with liver fibrosis when compared to healthy subjects. They concluded that U6 is not suitable for evaluation of serum microRNAs in liver diseases [5].

U6 levels also display a massive interindividual variability in healthy subjects. Benz et al. found that serum levels of U6 showed a high variability of up to eight cycles in RT-qPCR analysis between the various samples of healthy subjects [5]. Similarly, Xiang et al. demonstrated that U6 expression showed large fluctuations in the serum samples of 30 healthy individuals, with a ΔCt value of 3.29 between the highest expression level and the lowest [6].

We conclude that U6 is not stably expressed between different individuals and its serum levels are dysregulated in a disease-specific manner thus being far from an ideal normalizer. U6 needs further evaluation to use as a reference gene for microRNA expression studies.

Conflict of interest

None declared.

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Reply to: "The crucial need of internal control validation in the normalization of circulating microRNAs"

Dear Editor,

We appreciate the comments received from Madadi et al. on the selection of U6 as a reference control to normalize the quantification of target miRNA in our study. We quantified the levels of circulating miR-125b-5p in 326 patients with chronic hepatitis B (CHB) using U6 as the reference control.

Circulating miRNAs are considered as next generation biomarkers for various diseases. Their stability and cell-free form in the blood make them robust markers. The quantification of these biomarkers is dependent upon several factors like sample preparation, RNA extraction, and detection methodologies. Different methodologies have been employed to measure the levels of circulating miRNAs. A common method is quantitative real-time polymerase chain reaction (qPCR), which uses endogenous and/or exogenous reference genes for data normalization. However, there is no consensus on the use of reference genes, also called housekeeping genes. To our knowledge, more than ten normalization strategies have been reported. Endogenous reference genes are widely used to normalize the target miRNA to remove variations arising due to sampling methods and the quality of the samples. One study used miR-16 as an internal reference gene to normalize serum miRNA-210 levels in different groups of CHB patients [1]. Cheng et al. [2] used miR-25-3p as a reference control to quantify miR-122-5p and miR-151a-3p in the plasma of CHB patients with persistently normal levels of alanine aminotransferase. Tan et al. [3] detected serum miRNAs (miR-122-5p, miR-141-3p, and miR-26b-5p) using miR-24 as an internal control in patients with primary biliary cirrhosis. These reference genes are constitutively expressed under certain conditions, but their levels may change in different diseases. For example, miR-16 was expressed stably in CHB patients, but their levels were significantly lower in patients with hepatocellular carcinoma (HCC) [4].

To remove the interference due to technical variability, synthetic and exogenous spike-in miRNAs are employed in qPCR. The *Caenorhabditis elegans* miRNA, cel-miR-39, is added to the samples prior to reverse transcription to monitor the levels of the target miRNA [5,6]. Other than cel-miR-39, cel-miR-238 has also been used as a reference gene to quantify serum miRNAs (miR-122, miR-99a, miR-125b, miR-720, miR-22, and miR-1275) in both hepatitis B and hepatitis C patients [7]. Rahmel et al. [8] chose cel-miR-54 as a reference control to measure the serum miR-122 levels in patients with acute liver injury due to acute respiratory distress syndrome. The major drawback of using spike-in controls is that this method does not consider the quality of the tissue and body fluids and the purity of the extracted RNA [9]. Improper sample collection, preservation, or handling can lead to cell lysis and RNA degradation, which cannot be corrected in the spike-in method. Thus, global standard reference genes need to be established to ensure reliable miRNA quantification and to allow comparison across studies.

The gene encoding the small noncoding RNA U6 is frequently used as a qPCR normalizer. One study reported using U6 as a normalizer to evaluate the levels of circulating miR-21 in HCC patients [10]. Zhang et al. [11] investigated serum miR-143 and miR-215 levels using U6 as a reference control in patients with CHB and HCC. In our study, we selected U6 to normalize the miR-125b-5p levels in the serum of CHB patients. We observed that U6 expression indeed fluctuated among patients, as previously reported [12,13], and that our results were consistent with previous studies [14,15]. Besides, we recently used cel-miR-39 as a reference control for analyzing the levels of other serum miRNAs and found that its levels also varied in some serum samples, but the range of fluctuation was relatively small. We reported a similar tendency for variation in serum miR-125b-5p levels in some of our previous serum samples using cel-miR-39 as a reference gene. In future, we propose the use of more stable reference genes, including cel-miR-39 and miR-26a, to verify our previous results in a larger sample size.

In summary, U6 levels fluctuate in the serum and it is not the optimal reference gene for quantifying circulating miRNA by qPCR. An ideal reference gene that not only expresses stably in the serum/plasma in different diseases but also remains uninfluenced by the differences in sampling methods and RNA quality is urgently needed for future miRNA studies.

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Conflict of interest
None declared.

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