Introduction

Most of the discoveries from gene expression data are driven by a single study claiming an optimal subset of genes that play a key role in a specific disease within a particular clinical setting: diagnostic, prognostic, or response to treatment. However, poor agreement between the results of differentially expressed gene analysis from different gene expression experiments with a similar research question has been reported, which is possibly due to the well-known “curse of dimensionality” in microarray data.

Meta-analysis of the available datasets potentially helps in getting reliable results so that a real-life application may be more successful. Given the costs of experiments and the public availability of datasets, combining existing information from multiple gene expression experiments is an efficient tool to increase statistical power and to obtain more generalizable results. Guidelines in conducting a meta-analysis of microarray gene expression studies have been offered by Ramasamy et al. and recently by Gan et al. to specifically combine Affymetrix-based datasets. The proposed meta-analysis techniques have found their application in gene expression studies, eg, by Yi and Park, Li and Gosh, and Chang et al., as well as their application to find promising biomarkers.

The common goal of a meta-analysis is to increase the precision of the effect estimate. A cumulative meta-analysis combines studies in chronological order so that the change of the effect size estimate can be observed when a study is added to the analysis. However, in general, there is no adjustment for repeatedly testing the null hypothesis; nor can the power of the statistical analysis be quantified. As an alternative, sequential meta-analysis (SMA) has been proposed. SMA is not commonly applied yet, but it can be an efficient decision-making tool. In an SMA, we are able to see whether we already have enough evidence to draw a conclusion, a property that an “ordinary” meta-analysis does not have. This may be particularly useful if a series of experiments have already been done, to decide to start a new study or not and potentially save resources.

This study focuses on the application of SMA to find significant gene expression signatures across a number of microarray experiments. The sequential method in this study is applied to evaluate whether accumulated samples already show enough evidence for a certain effect size or whether more experiments should be initiated. Application of an SMA is illustrated by an example in acute myeloid leukemia (AML). We incorporated the between-study variance in the SMA to
adjust for the different nature of the experiments, such as the experimental setting and sample characteristics.

**Methods**

We describe the details of the proposed approach in this section and summarize them in Figure 1. We distinguished three major steps, namely data collection, data preparation, and data analysis. Raw gene expression datasets are obtained either as available data from different laboratories and/or from a systematic search in the online repositories. The raw datasets are then preprocessed prior to the data analysis. Finally, SMA is applied to combine the gene expression studies. As an illustration, we apply the proposed approach to find genes that are differentially expressed between normal controls and patients with AML. All statistical analyses described in this section were performed in R software.

**Step 1: Data collection.** In addition to finding a gene signature list, the sequential approach that was applied in this example also acts as a tool to evaluate the necessity of the next prospective experiment. A new experiment needs to be performed when we have not decided yet for all or at least a substantial number of observed genes to be either differentially or nondifferentially expressed, due to insufficient evidence from the experiments done so far to draw a conclusion for each gene. The datasets may be combined from different experiments/laboratories and/or collected from systematic search in online repositories. We recommend using raw data to reduce the source of variability due to different preprocessing procedures. We used the downloaded gene expression datasets from ArrayExpress by using *acute, myeloid, and leukemia* as keywords. We included experiments that had been done in *Homo sapiens* and had not used DNA by array assay technology. We left out experiments that had samples without class labels or that had no raw cell files. As a result, we found seven gene expression datasets, as summarized in Table 1 and briefly described below.

**E-GEOD-12662.** The main objective of this study was to characterize acute promyelocytic leukemia (APL), which is a subtype of AML. The experiment was conducted on 106 samples. The RNA samples were drawn from 76 de novo adult patients with AML and 30 healthy bone marrow donors.15

**E-GEOD-14924.** Peripheral blood samples from newly diagnosed patients with AML were used to observe the effect of having AML on the patients’ T cells. The peripheral blood T cells from healthy volunteers served as a control to be compared with patients with AML. The study used CD8 and CD4 T cells in the experiment for both patients with AML and normal controls, resulting in four groups with 10 samples each. In our analysis, we took the 20 samples from CD4 T cells from patients with AML and normal controls.16

**E-GEOD-17054.** Gene expression datasets were obtained from University of Michigan and Stanford University to study dysregulated pathways between normal bone marrow hematopoietic stem cells (HSC) and leukemic stem cells from patients with AML (AML LCC) samples. A set from Stanford University only was available and therefore included

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**Figure 1.** General proposed approach to apply sequential meta-analysis to gene expression datasets. The details for each step are described in the Methods section.

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**Table 1.** Characteristics of the seven selected microarray experiments.

| DATA ID     | ARRAYEXPRESS ID | YEAR | AFFY PLATFORM | SAMPLE SIZE (CONTROL: AML) | P₁ | P₁* | P₂ | P₂* | PSEG | RANGE (MIN; MAX) |
|-------------|-----------------|------|---------------|----------------------------|----|-----|----|-----|------|------------------|
| 1 E-GEOID-12662 | 2008            | HG-U133 Plus 2 | 106 (30;76)   | 54675                      | 19851 | 25323 | 11989 | 6085 | 1.80; 14.31 |
| 2 E-GEOID-14924 | 2009            | HG-U133 Plus 2 | 20 (10;10)    | 54675                      | 19851 | 35570 | 15319 | 4306 | 2.46; 14.23 |
| 3 E-GEOID-17054 | 2009            | HG-U133 Plus 2 | 13 (4;9)      | 54675                      | 19851 | 27504 | 13125 | 440  | 2.46; 14.38 |
| 4 E-MTAB-220   | 2011            | HG-U133 Plus 2 | 43 (10;33)    | 54675                      | 19851 | 38997 | 16432 | 882  | 2.15; 14.67 |
| 5 E-GEOID-33223| 2012            | HG-U133 Plus 2 | 30 (10;20)    | 54675                      | 19851 | 32472 | 14771 | 906  | 1.85; 14.93 |
| 6 E-GEOID-35010| 2012            | HG-1.0 st.v1   | 12 (6;6)      | 32321                      | 19878 | 19772 | 13688 | 501  | 2.09; 12.94 |
| 7 E-GEOID-37307| 2012            | HG-U133A      | 47 (17;30)    | 22283                      | 12496 | 19168 | 11355 | 1130 | 3.24; 14.22 |

**Notes:** P₁: The initial number of probesets. P₁*: The number of unique genes among P₁ (replicated genes were summarized by their median). P₂: The number of probesets after filtering. P₂*: The number of unique genes among P₂ (replicated genes were summarized by their median). PSEG: The number of differentially expressed genes determined by LIMMA and FDR 5% for the corresponding gene expression dataset. Range: The range of gene expression datasets after normalization and log transformation (1).
in our analyses, which contains nine AML LCCs and four normal HSCs.17

E-MTAB-220. C133+ cell fractions were isolated from the bone marrow of 33 patients with AML and 10 healthy donors, and their transcriptional profiles were assessed with Affymetrix HG U133 Plus 2.0. The experiment had been initiated to assess the association of WNT/β-catenin signaling with AML.18

E-GEO-33223. Thirty peripheral blood mononuclear cell (PBMC) samples were included in an experiment that was aimed at observing the factors that influence the prognosis in CEBPA-mutated AML. The participants were categorized into three groups, ie, control patients (n = 10), AML patients with multilineage dysplasia (MLD, n = 9), and AML patients without MLD (n = 11). We regrouped the samples into AML and normal controls.19

E-GEO-35010. Short-term (ST-HSC) and long-term HSC (LT-HSC), as well as granulocytic monocytic and progenitors (GMP) from patients with AML were compared in gene expression to healthy controls. The gene expression data from the GMP were used in our analysis, with six patients in the AML group and six healthy controls.20

E-GEO-37307. Microarray gene expression experiment was carried out in 30 AML patients and 19 normal HSC donors to identify genes that were differentially expressed between those groups. The gene expression data were obtained either from cryopreserved mononuclear cells or from testis cells. We excluded the two samples that had been obtained from testis cells.

Step 2: Data preparation. We followed a common procedure in preprocessing raw gene expression datasets for further analysis.

Preprocessing data. Methods are widely available for the preprocessing steps, eg, normalization, background correction, and logarithmic transformation. The choices for preprocessing microarray gene expression data have been widely discussed in the literature.21,22 A different choice of preprocessing methods may lead to a different result. However, this particular issue will not be covered in this study. The investigator may choose preprocessing methods by familiarity, with good knowledge of their properties. In the practical example of our proposed SMA approach, we normalized the raw datasets by quantile normalization, performed background correction according to manufacturer’s platform recommendation, and transformed the expression values to the log2 scale.23 For the Affymetrix platforms, median polish was used as a summarization method of probes into probesets, to deal with outlier probes.24

As is common in microarray studies, we also applied a filtering step to reduce the number of noninformative probesets. We only used detection call filtering to minimize the risk of excluding informative genes, ie, we retained all probesets whose log2 expression values were greater than 5 in at least 10% of the samples. The differentially expressed genes resulting from the SMA from filtered and nonfiltered data were then compared.

Gene annotation. Deciding the “objects” to be combined from multiple studies is another point to consider in pooling multiple studies, eg, combining expression values either at the probeset level or at the gene level. Due to the fact that different platforms may have different probeset names for the same genes, we mapped the probesets to the gene level to increase the agreement among the experiments. Since all the selected experiments had been performed on Affymetrix chips, we used the array-specific AffymetrixID by using the Bioconductor package (annotation packages: hgu133plus2.db, hgu133a.db, hugene10stprobeset.db, hugene10stproscript-cluster.db, and hugene10stv1probe).25 To deal with multiple probesets referring to the same gene, in each experiment we summarized the replicated genes by taking the median of their expression values.6

Step 3: Sequential meta-analysis. In each individual dataset, we performed a differential expression analysis by fitting a linear model using “limma” in R and controlling the false discovery rate at 5%, defined as the expected proportion of false rejection among the rejected hypotheses, using the Benjamini and Hochberg (BH) procedure.27 The resulting differentially expressed genes (DEGs) from each study were compared between studies.

Next, we applied an SMA following Whitehead’s boundaries approach for a double triangular test (TT).28 Sequential design and analysis was originally developed to monitor results of a randomized clinical trial (RCT) in order to draw a conclusion when enough evidence is available. There, the patient is the unit of analysis. The method can also be applied on a higher level, in the setting of a meta-analysis, where the unit of analysis is the study. The method can also be applied to nonrandomized studies. The application of SMA to microarray experiments is challenging, in the sense that we are dealing with thousands of end points (expression value of genes) that are analyzed simultaneously, and far more complex than sequential analysis in the clinical setting, where we usually have a single end point.

As previously described, the SMA is applied to each and every single gene by testing the null hypothesis that the average expression value is equal between two groups against the alternative hypothesis that there is a certain difference in average expression values between two groups. For gene i, the two-sided hypothesis testing is formulated as

\[ H_0 : \theta_i = 0 \]

\[ H_1 : |\theta_i| > \theta_k \]

where \( \theta_i \) is the standardized mean difference, also known as Cohen’s effect size, between average expression values in the two groups, and \( \theta_k \) is the prespecified relevant effect size.29 The same \( \theta_k \) is assumed for all the genes. The effect size of gene i in experiment j is estimated as
where $\bar{Y}_{j0}$ and $\bar{Y}_{j1}$ is the mean of (base 2) logarithmically transformed expression values of gene $i$ in Group 0 (1). Whitehead defined $s_j$ as the square root of the pooled variance estimate of the within-group variances. We, however, adopted the definition of $s_j$ as in the limma procedure, by borrowing information of variances from all tested genes. To be more specific, $s_j$ is the square root of the shrunken variance to a common variance by applying the empirical Bayes method. A common simple correction factor $f$ is

$$f_y = 1 - \frac{3}{4(n_{j0} + n_{j1}) - 9},$$

where $n_{j0}$ and $n_{j1}$ are the sample sizes in Group 0 and Group 1, respectively. The unbiased estimate of the effect size becomes $f_y \hat{\theta}_y$, and the variance estimate is redefined as $f_y^2 s_y^2$.

**Construction of the double triangular test.** The expression values of gene $i$ are analyzed in chronological order of experiments. In the TT test, each gene in each experiment contributes to two statistics, namely $Z$ and $V$, where $Z$ is the efficient score for $\theta$, and $V$ is Fisher’s information. The expression values for gene $i$ in experiment $j$ are transformed to $Z$ and $V$ values by the following formulas:

$$Z_y = \frac{n_{j0}n_{j1}(\bar{Y}_{j0} - \bar{Y}_{j1})}{s_{ij}^*} \text{ and } V_y = \frac{n_{j0}n_{j1}}{n_j} - \frac{Z_{ij}^2}{2n_j},$$

where $n_j$ is the total sample size in experiment $j$, i.e., $n_{j0} + n_{j1}$. We defined $s_{ij}^*$ as the square root of the shrunken variance to a common variance from a limma model with intercept only [while Whitehead defined the $s_j$ as the maximum likelihood (ML) standard deviation assuming equal means]. To incorporate heterogeneity, a weight is assigned to Equation (2), which is calculated by

$$w_y = \frac{1}{V_y + \hat{\tau}_y^2}.$$

The newly adjusted $Z$ and $V$ are then defined as $V_y = w_y$, and $Z_y = w_y \hat{\theta}_y$. Methods are available in practice to estimate the heterogeneity or between-study variance $\hat{\tau}_y^2$, e.g., the most commonly used method of moments (also known as Der Simonian–Laird (DL) method), restrictive maximum likelihood (REML), and the variance-component estimator. We used the recommended Paule–Mandel (PM) method to estimate the between-study variance.

The cumulative information from $k$ studies can be entered into a TT test as

$$Z_y = \sum_{j=1}^{k} Z_{ij}^* \text{ and } V_y = \sum_{j=1}^{k} V_{ij}^*,$$

Cumulative $(Z_Y, V_Y)$ values are then plotted in a TT plot (Fig. 2). The boundaries in a TT are based on three prespecified parameters, namely the type 1 error $\alpha$, the statistical power $1 - \beta$, and the relevant effect size to be detected $\theta_k$. We used the computer software PEST to calculate the boundaries for the TT with the aforementioned parameters.

A gene $i$ is declared differentially expressed if its sample path [ie, the path of $(Z, V)$-values] crosses the upper or lower red boundary, and we reject the null hypothesis. We do not reject the null hypothesis if the sample path crosses one of the blue dashed lines (Fig. 2). We are not able to draw a conclusion (yet) if the sample path falls within the boundaries. This also implies that more information is needed from future studies.

For a practical example of our proposed approach, we only took fully replicated genes, ie, the genes that appeared in all experiments, in order to visualize the sample path in the TT plot more clearly. As a result, we analyzed 12,211 unduplicated genes sequentially in the nonfiltered datasets. The boundaries of the TT were constructed with $\theta_k = 0.8$, $\alpha = 0.5\%$ and $(1 - \beta) = 80\%$ (this sequential design is named Design 1). Further, we applied a Bonferroni correction to $\alpha = 5\%$, resulting in $\alpha = 0.0004\%$. (with the same levels of $\theta_k$ and $(1 - \beta)$ as mentioned before) to construct a new sequential design, referred to as Design 2. Additionally, we also applied similar prespecified
parameters \( \theta_R = 0.8, (1 - \beta) = 80\% \), for each \( \alpha = 5\% \) and \( \alpha = 5\% \) with Bonferroni correction (equal to \( \alpha = 0.0007\% \)) in filtered gene expression data.

**Results**

The raw microarray gene expression datasets were collected from the ArrayExpress online repository by using the keywords *acute, myeloid, and leukemia*, yielding 377 experiments. The number of experiments reduced to 56 after excluding nonhuman experiments and had not used DNA by array assay technology (last checked on April 15, 2014). Manual screening of the retained studies resulted in seven microarray experiments that provided raw cell files and had class label in each individual sample. The raw datasets of those experiments were then downloaded. Five datasets were generated from gene expression experiments that had used Affymetrix HG-U133 Plus 2 (54,675 probesets), while the others had used Affymetrix HG-1.0 st. v1 (32,321 probesets) and HG-U133A (22,283 probesets), respectively. Due to different probeset names across platforms, we mapped probesets into the genes level. The number of genes in each dataset after the mapping process is presented in Table 1, as an additional column to the other basic information as well as the number of DEGs in each of seven experiments. We performed pairwise comparisons of the selected differentially expressed genes between the two datasets, and the results are shown in Figure 3. There is a high degree of overlapping informative genes that were obtained by Data 1 and Data 2, ie, 2,174 genes. Meanwhile, there are only 22 genes that were stated as differentially expressed genes by both Data 5 and Data 6. Although in general there is a considerable overlap in DEGs between two experiments, we found no gene that was stated as a DEG by all experiments, which confirms our motivation to aggregate the available information as accumulated evidence through SMA.

Based on the cumulative information of the seven experiments that were evaluated by TT using Design 1, with prespecified parameters \( \theta_R = 0.8, \alpha = 0.5\% \), and \( (1 - \beta) = 80\% \), there are 313 DEGs, 2,838 non-DEGs, and 9,060 genes that needed more experiments in order to draw a conclusion. Of the 12,211 tested genes, the selected \( \alpha \) level yielded an expected number of 62 false-positive genes. Methods for controlling the number of false-positive findings are widely available. For \( \alpha = 5\% \), with a conservative Bonferroni correction of \( \alpha = 0.0004\% \) (Design 2), 60 DEGs from the seven experiments were found, in which those were also stated as differentially expressed genes by Design 1. As compared to Design 1, Design 2 yielded far less DEGs, given the conservative correction in the type 1 error rate for 12,211 tested genes.

The TTs for the two designs in nonfiltered datasets are summarized in Figure 4. The figures are dominated by the orange region, which implies more experiments are to
be initiated in order to make a conclusion for the genes of interest, especially when Design 2 is used to construct the triangular test.

We selected four genes, presented in Figure 5, to show the sample paths of cumulative evidence based on SMA for Designs 1 and 2. The sample paths for the two different designs are identical in pattern but they do differ in the moment the conclusion can be drawn, due to the fact that the TTs have wider boundaries for the Bonferroni-corrected design. Considering gene G55704 for instance, two experiments are enough to decide that the gene is informative when it is evaluated by a TT that was constructed with Design 1. Meanwhile, we need cumulated samples from six experiments in order to draw a conclusion when Design 2 is used to evaluate the gene.

We filtered genes with low expression values and took 7,455 genes that appeared in seven experiments. We then performed TTs by applying Design 1. The sequential analyses detected 202 genes as DEG, while 1,392 and 5,861 genes were classified as uninformative and undecided, respectively. When the boundaries of the TT were constructed by $\alpha = 0.0007\%$ (Bonferroni correction for $\alpha = 5\%$ in 7,455 tests) with the same relevant effect size and statistical power as in Design 1, we found 40 DEGs with 580 genes classified as uninformative and 6,835 genes that needed more experiments in order to make a conclusion. As compared to the nonfiltered data, we found fewer DEGs in the filtered data, which might be due to the exclusion of potential informative genes during the filtering process. The comparisons of the DEGs found based on the nonfiltered and filtered data as well as with and without multiple testing correction are given in Table 2.

**Discussion**

This study has extended the application of SMA into the genomic field. We described and applied the proposed algorithm to find potential differentially expressed genes in AML by taking advantage of the public availability of gene expression datasets from published studies as suggested by the MIAME (Minimum Information About a Microarray Experiment) standards.

Table 2. Comparisons of the differentially expressed genes that were found with and without incorporating Bonferroni correction in the filtered and non-filtered gene expression data. The numbers represent the number of overlap differentially expressed genes between two different settings.

|                      | DESIGN 1 (FILTERED DATA, 202 DEGS) | DESIGN 2 (FILTERED DATA, 40 DEGS) | DESIGN 1 (NON-FILTERED DATA, 313 DEGS) |
|----------------------|-----------------------------------|-----------------------------------|----------------------------------------|
| Design 2             | 40                                |                                   |                                        |
| (Filtered data, 40 DEGs) |                                  |                                   |                                        |
| Design 1             | 125                               | 31                                |                                        |
| (Non-filtered data, 313 DEGs) |                                  |                                   |                                        |
| Design 2             | 39                                | 21                                | 60                                     |
| (Non-filtered data, 60 DEGs) |                                  |                                   |                                        |

Figure 5. Triangular tests of four selected genes. The boundaries were constructed for a pre-specified effect size $\theta_R = 0.8$, power $1 - \beta = 80\%$, and type 1 error $\alpha = 0.5\%$ (the upper row) or $\alpha = 0.0004\%$ (the lower row, Bonferroni correction for $\alpha = 5\%$). The $y$-axis and $x$-axis represent the $Z$ and $V$ score, respectively. More detailed explanation for the $Z$ and $V$ score is provided in the Methods section.
guideline.\textsuperscript{37} The systematic search in the ArrayExpress online repository and manual screening resulted in seven microarray studies to be analyzed further. We followed a published recommendation\textsuperscript{6} to conduct a meta-analysis in microarray gene expression data by downloading raw datasets in order to reduce the source of variation due to preprocessing procedures that might vary across experiments.

To the best of our knowledge, application of an SMA (following Whitehead's boundaries approach) to combine microarray gene expression studies has not yet been employed to detect differentially expressed genes. It is worth mentioning, however, that sequential analysis has been proposed in single microarray experiments for interim analysis.\textsuperscript{38} An "ordinary" meta-analysis is also a well-known method to combine information from different experiments in genome-wide association studies (GWAS). As mentioned in Introduction, the goal of a meta-analysis is to estimate the effect size (or fold change, in case of differentially expressed gene analysis) without evaluating the adequateness of cumulative evidence to draw a conclusion. The SMA approach could also be a useful tool to decide whether more experiments are needed to draw a conclusion for each and every gene of interest, a property that an "ordinary" meta-analysis lacks.

The effect size described in Equation (1) is similar to the \( t \)-statistic to assess the mean difference between two groups, where the denominator is the square root of the pooled variance. The estimation of variance is known to be unstable in small samples. Severe underestimation of variance would inflate the statistic, causing false-positive findings. On the other hand, large fold-changed genes would have small statistics if the variance is overestimated. In the analysis of microarray data, empirical Bayes moderated \( t \)-statistics came as one of the alternatives to produce stable variances by shrinking extreme variances toward the overall mean variance. Empirical Bayes \( t \)-statistics has been proven to outperform ordinary \( t \)-statistics.\textsuperscript{39} Hence, we adopted for the concept of variance estimation from empirical Bayes \( t \)-statistics in the estimation of the effect size for each and every gene.

The summaries of the TTs from the 12,211 genes show that almost all the genes need more than one experiment to be declared as either noisy or informative (Fig. 4). Further, the 271 samples from seven experiments are even not enough to draw a conclusion for 9,060 genes when evaluated by Design 1 and 10,994 genes by Design 2 in nonfiltered data. On the other hand, 274 and 55 genes are already classified as redundant genes by single experiments in Designs 1 and 2, respectively. This result also tells us that the signal of expression values differ across the genes. A gene may have a strong signal, so it is easy to be classified as an informative gene without involving a large sample size. Since microarray technology simultaneously measures thousands of genes, more experiments are needed to cumulatively gather information, particularly for indecisive genes.

Given the curse of dimensionality in microarray studies, filtering redundant genes is commonly applied in practice, eg, removing genes with low variations and/or low expression values.\textsuperscript{40} The filtering procedure has a risk of excluding the informative genes when gene expression datasets are cumulated across experiments via SMA. This was clearly shown by the identification of some differentially expressed genes on nonfiltered data that were not found in the result of SMA on filtered data. The consequence is even more severe when hard filtering by removing genes with low expression and low variance is applied. Hence, we recommend avoiding any filtering procedure if the computational resources allow doing so.

We kept analyzing every gene until information from all experiments was gathered, although the sample paths for some genes had already crossed one of the TT's boundaries. With the common sequential design, the analysis can be stopped once the sample path crosses a boundary for reason of efficacy or futility. However, investigators might also continue the sequential analysis although the boundary is crossed, in order to optimize the available information, a condition called overrunning.\textsuperscript{28,41} In some genes, the overrunning analysis has as a consequence inconsistency in conclusions. We found genes that were declared noisy genes (non-differentially expressed genes) by TT once they crossed a lower boundary, but then turned out to be informative genes (differentially expressed genes) as more information was accumulated. The inverse case was also found, ie, informative genes became noisy genes when more studies were included. Although more information was gathered and the conclusion changed, the overall fraction of rejected null hypotheses was close to the predetermined type 1 error rate. We provided examples of genes that changed the conclusion when the overrunning analysis was performed (Fig. 6). We also noticed that the phenomenon of a switched conclusion happened only for the genes that had a sample path close to the inner boundary of the TT. We found no gene that crossed both upper and lower boundaries during the sequentially cumulated process.

FMS-related tyrosine kinase 3 (FLT3) is an important gene in the development of AML. However, all designs in TTs could not classify FLT3 as being differentially expressed, since the selected cumulative samples could not provide enough information to make a conclusion for this particular gene (Fig. 7). Further, the selected studies\textsuperscript{15–20} also did not mention this particular gene as a potential biomarker for distinguishing patients with AML from normal healthy controls.

The boundaries of a TT depend on prespecified parameters, namely the effect size, type I error rate, and statistical power. In this study, we used \( \theta_r \) equal to 0.8, which is a relatively large effect size in epidemiological settings.\textsuperscript{29} It is important to keep in mind that the gene expression data was analyzed on the \( \log_2 \) scale, so that our chosen \( \theta_r = 0.8 \) is equal to a fold change of 1.7 on the original scale. This reference fold change is relatively low compared to the common
Figure 6. Triangular tests of four selected genes that have inconsistent conclusions. The boundaries were constructed for a relevant effect size $\theta_R = 0.8$, power $1 - \beta = 80\%$, and type 1 error $\alpha = 0.5\%$. The sequential analyses were continued although the sample paths crossed the boundaries (so-called overrunning). The $y$-axis and $x$-axis represent the Z and V score, respectively. More detailed explanation for the Z and V score is provided in the Methods section.

Figure 7. Triangular tests for the FLT3 gene. The boundaries were constructed for a prespecified effect size $\theta_R = 0.8$, power $1 - \beta = 80\%$, and type 1 error $\alpha = 0.5\%$ (the first column) or $\alpha = 0.0004\%$ (the second column, Bonferroni correction for $\alpha = 5\%$). The first (second) row is the triangular tests when full (filtered) data is used for analysis. The $y$-axis and $x$-axis represent the Z and V score, respectively. More detailed explanation for the Z and V score is provided in the Methods section.
There is enough evidence at a certain time point to draw formative. This study showed a method to determine whether likely not enough to classify a gene as informative or noninformative testing if the effect size is real or if no relevant difference exists. However, TT minimizes the maximum amount of information needed to come to a conclusion compared to the SPRT. We refer to van der Tweel and van Noord for further details regarding the comparison of TT and SPRT particularly in the case-control study setting. We analyzed the gene expression data also by SPRT and found comparable results with the TT (results are not shown).

We tested gene expression values from different microarray experiments with a group sequential method. Further, we showed that the time to make a decision varies across the tested genes. This study shows the application of a sequential method in continuous outcome data. Such application may also be extended to count data (Poisson-distributed outcome data, such as in RNA sequencing) or survival outcome data.

Conclusion

We have shown that samples from one experiment are most likely not enough to classify a gene as informative or noninformative. This study showed a method to determine whether there is enough evidence at a certain time point to draw a conclusion for a particular gene or to hold the conclusion until the evidence is adequate to make conclusion for all the genes under study. SMA following Whitehead's boundaries approach offers an alternative method to find a gene signature list by evaluating the adequacy of the accumulated evidence.

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Author Contributions

Conceived and designed the experiments: PWN, MJCE, IvdT. Analyzed the data: PWN. Wrote the first draft of the manuscript: PWN. Contributed to the writing of the manuscript: PWN, IvdT, VLJ, KCBR, MJCE. Agreed with the results and conclusions: PWN, IvdT, VLJ, KCBR, MJCE. Jointly developed the structure and arguments for the paper: PWN, MJCE, IvdT. Made critical revisions and approved final version: PWN, IvdT, VLJ, KCBR, MJCE. All authors reviewed and approved of the final manuscript.

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