Housekeeping genes for studies of plasma microRNA: A need for more precise standardization

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Introduction. *Plasma microRNAs (miRNAs) are promising biomarkers for many forms of cancer in humans; however, a fundamental concern is the lack of standardization in current data acquisition and reporting. Part of this problem lies in the use of numerous, different housekeeping genes (HKG) for the acquisition of real-time polymerase chain reaction data. This existing practice of using different HKGs generally is accepted, but reproducibility of data for comparison and validation between different laboratories calls for improvement. The need for data reproducibility standardization is crucial. An ideal plasma HKG (1) should be expressed in all samples, (2) have medium-to-high levels of expression, and (3) have consistently measurable levels of expression.*

Methods. *Total RNA was extracted from 200-μL plasma samples via a modified miRNeasy (QIAGEN) extraction technique with yeast carrier. Total RNA purity was assessed with a Nanodrop 2000 spectrophotometer (Thermo Scientific). The cycle threshold (Ct) was fixed at 0.03 for all samples. We investigated 10 potential HKGs based both on reports in the literature and our previous data. The potential HKGs were Let-7a, Let-7d, Let-7g, miR-16, RNU6, RNU48, miR-191, miR-223, miR-484, and miR-520d-5p. Once all samples were run for each potential HKG, the mean Ct and SD was calculated for all sample groups, allowing for comparison among HKGs.*

Results. *We screened 380 miRNAs by using microfluidic array technology (Applied Biosystems) in a discovery cohort of 20 colorectal cancer (CRC) patients, 10 patients each with breast cancer (BC), lung cancer (LC), pancreatic cancer (PC), 11 patients with colorectal adenoma, and 12 controls. The mean Ct and SD was calculated for RNU6, miR-520d-5p, miR-16, miR-191, miR-223, and miR-484, which were expressed in all samples. Let-7a, Let-7d, Let-7g, and RNU48 were only expressed in 26%, 7%, 10%, and 8% of samples, respectively, and therefore were deemed to be insufficiently reliable HKGs. Only miRNAs with >50% expression were included in this statistical analysis. U6 and miR-520d-5p had the most consistent Ct as well as the least SD. The use of both RNU6 and 520d-5p as HKGs provided reliable results.*

Conclusion. *Among HKGs that were expressed in all samples, we suggest that RNU6 and miR-520d-5p were the best candidates for HKGs for studies of plasma miRNA because of the consistent and high Ct in all samples and a very narrow, reproducible SD. (Surgery 2015;158:1345-51.)*

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Since their initial description in the early 1990s, microRNAs (miRNA) have been used increasingly as biomarkers for many human diseases. Certain miRNAs have been described to be present not only in tissue but in a variety of body fluids, including plasma, urine, amniotic fluid, pleural effusions, and others.[1] It is estimated that miRNAs regulate the function of the majority of protein-coding genes in mammals by repressing translation. These miRNAs have been proposed as biomarkers for many cancers, because they are inherently stable, involved

in numerous regulatory pathways, and have been shown in other disease states to be sensitive and specific biomarkers. Certain miRNAs have been evaluated in many common human cancers, including esophageal, lung, colon, breast, pancreas, adrenocortical, and thyroid cancer.[2-13] All of these cancers have miRNAs that are relatively sensitive and specific for that type of cancer. Therefore, miRNAs in the future may provide surgeons assays to screen patients for the presence of a disease, as well as assays to monitor patients for recurrence after resection. Unfortunately, with the great increase in reported studies of miRNA, there has been little standardization of methodology, making it difficult to compare different studies with each other.

Most plasma miRNA-based research requires ultimately the use of a housekeeping gene (HKG) as a reliable, reproducible marker or reference with which to compare the expression of other miRNAs. Such reliability and reproducibility is essential when considering clinical tests that may become ultimately an integral part of clinical practice.[13] A test should not be used unless it is consistent and reproducible from laboratory to laboratory. Most tests that investigate plasma miRNAs currently use HKGs as an internal reference against which to base their results.[14-19] With an external control, a known quantity of another gene is added to a sample as reference. From a recently published systematic review, 42% of the plasma miRNA studies published during a 1-year period used an external control, whereas 43% used an internal control (HKG), and 16% did not specify what type of reference was used.[20] The HKGs must be present in all subjects at a consistently detectable level, which allows for reference normalization of other miRNAs that could have variable expression in different disease states.

HKGs themselves usually are involved in some underlying basic cellular function.[21-32] Accordingly, HKGs usually are expressed constitutively at some baseline level in all cells under normal and abnormal conditions. This expression is essential for the researcher when providing a baseline against which to compare abnormal levels. Interestingly, the importance of standardization of HKGs in studies of plasma miRNA has not been discussed widely. In contrast, reproducibility recently has been a concern in miRNA research.[33] Because the use of miRNA is a relatively new field, the use of many different extraction techniques and HKGs creates an intellectual free-for-all. This being the case, one must seek HKGs that are reliable to use for any laboratory, which will be important if miRNAs eventually become excellent tumor markers for multiple types of cancers; HKGs that are reproducible and reliable are essential.

Table I. Housekeeping gene and known function in humans

Housekeeping gene	*Function*
Let-7a	Tumor suppressor gene[23]
Let-7d	Tumor suppressor gene[23]
Let-7g	Tumor suppressor gene[23]
miR-16	Tumor suppressor gene[29]
miR-191-5p	Oncogenic regulator[22,30,34]
miR-223	Oncogene[24,26,27]
miR-484	Protein translation suppressor[32,35]
miR-520d-5p	Metastasis regulator[31]
RNU6	Transcription gene promoter[28,36]
RNU48	Ribosome biogenesis methylation director[21,25]

HKGs that are used commonly currently in plasma miRNA research include Let-7a, Let-7d, Let-7g, miR-16, RNU6, RNU48, miR-191, miR-223, miR-484, and miR-520d-5p, among others (Table I). This list gives investigators many options but creates confusion when one is trying to interpret published work and reproduce the experiments of other scientists using different HKGs. Many, including our laboratory, have used RNU6 as a HKG. The selection of HKG may, however, need to change with type of specimen analyzed (ie, specific disease, type of tissue, plasma, or other body fluids).

MATERIALS AND METHODS

Patient population. This study was approved by the University of Louisville Institutional Review Board, and written informed consent was obtained from all subjects. All subjects were from a large university surgical and medical practice or from the University of Louisville Biorepository. Plasma samples of patients with breast cancer (BC), lung cancer (LC), and pancreatic cancer (PC) were obtained from the University of Louisville Biorepository, whereas plasma samples of patients with colorectal cancer (CRC) came from both our surgical practice and the Biorepository. Stage and distribution of sex for cancer patients is shown in Table II. Plasma samples from patients with colorectal adenomas and controls were obtained exclusively from our clinical practice. Control plasma samples were derived from individuals undergoing colonoscopy who were free of colon cancer, colorectal adenomas, or inflammatory bowel disease.