Type 1 Diabetes in Children With Genetic Risk May Be Predicted Very Early With a Blood miRNA

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Progression to clinical type 1 diabetes is monitored through the appearance of islet autoantibodies against pancreatic β-cell antigens, and most children with two or more autoantibodies progress to disease (1). However, autoantibodies indicate already active islet autoimmunity, and by that time, loss of immune tolerance may have reached a point of no return. Thus, there is an urgent need for biomarkers that would predict the disease before the appearance of islet autoantibodies and provide a longer window for intervention. New biomarkers might help identify optimal sets of subjects for clinical trials or a subgroup of patients who may be more likely to benefit from a given therapy.

MicroRNAs (miRNAs) secreted in extracellular vesicles have been detected in blood and may have biomarker potential (2). Several studies have shown the usefulness of miRNAs as biomarkers for many diseases (reviewed in Das et al. [3]). Aberrant miRNA expression has been observed in sera of patients with type 1 diabetes (reviewed in Ventriglia et al. [4]). However, most miRNA studies have analyzed samples at or after the onset of clinical type 1 diabetes.

Here, we analyzed whether we can detect changes in miRNA levels before and during islet autoimmunity in whole-blood samples from children with HLA-conferred risk of type 1 diabetes participating in the Type 1 Diabetes Prediction and Prevention (DIPP) study (5). Children from the DIPP study with high HLA-conferred risk were followed up, and whole-blood samples were collected at multiple time points. Case-control matching was based on HLA-DQB1 genotype, date and place of birth, and sex, as described earlier (5). To study genome-wide miRNA profiles before the clinical presentation of type 1 diabetes, we first performed miRNA sequencing (miRNA-seq) on 87 longitudinal samples collected from four multiple-autoantibody–positive case subjects and matched autoantibody-negative control subjects (Fig. 1*A*, miRNA-seq discovery cohort) using the Illumina HiSeq 2500 platform. A linear mixed-effects model for each miRNA was used to test differential expression between case and control subjects. The most significantly upregulated miRNA in case subjects was hsa-miR-6868-3p ($P < 0.001$), which was not previously associated with type 1 diabetes. Interestingly, hsa-miR-6868-3p was upregulated before seroconversion (Fig. 1*B*). We confirmed the finding ($P < 0.001$) on these and 10

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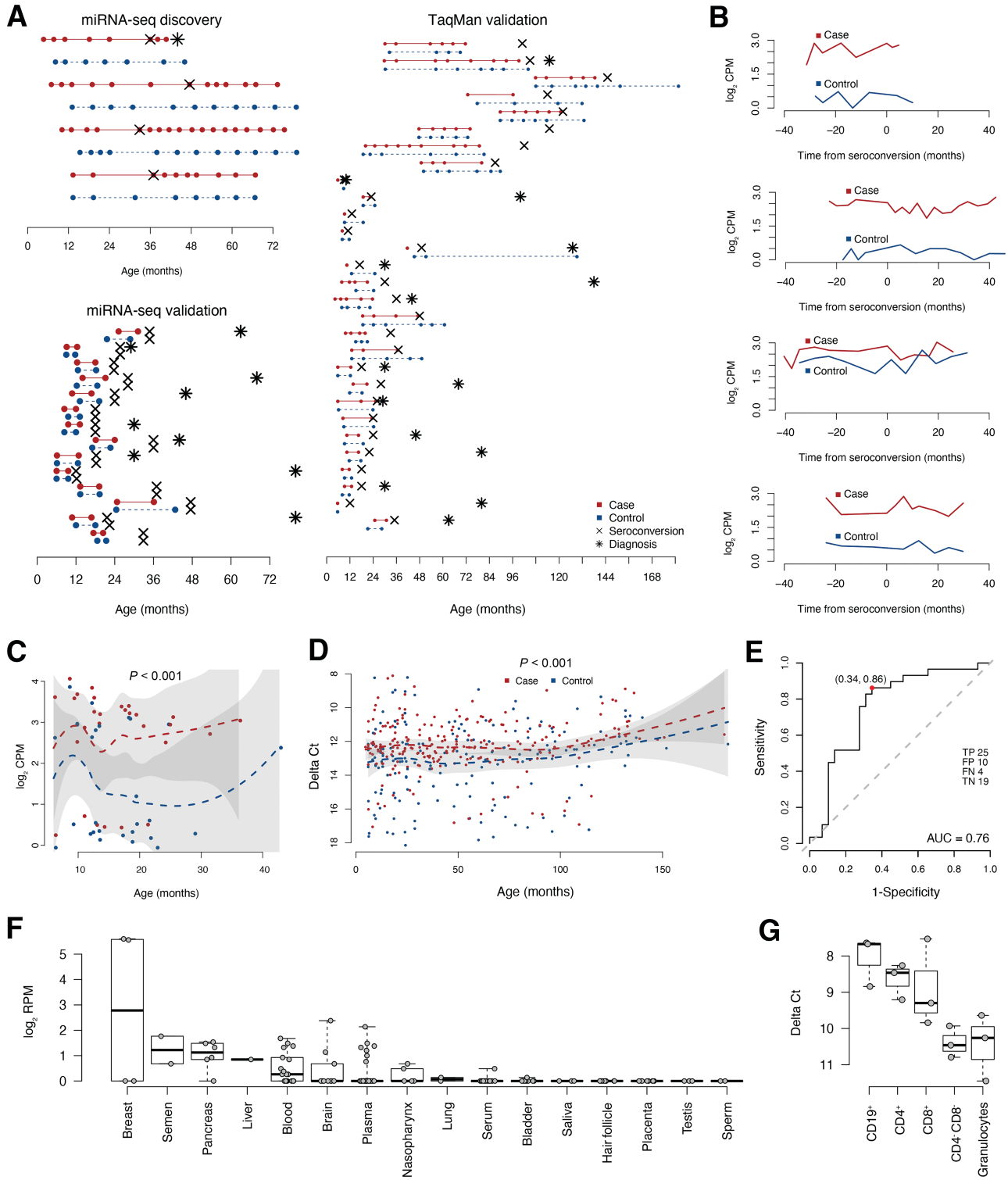

Figure 1–hsa-miR-6868-3p as early marker for type 1 diabetes. *A*: The samples from the miRNA-seq discovery cohort (top left), the miRNA-seq validation cohort (bottom left), and the TaqMan validation cohort (right). Each line is an individual, and each dot is a PAXgene sample. *B*: The line plots showing the expression profiles of hsa-miR-6868-3p for the four case-control pairs of the discovery cohort. The plots are seroconversion centered. CPM, counts per million. *C*: Line plots showing the average longitudinal case-control profiles of hsa-miR-6868-3p in the validation cohort. Each red and blue dot shows a case or a control sample, respectively. The dashed lines show average expression, and the gray area shows a 95% confidence interval. *D*: TaqMan expression of hsa-miR-6868-3p over time in 29 case-control pairs. The *P* value shown at the top was obtained from the linear mixed-effects model. *E*: ROC analysis showing the performance of hsa-miR-6868-3p in differentiating the DIPP case subjects from control subjects in samples before the appearance of islet autoantibodies. The delta Ct values were adjusted using linear regression with HLA type (DR3/DR4, DR3/DR3, DR4/DR4, DR3/other, and DR4/other) and TaqMan plate as explanatory variables. The resulting residuals were used to normalize the possible effects of HLA and plate. True positives (TP), true negatives (TN), false positives (FP), and false negatives (FN), as predicted by the model, are shown on the plot. AUC, area under the curve. *F*: The expression profile of hsa-miR-6868-3p across different tissues. The reads per million (RPM) data were taken from miRmine database (https://guanfiles.dcmb.med.umich.edu/mirmine/index.html). *G*: Expression of hsa-miR-6868-3p in blood cells of healthy donors (n = 3). Each dot represents an individual.

additional case-control pairs using miRNA-seq of two time points before seroconversion (Fig. 1*A*, miRNA-seq validation cohort, and Fig. 1*C*).

We further confirmed the miRNA upregulation by TaqMan quantitative RT-PCR assay in samples collected from 29 case-control pairs, of which 14 were included in the miRNA-seq analysis (Fig. 1*A*, TaqMan validation). A strong correlation (r = 0.75) was observed between the sequencing and TaqMan results for hsa-miR-6868-3p expression. The TaqMan data showed higher hsa-miR-6868-3p expression in cases than controls ($P < 0.001$) across the time points (Fig. 1*D*), convincingly recapitulating the miRNA-seq result.

Given the early upregulation of hsa-miR-6868-3p, we tested whether the miRNA can distinguish the 29 case subjects from control subjects before seroconversion, using the average expression before seroconversion for a given child. The changes in threshold cycle (delta Ct) expression values, calculated relative to hsa-miR-191-5p, were adjusted for individual HLA type and TaqMan plates using a linear model. The area under the receiver operating characteristic (ROC) curve was 0.76 (Fig. 1*E*), suggesting that the miRNA may indeed serve as a screening biomarker for the stratification of children at increased genetic risk for type 1 diabetes.

In addition to being expressed in blood, breast, and brain, the miRNA is expressed in the pancreas (Fig. 1*F*), presenting an interesting possibility that its upregulation in blood samples of case children originates from the pancreas and circulates through blood under inflammatory conditions. It is important to note that this miRNA may also come from blood lymphocytes, given its expression in these cells (Fig. 1*G*).

The ROC analysis implied that hsa-miR-6868-3p may serve as a screening biomarker for the stratification of children at risk for islet autoimmunity. However, the small cohort size of 58 study subjects is a limitation of the study, and the finding should be validated in whole-blood samples of an independent, preferably larger cohort. For a screening biomarker, high sensitivity is preferred over high specificity (i.e., false negatives are of more concern than false positives). The current model reaches a higher sensitivity of 0.86 at the specificity of 0.66. It remains to be seen whether combining hsa-miR-6868-3p with other miRNAs or mRNAs will improve the performance of the predictive model. It will also be interesting to determine whether the miRNA expression correlates with the time from seroconversion to clinical disease.

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Author Contributions. T.S. and U.U.K. designed experiments, analyzed the data, prepared the figures, and wrote the manuscript. O.R. designed experiments, analyzed data, and wrote the manuscript. A.L., H.K., M.V.-M., M.N., J.M., and T.H. contributed to the design of the study and analysis. H.H., J.I., R.V., J.T., and M.K. were responsible for the DIPP cohort. R.V. and M.K. were responsible for the islet autoantibody analyses. All authors contributed to the final version of the manuscript. R.L. initiated, designed, and supervised the study. L.L.E. participated in the design of the study and analysis and supervised the study. R.L. and L.L.E. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Data and Resource Availability. Analyzed count data from miRNA-seq of the discovery cohort (87 samples from four case-control pairs) and the validation cohort (56 samples from 14 pairs) can be accessed from the ArrayExpress database (https://www.ebi.ac.uk/arrayexpress/) using the accession codes E-MTAB-10959 and E-MTAB-10968, respectively. Other data generated during the current study are available from the corresponding author on reasonable request.

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