Reply to “Need for Rigor in Design, Reporting, and Interpretation of Transcriptomic Biomarker Studies”

Pooling samples in biomedical studies has become frequent practice (8, 9, 13). For example, >15% of the data sets deposited in the Gene Expression Omnibus Database derive from pooled RNA samples (4). One reason in support of a pooling strategy is that the practice of pooling biological samples not only reduces biological variation but also minimizes the size and number of data files subjected to computer-intensive comparative analyses (7). A pooling strategy therefore remains valuable and is recommended in many settings, especially those which are resource constrained. Moreover, several recent publications have addressed the risk that pooling samples might potentially hide biological variance and give false confidence concerning the inferred significance of experimental data. For instance, Kendzior-ski et al. (4) showed that even when biological averaging does not hold, pooling can be useful and inferences regarding differential gene expression are not adversely affected by pooling.

In our recent study (2), a pooling strategy was used in analyzing the circulating microRNA (miRNA) population and the results suggested that miRNAs likely regulate the human immune response to *Mycobacterium tuberculosis*. In order to reduce biological variation, all subjects (there were no differences in age and sex between tuberculosis [TB] patients and healthy controls) were very carefully selected and all samples were very carefully prepared to ensure that individual samples contributed equally to the pool (2). The results of our pretest indicated that the miRNA profiles of pooled samples in general mirrored individual samples. So, to reduce biological variation and material costs, a pooled strategy was used for microarray analysis in our study (2). Moreover, to validate the microarray results, individual samples from controls and active TB cases were used for quantitative real-time reverse transcription-PCR (RT-PCR) analysis (1, 10).

Up to now, very stable internal controls of plasma miRNAs have not been reported (5), and many researchers are trying to discover appropriately stable internal controls for miRNA expression in serum. Although miR-375 has been used as an internal control in plasma (5), levels of miR-375 were increased in TB serum in our study, eliminating it as an appropriate internal control (2). U6 has also been used previously as a serum control (3, 6, 14). In our study, we detected U6 expression and found that there was no difference in U6 levels between TB cases and controls (U6 threshold cycle [C_T] levels, 21.32 in control serum and 21.29 in TB serum; these data represent the average values of $n = 30$). For this reason, U6 was used as the internal control in serum to normalize miRNA expression in real-time PCR analysis (2).

Pulmonary TB is a life-threatening infection generally accompanied by extensive tissue destruction and a systemic inflammatory state. These drastic physiological changes undoubtedly alter the serum miRNA expression profile of patients with active TB. The goal of our study was to identify differentially expressed miRNAs in the sera of patients with active pulmonary TB in comparison to those in the sera of matched healthy controls (11, 12). Moreover, our results initially showed that a number of miRNAs were differentially expressed during active pulmonary TB infection and it was difficult to draw definitive conclusions as to the

miRNAs involved in the pathogenesis of active pulmonary TB based on this single study.

Accordingly, in response to the points raised by Dr. Walter and colleagues in the [foregoing letter](#), we conclude that the perfect design of a biomarker study for the diagnosis of TB may never exist, no strategy is perfect, and additional studies that effectively challenge the specificity of TB markers would be exceedingly valuable. Further longer-term investigations are needed to determine whether differentially expressed microRNAs represent host responses that are specific or not to TB infection.

ACKNOWLEDGMENTS

This work was supported by grants from the National Science Foundation of China (30972639) and the Project of Shandong Province Higher Educational Science and Technology Program of China (J09LF20).

The microarray experiments were performed by KangChen Bio-tech, Shanghai, China.

REFERENCES

1. Diz AP, Truebano M, Skibinski DO. 2009. The consequences of sample pooling in proteomics: an empirical study. *Electrophoresis* 30:2967–2975.
2. Fu Y, Yi Z, Wu X, Li J, Xu F. 2011. Circulating microRNAs in patients with active pulmonary tuberculosis. *J. Clin. Microbiol.* 49:4246–4251.
3. Ji F, et al. 2011. Circulating microRNAs in hepatitis B virus-infected patients. *J. Viral Hepat.* 18:e242–e251.
4. Kendzior-ski C, Irizarry RA, Chen KS, Haag JD, Gould MN. 2005. On the utility of pooling biological samples in microarray experiments. *Proc. Natl. Acad. Sci. U. S. A.* 102:4252–4257.
5. Komatsu S, et al. 2011. Circulating microRNAs in plasma of patients with oesophageal squamous cell carcinoma. *Br. J. Cancer* 105:104–111.
6. Kuwabara Y, et al. 2011. Increased microRNA-1 and microRNA-133a levels in serum of patients with cardiovascular disease indicate myocardial damage. *Circ. Cardiovasc. Genet.* 4:446–454.
7. Leparc GG, Mitra RD. 2007. A sensitive procedure to detect alternatively spliced mRNA in pooled-tissue samples. *Nucleic Acids Res.* 35:e146. doi:10.1093/nar/gkm989.
8. Li LM, et al. 2010. Serum microRNA profiles serve as novel biomarkers for HBV infection and diagnosis of HBV-positive hepatocarcinoma. *Cancer Res.* 70:9798–9807.
9. Moussay E, et al. 2011. MicroRNA as biomarkers and regulators in B-cell chronic lymphocytic leukemia. *Proc. Natl. Acad. Sci. U. S. A.* 108:6573–6578.
10. Plymoth A, et al. 2006. Rapid proteome analysis of bronchoalveolar lavage samples of lifelong smokers and never-smokers by micro-scale liquid chromatography and mass spectrometry. *Clin. Chem.* 52:671–679.
11. Stern JN, et al. 2009. Molecular signatures distinguishing active from latent tuberculosis in peripheral blood mononuclear cells, after in vitro antigenic stimulation with purified protein derivative of tuberculin (PPD) or Candida: a preliminary report. *Immunol. Res.* 45:1–12.
12. Wang C, et al. 2011. Comparative miRNA expression profiles in individuals with latent and active tuberculosis. *PLoS One* 6:e25832. doi:10.1371/journal.pone.0025832.

Address correspondence to Yurong Fu, yufuyurong@163.com.

This is a response to a letter by Walter et al. (doi:10.1128/JCM.06775-11).

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JCM.06845-11

13. **Zanette DL, et al.** 2007. miRNA expression profiles in chronic lymphocytic and acute lymphocytic leukemia. *Braz. J. Med. Biol. Res.* **40**:1435–1440.
14. **Zhang Y, et al.** 2011. Altered expression levels of miRNAs in serum as sensitive biomarkers for early diagnosis of traumatic injury. *J. Cell Biochem.* **112**:2435–2442.

Yurong Fu

Department of Medical Microbiology
Shandong Provincial Key Laboratory of Clinical Laboratory Diagnostics
Weifang Medical University
Weifang, China

Zhengjun Yi

Department of Laboratory Medicine
Shandong Provincial Key Laboratory of Clinical Laboratory Diagnostics
Weifang Medical University
Weifang, China

Xiaoyan Wu

Department of Medical Microbiology
Shandong Provincial Key Laboratory of Clinical Laboratory Diagnostics
Weifang Medical University
Weifang, China

Jianhua Li

Fuliang Xu

Department of Laboratory Medicine
Shandong Provincial Key Laboratory of Clinical Laboratory Diagnostics
Weifang Medical University
Weifang, China