstanding reference gene for normalization of gene expression in the breast cancer samples. The results also proposed a probable relationship between RPL13A decreased expression with p53 and HER2/neu expression in the breast cancer. Since HER-2/neu overexpression is a poor prognostic factor for breast cancer patients, it is worthy to confirm the results of this study by including larger sample numbers and finding exact interaction pathways in future studies.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Tabriz University of Medical Sciences (Ethical code: TBZmed. REC.1397.022). Informed consent was taken from all participants.

Human and animal rights

No animals were used in this study. All reported experiments on humans were followed in accordance with the ethical standards of the committee responsible for human experimentation (institutional national), and with the Helsinki Declaration of 1975, as revised in 2013.

Consent for publication

Informed consent was obtained from each participant.

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Conflict of interests

The authors declare that there is no conflict of interests regarding the authorship of the presented manuscript.

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