Low and high \textit{BAALC} expression groups for gene-expression and miRNA-expression array comparisons

For the 89 patients included in this study that were enrolled on CALGB 19808, 77 patients were analyzed by microarrays. Of these 77 patients, those in the lowest third (n=26) of the distribution of \textit{BAALC} expression values measured by real-time RT-PCR were compared with those in the highest third (n=26) of the distribution in order to derive expression signatures associated with \textit{BAALC}. Samples from 48 of these 52 patients were measured by both gene-expression and miRNA-expression arrays. Of the remaining four patients, two with low expression of \textit{BAALC} lacked gene-expression array analysis and two with high expression of \textit{BAALC} lacked miRNA-expression array analysis; the lacking array analyses were primarily due to insufficient RNA concentration. Consequently, the gene-expression array comparison was based on 24 low and 26 high \textit{BAALC} expressers while the miRNA array comparison was based on 26 low and 24 high \textit{BAALC} expressers.

Probe signal normalization for miRNA-expression array comparison

The signal intensity was calculated for each spot without adjusting for local background. Spots with a low signal-to-noise ratio were considered as missing values. Intensities were log-transformed and log-intensities from replicate spots were averaged. A median-centering normalization was performed based on all human miRNA probes represented on the array. miRNA probes with a low signal-to-noise ratio on 50% or more of arrays were excluded from subsequent analyses, reducing the number of examined human miRNA probes in the training set to 305. For each miRNA probe, an adjustment was made for batch effects (ie, differences in expression related to the batch in which arrays were hybridized). The batch adjustment was made by fitting a linear model for the expression values of each miRNA probe with array batch as the factor. A correction to the expression values was then made for the measured batch effects.

Quantitative real-time PCR assay for miRNA-expression analysis

First-strand cDNA synthesis for the quantitative miRNA real-time PCR assay was carried out from 50 ng of total RNA in 20 µl of final volume containing 50 nM stem-loop primer for miR-148a and RNU6, 1xRT buffer, 3.33 U/µl MultiScribe reverse transcriptase and 0.25 U/µl RNase inhibitor. The mix was incubated at 16°C for 30 min, 42°C for 50 min, 85°C for 5 min and then held at 4°C.

Real-time PCR was performed using 2 µl of RT product, 1× Universal TaqMan Master Mix and 1× TaqMan probe/primer mix in a total reaction of 10 µl and run on the Applied Biosystems 7900HT Fast Real-Time PCR system (all reagents, primers and probes were obtained from Applied Biosystems). All RT reactions including water controls were run in triplicate. RNU6 was used as an endogenous control and data were normalized against RNU6 expression. The relative amount of transcript was calculated using the comparative Ct method.