

## Geometric Mean of 5S rRNA and MiR-16 as a Suitable Normalizer in Esophageal Cancer

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### Abstract

Esophageal squamous cell carcinoma (ESCC) is a deadly cancer with poor prognosis. In this regard, early diagnosis is of vital importance to cure the tumor in its early stages. Novel cancer diagnostic and therapeutic approaches have been recently introduced based on microRNAs (miRNAs). Also, accurate normalization using appropriate reference genes is a critical step in miRNA expression studies. In this study, we aimed to identify appropriate reference genes for miRNA quantification in serum samples of ESCC. In this case and control experimental study, two statistical algorithms including GeNorm and NormFinder were used to evaluate the suitability of miR-16 and 5S rRNA and their geometric mean as reference genes. Then, relative expression of miR-451 and miR-24 were evaluated while different normalizer including miR-16, 5S rRNA and their geometric mean were applied. Both GeNorm and NormFinder analyses showed that geometric mean of miR-16 and 5S rRNA is the most stable reference gene in these samples. Also, our data showed that choosing an inappropriate normalizer could change the relative expression of target genes of miR-451 and miR-24 in ESCC samples which emphasize on the importance of selecting a reliable internal control in expression analyses. We demonstrated that geometric mean of two reference genes could increase the reliability of normalizers and also by using geometric mean as reference gene, relative expression of different target is closer to reality.

**Keywords:** Esophageal cancer, MicroRNA, qRT-PCR, Reference genes

### Introduction

Esophageal cancer is one of the most common malignancies in the world which ranked eighth in cancer incidence and sixth in cancer mortality (Zhang, 2013). There are two major types of esophageal cancer, esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC) (Xu et al., 2012). Golestan province in northern Iran is known as an area with a very high incidence of esophageal cancer (Islami et al., 2009). ESCC is a deadly cancer with poor prognosis which its early diagnosis is of vital importance to cure the tumor in its early stages (Fitzmaurice et al., 2015). Novel cancer diagnostic and therapeutic approaches have been recently introduced based on miRNAs (Monroig and Calin, 2013). MiRNAs are short RNA molecules that bind (generally) to 3' UTR sequences of target messenger RNAs (mRNAs), thereby modulating their expression patterns. This modulated gene expression is regulated either as translational repression (Lai, 2002), or mRNA degradation (Engels and Hutvagner, 2006).

MiRNAs play major roles in governing diverse biological processes such as differentiation, proliferation, and apoptosis (Chen et al., 2004; Croce and Calin, 2005). Individual miRNAs have oncogenic and tumor suppressor functions (Esquela-Kerscher and Slack, 2006), and aberrant miRNA expression has been implicated in many malignancies.

Several methods have been employed in miRNA expression studies including Northern blotting, microarrays, reverse transcription-qPCR (RT-qPCR), and sequencing. RT-qPCR possesses advantages in terms of high sensitivity, wide dynamic range and low template requirements (Pritchard et al., 2012). Appropriate normalization of RT-qPCR data using stably expressed reference genes is critical to ensure accurate and reliable results, because data normalization with an inappropriate reference gene would lead to twisted and biased results (Chang et al., 2010; Das et al., 2016; Ferdous et al., 2015). So, in order to achieve

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accurate, reproducible and biologically relevant miRNA RT-qPCR data and also to remove any non-biological sample-to-sample variation, appropriate reference genes should be selected in each study design (Liu et al., 2014).

A candidate reference gene should meet certain criteria before being applied as a reliable normalizer in expression studies. These criteria include having comparable length, expression level and quantification efficiency to the target gene, and most importantly displaying an invariable expression level across all samples under study (DAS et al., 2016; Schaefer et al., 2010; Shen et al., 2011).

The aim of this study was to investigate the suitability of two widely used reference genes in serum samples of patients with esophageal cancer. An additional goal of this study was to assess the impact of exploiting different reference genes on miRNA quantification in ESCC disease.

In this regard, the stability of miR-16 and 5S rRNA was evaluated in serum samples of ESCC. Then, different algorithms were used to determine the most stable reference genes in these samples. Finally, miR-451 and miR-24 expression were normalized to miR-16 and 5S rRNA and also to their geometric mean.

## Materials and Methods

### Clinical Sample Collection

39 serum samples from patients with ESCC and 39 serums from normal individuals were provided by Atrak Hospital (Golestan University of Medical Sciences, Iran). The histo-pathological characteristics of the ESCC patients are summarized in Table 1.

### Ethics Statement

This study was reviewed and approved by the Ethical Committee of Golestan University of Medical Sciences. All samples were collected according to the institutional policies and patients informed consent.

### RNA Extraction From Esophageal Serum

All serum samples were first centrifuged for 5 minutes at 3000 rpm to remove any cell contamination or cell debris. Next, in order to remove protein contamination, all serum samples were treated with Proteinase K (Fermentas, Lithuania) for 3 hours at 54°C. RNA was then extracted from serum samples with TRIzol LS reagent (Invitrogen, USA) according to the manufacturer's instructions. The resultant RNA pellets were dissolved in 20 ul of RNase free water

and were kept in -80°C for further analyses.

### miR-451 and miR-24 Quantification by Quantitative RT-PCR

Due to the presence of inhibitors in serum, as recommended by Gharbi and colleagues, different volumes of RNA samples should be tested for cDNA synthesis (Gharbi et al., 2014).

Firstly, 5 µl of total RNA sample was polyadenylated by poly (A) polymerase and then 1.5 µl of poly-A-adenylated RNA which has the least amount of inhibitors, was subjected to reverse transcription reaction in a total volume of 10 µl. This reaction was performed using RT-PCR kit, miR-451 and miR-24-specific primer bearing a 3' universal tag (ParsGenome, Iran).

Real-time PCR was performed using each miRNA specific primers, universal reverse primer (complement to the universal tag added in RT-PCR step to the end of each miRNA), and SYBR Green Premix (ParsGenome, Iran) using Step One real-time PCR machine (Applied Biosystems, USA).

### Data Analyses

qRT-PCR data analyses were performed using  $\Delta\Delta Cq$  (quantitation cycle) method and gene expressions were normalized to the expression levels of different reference genes in each experiment. All experiments were performed in duplicates.

The t test statistic was used for comparison of the distribution of the reference genes expression between the control and patient groups (significance level=0.05). Data was analyzed using SPSS 16.0 (SPSS Inc, USA) and GraphPad Prism 6 softwares. GeNorm (GenEX software) and NormFinder (GenEX software) were used to analyze the stability of the examined reference genes in all tested samples including cases and controls. The GeNorm software ranks the tested genes based on their expression stability (M value) and introduces the two most stable reference genes among all tested genes.

The M value describes the mean pairwise variation of a candidate gene compared with all other candidate genes. The stability ranking of each candidate gene was then determined by stepwise exclusion of the gene with the highest M value, followed by recalculation of average expression stability for the remaining genes until the two most stable genes were found.

The NormFinder uses a different mathematical model compared to GeNorm and takes into account the intragroup and intergroup variation. In addition, NormFinder calculates a stability value for each reference gene and ranks the candidate genes based on accumulated standard deviation (Acc SD).

**Table 1.** Histopathological characteristics of the patients

Age (year)	Sex		Tumor grade			Normal esophagus				Mucosal biopsy	
	Female	Male	Low	Moderate	High	No changes	Mild esophagitis	High grade squamous dysplasia	Low grade squamous dysplasia	Mild chronic gastric	<i>H. pylori</i> associated chronic gastric
62.77%	66.6 %	33.4 %	77.8 %	16.7 %	5.5%	55.5 %	33.5 %	5.5 %	5.5 %	76.7 %	23.3%

**Results**

**Expression Pattern of Candidate Reference Genes In Serum**

The average Cq values of both reference genes and their geometric mean are shown in table 2. As shown in this table, SD of reference genes was decreased when geometric mean of two reference genes is used.

**Table 2.** Descriptive statistical values of Cycle of quantification of reference genes in 39 tested samples

Rank	Gene	Min	Max	Average	Median	SD
1	miR-16	26.91	34.95	30.93	31.67	1.94
2	5S rRNA	23.42	29.15	26.28	26.30	1.62
3	Geometric mean	26.22	29.78	28	29.08	1.41

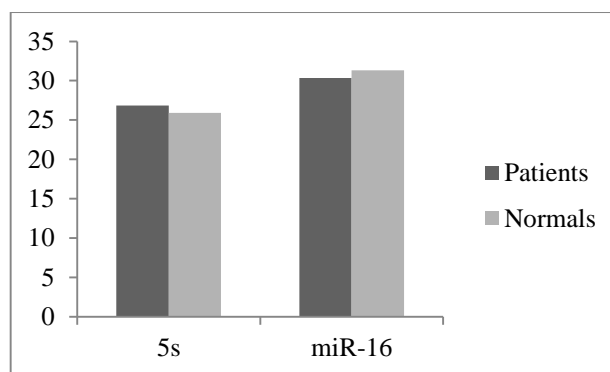
**Expression Levels of Candidate Reference Genes In Patient And Healthy Groups**

None of the reference genes were affected by the disease state since observed Cq values did not show any significant difference between healthy and patient groups (Fig.1). In addition, the result of t test with  $P > 0.05$  for both reference genes indicated that the tested populations of patients and controls have equal variance. This is an essential step before evaluating the stability of reference genes.

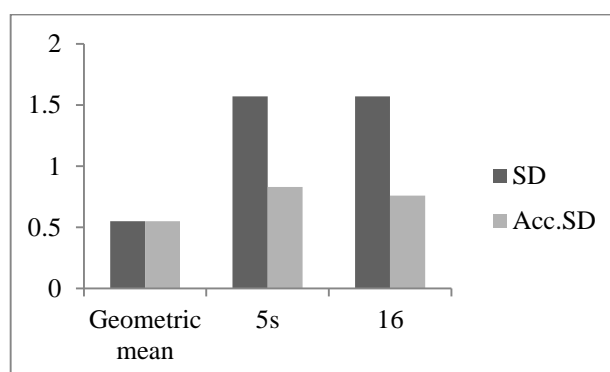
**Expression Stability of Candidate Reference Genes**

Expression stability of the candidate genes were assessed using comparison of their standard deviation and also using two algorithms of GeNorm and NormFinder as shown in table 2. miR-16 had the most variation and geometric mean had the last SD

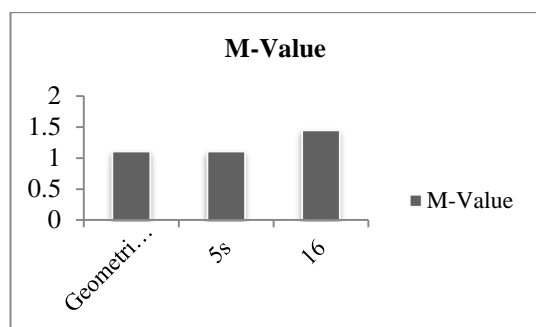
in these samples. GeNorm analysis showed that geometric mean and 5S rRNA had the same stability in these samples (Fig 2), but NormFinder analyses ranked geometric mean as the most stable and adequate reference genes in these samples (Fig 3). In total, our analyses revealed that the stability of the geometric mean normalizer is significantly higher than each candidate individually.



**Figure 1.** Cq values of candidate genes in tested samples. No differences were found between the control and patient groups ( $P > 0.05$ ). Cq; Cycle of Quantification.



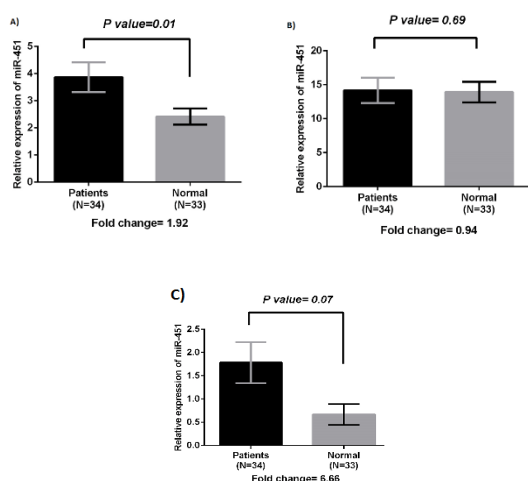
**Figure 2.** Normfinder analysis of 5S rRNA and miR-16 after applying geometric mean as a separate normalizer. The expression stability of each candidate is shown by SD in NormFinder. The lower value of SD represents higher gene stability. SD; Standard Deviation.



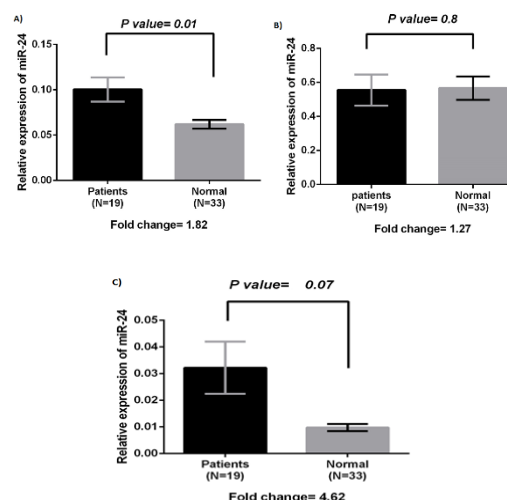
**Figure 3.** GeNorm analysis of 5S rRNA after applying geometric mean as a separate normalizer.

### Target Gene Expression Normalized With Different Reference Genes

We evaluated the expression level of two target genes including miR-451 and miR-24 in these samples and normalized them with miR-16, 5S rRNA and their geometric mean as reference genes (Fig. 4 -5). As shown in these figures, relative expression and P-value of target genes is different when normalized with different reference genes and to their mean. In case of miR-451, the fold changes were 1.92, 0.94 and 6.65 normalized to 5S rRNA, miR-16 and geometric mean respectively. This results showed that by applying the geometric mean as the most stable normalizer instead of miR-16, the fold change was remarkably changed from 0.94 (P value= 0.69) to 6.65 (P value=0.07). The same results obtained in the case of miR-24 as well which means normalization with different normalizer could change the fold change in an opposite direction and also it can influence the experiment significance.



**Figure 4.** Relative expression of miR-451 in serum samples with ESCC compared to normal samples. miR-451 relative expression when normalized with: A) Geometric mean of miR-16 and 5S rRNA (Fold change= 1.92; P-value=0.01). B) miR-16 (Fold change=0.94; P-value=0.69). C) 5S rRNA (Fold change= 6.65; P-value= 0.07).



**Figure 5.** Relative expression of miR-24 in serum samples with ESCC compared to normal samples. miR-24 relative expression when normalized with: A) Geometric mean of miR-16 and 5S rRNA (Fold change= 1.82; P-value=0.01). B) miR-16 (Fold change=1.27; P-value=0.8). C) 5S rRNA (Fold change= 4.62; P-value= 0.07)

### Discussion

MicroRNAs represent an important new class of biomarkers with critical role in post-transcriptional gene regulation, where their aberrant expressions have been observed in various diseases states. These tiny molecules regulate a large number of important biological processes including early development, cell proliferation, differentiation, apoptosis, fat metabolism, and oncogenesis (Lu et al., 2005). Reverse transcription quantitative real-time PCR (RT-qPCR) is the most sensitive and reliable method of choice which has been widely used in microRNA expression studies. To achieve accurate and reproducible RT-qPCR data, appropriate normalization is critical to correct technically, non-biological variations (Peltier and Latham, 2008). More recently, it was advised to validate the suitability of a panel of internal control genes in each sample. Inappropriate normalization of data can lead to incorrect conclusion and can conceal or magnify biologically meaningful changes of microRNAs (Vandesompele et al., 2002). Vandesompele et al proposed to evaluate the suitability of one to three commonly reference genes for each specific study (Lu et al., 2005). Here, 5S rRNA and miR-16, two commonly used reference genes in microRNA studies of serum samples, were selected and their expression was evaluated using q-RT PCR in serum samples of esophagus cancer.

As a pre-requirement of our analysis, we showed that

expression pattern of miR-16 and 5S rRNA is the same in the case and control groups. Lack of difference in this pattern showed that the tested candidate genes are not influenced by physiological state of the disease.

Data analyses on these samples using GeNorm and NormFinder showed that the geometric mean of miR-16 and 5S rRNA has the least variation compared to each of the mentioned reference genes individually. This result is consistent with the results of Vandesompele et al which showed the geometric mean of three reference genes results in more accurate data (Vandesompele et al., 2002). Gharbi and colleagues in their studies on serum samples showed that geometric mean of two reference genes is the most stable normalizer. They also showed that geometric mean of two reference genes represents the least variation even when one of these reference genes has the least stability in the samples (Gharbi et al., 2015). Then, expression level of miR-24 and miR-451 was evaluated in serum samples of esophageal cancer compared to control group. The normalization factor was 5S rRNA, miR-16 and their geometric mean. We observed that miR-24 and miR-451 were significantly over expressed when the data was normalized to 5S rRNA and geometric mean. But applying miR-16 as normalizer, the expression level between case and control group was not significant. Up regulation of miR-24 and miR-451 in biological fluids of esophageal cancer has been reported in some studies (Gu et al., 2014; Murata et al., 2013; Zhu et al., 2014). This variation in the result reveals the importance of selecting a reliable internal control in expression studies. Lim and colleagues assessed the suitability of a panel of internal controls in neuronal differentiation. They showed that normalization to an unsuitable reference gene could under-estimate the up regulation of miR-125 and miR-211 (Lim et al., 2011).

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