1 copy/reaction has to be questioned. Kawano et al do not present any controls for the PCR (-RT, no template control), nor for the specificity of the amplicons. Visualization by gel electrophoresis would help to exclude nonspecific products, but this is not provided.

Kawano et al calculate a relative microRNA expression between $10^{-2}$ and $10^{-8}$. This range corresponds to a large difference between the ΔCt-values. An expression of $10^{-4}$ requires 10 PCR cycles and an expression of $10^{-7}$ requires 20 PCR cycles. A ΔCt of 20 or more cycles reflects the detection limit of the PCR, and nonspecific amplifications have to be expected. A cutoff value to discriminate between positive and negative results is not provided by the authors.

The authors describe a correlation between EBV viral load and the expression of microRNA. Detailed data or a figure supporting this statement are not provided. Figure 5A in the article indicates no correlation between microRNA and viral load [1].

The observation that the authors detected EBV-microRNA in seronegative patients in whom no viral load was detected is not plausible and raises doubts regarding the specificity of the PCR. We are convinced that these results have to be interpreted as nonspecific amplifications. The corresponding relative expression in these patients was calculated to be $10^{-7}$. This would mean that the PCR cutoff is at least above this value. Furthermore, the microRNA BART-7 in seronegative patients was detected at a relative expression of $10^{-4.5}$. This value is significantly above the expression level of some microRNAs in seropositive patients with active EBV replication. In the article by Kawano et al, expression of BART-7 reaches very high levels in all patient subgroups and higher levels than all other microRNAs. This is in line with a study by Gourzones and colleagues [4] that used the same TaqMan-assay provided by AppliedBiosystems. Therefore, it cannot be excluded that primer and probes for BART-7 reveal nonspecific results, possibly due to an overlap between stem-loop and forward primer (Figure 1).

In conclusion, detection of RNA fragments as short as microRNA is a challenge and requires the use of stem-loop-primers. However, these primers should be evaluated carefully for each individual microRNA (cutoffs). Without individual validation, expression levels of different microRNAs cannot correctly be related to each other.

Notes

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and the expression levels of microRNA were analyzed using Pearson correlation coefficient analysis. A significant correlation was detected between the plasma EBV DNA copy number and the level of each microRNA, except for that of miR-BHRF1-3. The $r$ values for miR-BART13, miR-BART2-5p, and miR-BART15 were .630, .595, and .735 (all $P < .01$), respectively (Figure 1).

Notes

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Influenza Vaccination Effectiveness, Unmeasured Confounding, and Immunomodulatory Treatment

To the Editor—Inactivated influenza vaccines have been the foundation for public health strategies for the control of influenza for almost 50 years. Despite increasingly broad vaccination recommendations and expanded programs for vaccine delivery in many countries, there is still widespread public discussion and often partisan debate among experts about the effectiveness of annual vaccination in reducing influenza-related acute respiratory illness, hospitalization, and mortality. The recently introduced case test-negative method provides another way of evaluating influenza vaccination effectiveness [1]. With this method, patients with influenza-like illness are tested for influenza virus infection by reverse-transcription polymerase chain reaction; cases are those who are test-positive and controls are those who are test-negative. Several studies, including that by Bateam et al [2], have used this method to estimate vaccination effectiveness in preventing acute respiratory illness and hospitalization with influenza-like illness in older adults [2–4]. While the case test-negative method offers greater sensitivity, it has several potential shortcomings, including delayed or incomplete virological testing and inadequate ascertainment of vaccination status. In addition, like case-control and other observational study designs, it too depends on statistical adjustment for confounding factors, such as chronic medical conditions, functional status, and patterns of healthcare-seeking behavior. Whether investigators examine groups of individual patients or large administrative databases, accurate adjustment for confounding variables remains an essential requirement for any valid observational study. All would agree that unmeasured confounding potentially affects the validity of any attempt to estimate influenza vaccination effectiveness.

Observational studies of influenza vaccination effectiveness in older adults report that a high proportion of study subjects have chronic medical conditions, and investigators routinely adjust for these conditions in their analyses. These patients also take a large number of medications for these conditions. In the Canadian study cited above, the average number of prescriptions received by each study subject during the preceding year was >15 [4]. In the United States, the National Center for Health Statistics has reported on current use of selected prescription drug classes for persons ≥65 years of age for the period 2007–2010 [5]. Among those with hyperlipidemia, 46.7% were receiving treatment, with most probably taking statins. For those with heart disease and high blood pressure, 21.9% were taking angiotensin-converting enzyme (ACE) inhibitors, and 12.2% were taking angiotensin receptor blockers (ARBs). For those with diabetes, 18.4% were being treated, with metformin probably used in most cases. These drugs have broad antiinflammatory and immunomodulatory (pleiotropic) effects and might be useful in treating influenza and other forms of acute critical illnesses [6].

Over the past decade, numerous observational studies have suggested that outpatient statin treatment reduces the risk of hospitalization and death due to pneumonia and sepsis [6]. A recent propensity-matched case-control study of 23 000 adults ≥65 years of age hospitalized with community-acquired pneumonia showed that inpatient statins, ACE inhibitors, and ARBs were associated with 32%–53% reductions in 30-day all-cause mortality [7]. In this study, 30% of subjects were receiving statins, 30% were receiving ACE inhibitors, and 4% were receiving ARBs. In another study, which examined 3043 patients hospitalized with laboratory-confirmed seasonal influenza, statins reduced 30-day mortality by 41% [8]. This reduction was in addition to any that might have been attributed to previous influenza vaccination or antiviral treatment. Moreover, a randomized controlled trial involving only 100 statin-naive patients hospitalized with sepsis showed that inpatient atorvastatin treatment begun on the first hospital day reduced progression to severe sepsis by 83% [9]. These and many other studies suggest the possibility that statins, ACE inhibitors, ARBs, and other immunomodulatory agents might reduce mortality due to seasonal and pandemic influenza [6]. They also suggest that these agents might be important confounders in observational studies of influenza vaccination effectiveness [6].

None of the case test-negative studies mentioned above, nor earlier case-control and cohort studies of influenza vaccination effectiveness, have considered statins, ACE inhibitors, ARBs, or other immunomodulatory agents as potential confounding variables. We do not yet know whether it is essential to consider these agents in adjustment strategies, independent of the underlying medical conditions for which they have been prescribed. However, long-term adherence to treatment with statins and these other agents is often poor, and nonadherence can increase risks of hospital readmission and death [10]. Because these agents are often used to treat older adults, and because such treatment could affect rates of influenza-related hospitalization and mortality, estimates of influenza vaccination effectiveness based on studies that have not considered them as potential confounders should probably be regarded as imprecise.