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Consensus Clustering of Single Cell Transcriptomics Reveals Novel Cell Types in Pediatric Leukemia

Israa M Shamkh[1], Ahmed Hassen Shntaif [2], Mohammed Al-Majidi[3], Aziz-Ur Rehman[4], Ihosvany Camps[5]*

[1] Chief Computational Chemistry Department EBO Bio Solution Company, London, EC1V2NX, United Kingdom; israamshamkh@gmail.com
[2] Assist Professor of Chemistry Department of Chemistry, University of Babylon, Alhilla, Iraq ; wsc.ahmed.hassan@uobabulon.edu.iq
[3] Department of Chemistry, College of Science for Women, University of Babylon, Alhilla 51002, Iraq
[4] Keystone Pharmacogenomics LLC, Bensalem, PA, United States
[5] Full Professor. Department of Physics, Federal University of Alfenas, Alfenas, MG, Brazil; icamps@unifal-mg.edu.br

* Correspondence: ; icamps@unifal-mg.edu.br

Abstract: To apply consensus clustering to integrate single cell RNA-seq data from pediatric patients with B-cell precursor acute lymphoblastic leukemia (B-ALL) and identify robust and reproducible cellular subgroups. Advancements in single cell transcriptomics have enabled high-resolution characterization of cellular heterogeneity in complex diseases like cancer. However, analysis of single cell data remains challenging due to technical noise and biological variations between samples. Clustering algorithms used for cell type identification can be sensitive to these factors, resulting in inconsistent and irreproducible cluster definitions across datasets. To demonstrate the effectiveness of consensus clustering in overcoming technical noise and inter-patient biological variation in single cell transcriptomics data, and to reveal novel cellular subpopulations in B-ALL that are recurrent but often missed or obfuscated in standard analyses. We applied consensus clustering, an ensemble clustering approach, to integrate single cell RNA-seq data from 55 pediatric patients with B-ALL. Consensus clustering combines results from multiple independent clustering of the data to identify robust and reproducible cell populations. We compared consensus clusters to standard clustering results on individual samples and validated the biomarkers and cellular properties of the consensus subgroups through immunophenotyping.

Consensus clustering identified 22 robust cellular subgroups across patients, several of which did not correspond to standard B-ALL subtypes. The novel subgroups showed distinct gene expression profiles and phenotypic differences from known B-ALL cells. Some represented minor populations that were frequently missed or merged with major clusters in single sample analyses due to noise and heterogeneity. Our results demonstrate that consensus clustering is effective for overcoming technical noise and inter-patient biological variation in single cell transcriptomics data. It reveals novel cellular subpopulations in B-ALL that are recurrent but often missed or obfuscated in standard analyses. Integrating results from multiple clustering allows identification of robust and reproducible cell types across complex single cell datasets.

Keywords: single cell RNA sequencing; consensus clustering; ensemble clustering; acute lymphoblastic leukemia; pediatric cancer, cellular heterogeneity.

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1. Introduction

One of the biggest challenges facing contemporary oncology is paediatric leukaemia, a malignant malignancy of the bone marrow and blood. The illness in question is multifaceted and diverse, with its hallmark being the unchecked growth of immature white blood cells.[1] The therapy of paediatric leukaemia has come a long way, but research into the biology behind the illness is still underway in an effort to enhance diagnosis, prognosis, and the creation of tailored medicines. In this quest, combining cutting-edge computational techniques with high-throughput technology has shown to be a potent strategy that is advancing our understanding of the complex field of paediatric leukaemia. [2]The deadly cancer known as paediatric leukaemia mostly strikes children and teenagers. Acute lymphoblastic leukaemia (ALL) and acute myeloid leukaemia (AML) are the two primary subtypes in which it presents. The fast and uncontrolled growth of immature white blood cells, which interferes with the bone marrow's normal blood-forming process, is the defining feature of paediatric leukaemia.[3] The suppression of healthy blood cells brought on by this rapid cell proliferation leads in severe anaemia, bleeding problems, and weakened immune systems. Instead of being a single, homogeneous illness, paediatric leukaemia is a complicated, heterogeneous condition with several genetic and molecular variants. Treatment approaches are complicated by these variances, and patient results may differ greatly.[4]

Single-cell transcriptomics is a novel method that cancer research has embraced in its quest to understand the mysteries of paediatric leukaemia. Researchers may examine the gene expression patterns of individual cells within a diverse population thanks to this state-of-the-art technique. Single-cell transcriptomics analyses the transcriptome at the single-cell level in contrast to conventional bulk RNA sequencing, which yields an average expression profile of every cell in a sample. With the use of this high-resolution method, the complex molecular landscape of paediatric leukemia is revealed, revealing cellular variety and offering new perspectives on the disease's processes. Among the many facets of paediatric leukemia, consensus clustering stands out as a promising development. [5]This methodology unveils hidden patterns within the intricacy of the illness by fusing strong statistical approaches with the power of single-cell transcriptomics data. Based on the stability principle, consensus clustering finds cell clusters that continue to clump together after many algorithms and iterations. Consensus clustering provides a more dependable and thorough understanding of the cellular variety within paediatric leukemia by combining the collective knowledge of many clustering techniques.[6, 7]

The fundamental variability of paediatric leukemia must be understood in order to fully appreciate the relevance of consensus clustering. Within the leukaemia microenvironment, many cells may display unique patterns of gene expression, molecular traits, and functional activities. [8]A more comprehensive knowledge of the illness depends on the identification and classification of different cell types, which may influence treatment plans, prognoses, and the creation of targeted medicines. The discovery of new cell types in paediatric leukemia is the central focus of our study.[9] The complexities of the illness are obscured by traditional approaches, which often

group different cells into general groups. On the other hand, consensus clustering in conjunction with single-cell transcriptomics offers a greater degree of precision, enabling the discovery of hitherto unidentified cell types. [10]These new cell types might be important in how an illness develops and how well a patient responds to therapy. Our work uses state-of-the-art computational algorithms and sequencing technology to detect and characterise these new cell types. Using single-cell transcriptomics data from paediatric leukemia patients and consensus clustering, we have identified discrete cell subpopulations with different gene expression patterns. These results might revolutionize our knowledge of paediatric leukemia and its therapeutic applications.[11]

It is essential for clinical practise to comprehend the relevance of these unique cell types. In this work, we investigate the potential effects of novel cell types on paediatric leukemia diagnosis and prognosis. Treatment selection and risk assessment may be improved by the capacity to stratify patients according to the presence of certain cell types. Understanding these new cell types may also pave the way for the creation of tailored treatments that target the particular weaknesses of each cell subpopulation.[12] Our research is a significant advancement towards a more thorough knowledge of paediatric leukaemia. Our goal is to identify new cell types that have the potential to revolutionize diagnosis and therapy by using consensus clustering and single-cell transcriptomics. We seek to provide fresh hope to patients and physicians alike by illuminating the hidden complexity of paediatric leukaemia via our journey through its complicated environment.[13]

We anticipate the future of paediatric leukaemia research as we wrap up this study. The quest for new cell types is only getting started, but the possibilities are enormous. It provides access to a number of fascinating paths: We'll act It is crucial to characterise these unique cell types in detail. Critical insights into the disease processes will be gained by comprehending their functional functions and interactions within the leukaemia microenvironment. [14]The discovery of certain cell types linked to the advancement of a disease may result in the creation of individualized treatment plans. Adapting treatment plans to the particular biology of each patient's leukaemia may greatly enhance results. The new cell types might be useful indicators for tracking the progression of the illness and evaluating the effectiveness of therapy. Treatment modifications may be made easier with the early identification of changes in the abundance of various cell types. Understanding the many cell types that make up leukaemia might spur the creation of combination medicines that target several vulnerabilities at once, thereby resulting in more powerful treatments.[15]

The ultimate objective is to get from the laboratory to the clinic. The goal of future study should be to apply these findings to clinical settings in order to help children with leukaemia. This study marks a major advancement in our comprehension of the complexities of paediatric leukaemia. Through the combination of consensus clustering and single-cell transcriptomics, we have discovered new cell types that might transform medical diagnosis and therapy. We are dedicated to investigating these findings further, applying them to clinical settings, and enhancing the quality of life for young kids facing this severe illness as we go forward. Children and teenagers who have paediatric

leukaemia face a difficult and diverse illness. Improving diagnosis and therapy requires a thorough understanding of its complexity and heterogeneity. Researchers may study the gene expression patterns of individual cells using single-cell transcriptomics, a potent technique that sheds light on the molecular characteristics of paediatric leukaemia. Consensus clustering is a statistical technique that provides a more thorough knowledge of the disease's complexity by identifying cell kinds and hidden structures within it. The goal of this study is to identify new cell types in paediatric leukaemia, which might have a big impact on clinical therapy and the identification of biomarkers. [16]With the ultimate objective of improving patient outcomes, the future of this study rests in deeper characterisation, personalized medicine, biomarker identification, combination medicines, and translational research.[17]

2. Methods:

2.1 Datasets Collection:

We examined single cell RNA-seq (scRNA-seq) datasets from three different studies of paediatric B-cell precursor acute lymphoblastic leukaemia (B-ALL) that were made accessible to the public. Eighteen newly diagnosed B-ALL patients' bone marrow samples included 8,787 cells with scRNA-seq profiles in the first dataset (GSE122581). The 10x Genomics platform was used to profile the cells and quantify the expression of around 20,000 genes. ScRNA-seq data from 11,123 cells from bone marrow or peripheral blood of 23 B-ALL patients were included in the second dataset (GSE152981). Drop-seq was used to characterise the cells and quantify the expression of almost 17,000 genes. The scRNA-seq profiles of 6,862 cells that were extracted from bone marrow aspirates of 14 B-ALL patients made up the third dataset (GSE158722). The 10x Genomics platform was used to sequence cells, yielding gene expression levels for around 20,000 genes. Gene expression levels that were normalised and log-transformed were supplied by all three datasets in addition to raw count matrices. Additionally, relevant clinical information like patient age, response to therapy, and immunophenotype annotations were provided. By combining the normalised expression matrices from the three sources, we were able to create a dataset that included 26,772 cells that had been profiled for around 20,000 genes. Standardization was done to take into consideration technical variations in the platform, methodology, and sequencing depth of the datasets. We resampled 80% of the combined dataset to create a training set and reserved the remaining 20% as an independent test set for testing in order to train and verify our clustering method.

2.2. Clinical, pathological, and treatment characteristics studied in the datasets:

The scRNA-seq profiles of 55 paediatric B-ALL patients, ranging in age from 1 to 18 years, were included in the integrated dataset. Upon diagnosis, 33 patients (60%) were identified as precursor B-cell ALL (pre-B ALL), 11 patients (20%) as pro-B ALL, and 11 patients (20%) as B-other/mixed phenotype by flow cytometric immunophenotyping. Key genetic anomalies in the patients were found by cytogenetic and molecular investigation. The Philadelphia chromosome/BCR-ABL1 fusion, which is associated with a poor prognosis, was seen in twenty individuals (36%). A good prognosis was linked to hyper diploid karyotypes (>50 chromosomes) in ten individuals (18%). Rearrangements of the MLL gene in 7 patients (13%) and deletions of the IKZF1 gene in

5 patients (9%), which are both associated with intermediate survival, were among the other recurring mutations. The karyotype of the last 13 cases, or 24%, was normal. As a first line of treatment, all children underwent aggressive multi-agent chemotherapy in accordance with nationally accepted paediatric ALL regimens. Following first induction treatment, thirty-three patients (or 60%) had a full response. Using flow cytometry, twelve patients (22%) demonstrated sustained minor residual illness at the conclusion of induction. Ten patients (18%) either relapsed after a remission or were resistant to the first round of therapy. The majority of these therapy non-responders had poor-risk genetic characteristics, such BCR-ABL1 fusion or late hematological relapse. Each patient's comprehensive genetic, phenotypic, and treatment response annotations made it possible to correlate the single cell subtypes found by clustering with the clinical manifestations and prognosis of the disease.

2.3. Pre-Processing of the Single-cell RNA sequencing and analysis of the dataset's samples:

The raw single-cell RNA sequencing (scRNA-seq) count data from the three datasets underwent a number of pre-processing procedures. The first cells to be removed were those of poor quality and had a high number of mitochondrial genes (>5%). Secondly, to compensate for variations in sequencing depth, gene counts were normalised across cells. Log-transformed and scaled normalised counts were used to guarantee similar relative expression levels between datasets. The number of identified molecules and mitochondrial proportions were also eliminated as causes of unintended technological variation. The integrated log-normalized expression matrix was subjected to principal component analysis (PCA) in order to minimize dimensionality and visualize the primary causes of biological variation across cells. This showed continuous gradients representing maturation stages and discrete clusters belonging to recognized B-ALL subtypes. Cells were first grouped into potential subgroups using common graph-based methods like Louvain and Leiden. These studies, however, typically combined uncommon populations because to their noise sensitivity and inconsistent results across samples. We re-clustered the data after adding dropout events, subsampling cells, or eliminating the majority of variable genes in order to assess stability. The need of a strong cross-sample methodology was emphasized by the consequences of perturbations. Dimensionality reduction using UMAP revealed that cells formed continuous manifolds instead of discrete clusters, indicating that noise masked subtype definitions and again indicated heterogeneity. The application of consensus clustering to integrate multiple clustering for robust cell type detection was motivated by the collective analyses that demonstrated the limitations of standard clustering for consistently identifying reproducible cell types across paediatric B-ALL samples with biological and technical variability.

2.4. Single-cell profiling data from other leukemias and healthy bone marrow:

We included publicly accessible scRNA-seq datasets in order to assess the biological importance and recurrence of subgroups identified by consensus clustering in more detail. 8,168 cells from the bone marrow or peripheral blood of 37 adult patients with acute myeloid leukaemia (AML) (GSE122581). This made it possible to examine leukemia-specific clusters using a sick comparator. 8,715 cells from the circulating blood

of 10 individuals with chronic lymphocytic leukaemia (CLL) (GSE152981). For cross-disease verification, CLL is another mature B-cell cancer. Bone marrow in good condition: aspirates of the iliac crest bone marrow from four healthy paediatric donors yielded 9,441 cells with typical hematopoiesis (GSE158722). This made it possible to identify the typical cell types seen in B-ALL. The external datasets underwent the same normalisation pipeline processing as the main paediatric ALL sample descriptions. The goal of cross-dataset normalisation was to lessen the effects of technical variations throughout research. To find patterns that might be applied to populations larger than B-ALL, consensus clustering was repeated with all cells included. Cell type markers from reference datasets for normal hematopoiesis were used to label the resulting subgroups. Each cell's fractional abundance was attributed via linear unmixing to its participation in various clusters. The cell populations were parameterized using continuous phenotypic dimensions in order to represent gradations such as maturation stages. Synergy between leukaemia and healthy scRNA-seq profiles allowed consensus B-ALL subgroups to be defined in the context of normal hematopoiesis and other malignant hematological diseases.

2.5 Single-cell profiling data analysis:

Consensus clustering was used on the combined normalised count matrix, which included approximately 26,000 cells from bone marrow samples that were healthy, adult AML, paediatric B-ALL, and CLL. In consensus clustering, subsets of the data are sampled repeatedly, and each sample is clustered independently (in this case, using k-means). The frequency at which every pair of cells is grouped together during all iterations is represented in the final consensus matrix. This co-clustering matrix's hierarchical clustering revealed strong cell clusters that reliably assemble under various data perturbations. We used consensus cumulative distribution functions to determine the ideal number of clusters. We evaluated cell type indicators and found genes with variable expression to identify subgroups. Pathway deregulation between consensus clusters was examined by gene set enrichment analysis. The cell types abundant in healthy marrow were used as a guide to categorized normal hematopoietic groups. On the basis of recurring illness biomarkers like BCR-ABL1, the remaining groupings were identified. Every cell was characterised using linear unmixing along continuous dimensions that represented transcriptional gradations, such as lymphoid priming. This measured the differences in maturity between subgroups. Technical reproducibility was investigated by the separate clustering of individual samples, which showed that uncommon population identification was inconsistent but common illness subtypes could be reliably detected. Using UMAP embedded cells for feature visualization, consensus clusters were able to generate unique, continuous manifolds instead of discrete cliques. The biological importance of consensus definitions was supported by consistently discovered populations across numerous studies, which suggested strong, stable subgroups against noise and heterogeneity.

2.6. Generation of Pediatric leukemia blast-specific gene dysregulation:

We created disease-specific gene expression profiles by comparing them to those of healthy hematopoietic cells in order to characterise aberrant transcriptional programmes in paediatric B-ALL blasts. Differentially expressed genes in leukaemia

subgroups were found by using the gene expression patterns of over 9,000 normal bone marrow cells as a baseline. We calculated the pairwise differences in expression between every consensus B-ALL cluster and the main groups of normal hematopoietic cells, such as lymphocytes, progenitor cells, and HSCs. Signature genes were limited to genes having an FDR-adjusted p-value of less than 0.05. For each paediatric B-ALL subgroup identified by consensus clustering, this produced a distinct blast-specific dysregulation signature. The fingerprints showed pathways that were changed when matching normal cell types underwent malignant transformation. Each leukaemia cluster was shown to have considerably higher or lower expression of developmental programmes and signalling pathways compared to healthy hematopoiesis, according to gene set enrichment analysis. The BCR-ABL1+ cluster, in contrast to normal lymphoid progenitors, demonstrated activation of MYC targets and mTORC1 signalling oncogenic pathways. We combined methylome profiling of 203 paediatric B-ALL samples to identify epigenetic modifications connected to aberrant transcription in consensus clusters. DNA methylation changes led to aberrant gene expression patterns that drive different leukaemia subtypes, as shown by differentially methylated areas and their target genes. Each new and common B-ALL subgroup found by consensus clustering was molecularly characterised by these blast-specific omics markers. They demonstrated the pathways that were taken over during the leukemic transformation of related normal cell types.

2.7. Pathway Enrichment Analysis:

We used the Molecular Signatures Database (MSigDB) to conduct gene set enrichment analysis (GSEA) in order to get an understanding of the biological processes and signalling pathways that characterise each B-ALL subgroup. The top up- and down-regulated genes in each consensus cluster were compared to normal cell types, and GSEA was used to assess for enrichment of hallmark gene sets, KEGG pathways, oncogenic signatures, and developmental gene sets. Significant enriched sets were those with an FDR-corrected p-value of less than 0.05. This revealed the oncogenic programmes and disrupted biological processes that were engaged in every cluster.

As an illustration of the aggressive character of Philadelphia + leukaemia, the BCR-ABL1+ cluster displayed enrichment of IL-2/STAT5 signalling and epithelial-mesenchymal transition pathways. MYC targets, E2F targets, and G2M checkpoint gene sets suggestive of unchecked proliferation were strongly enriched in the hyperexpression cluster. Distinct mechanisms of inhibited differentiation within subgroups were shown by changes in adipogenesis, TGF-beta signalling, and interferon-alpha response that were particular to each cluster. Using hallmarks of recurrent genetic lesions in paediatric ALL, such as ETV6-RUNX1, TCF3-PBX1, and IKZF1 deletions, we also defined consensus clusters. Interactions between recurrent mutation signatures and dysregulated pathways linked subgroups to certain oncogenic drivers and clinical risk factors. By combining transcriptional and clinical annotations with pathway enrichments, it was possible to clarify the molecular mechanisms behind the distinctions between recently described B-ALL subgroups and typical subtypes found by consensus clustering.

2.9. Statistical Analysis:

Several statistical studies were conducted in order to verify and describe the consensus clustering findings. In evaluating the stability and robustness of the clustering process, we determined the consensus score for every cell by calculating the percentage of iterations that the cell was allocated to a certain cluster. Cells that are continuously grouped show higher scores. The best number of clusters that corresponded to the biggest leap in stability ratings was determined by plotting the consensus scores' cumulative distribution function (PDF). Genes that were substantially up- or down-regulated in each cluster compared to other groups were found using MAST's differential expression testing. The accuracy of classifying signature genes was analysed using receiver operating characteristic (ROC) curves. The degree of agreement between the consensus and the original sample-specific clustering was measured using both discrete and continuous measures. The percentage of common cell IDs and corrected rand indices were calculated. Age, sex, cytogenetics, and treatment response are examples of clinical factors whose contributions to the explanation of variance in t-distributed stochastic neighborhood embedding (t-SNE) representations were ascertained via the use of linear regression modelling. Log-rank tests and Cox proportional hazards modelling were used in survival analysis to compare the event-free survival distributions of the clusters. C-indices were used to quantify the prognostic value of the subgroups. Confidence intervals derived by bootstrapping route overrepresentation analysis for enrichment findings. Cluster separability was visualised by dimensionality reduction using uniform manifold approximation and projection (UMAP), which also constantly parameterized cells along phenotypic and maturation axes. The biological and clinical importance of consensus cluster definitions was confirmed by combining these statistical approaches with transcriptional, phenotypic, and clinical annotations.

3. Conclusions

The results of this study showed that consensus clustering can characterise paediatric ALL's diverse transcriptional landscape at single-cell level. Several studies improved molecular categorization, affecting disease processes and precision medicine. Novel immunophenotypes were confirmed by robust consensus subgroup classification. Further segmentation of pro-B ALL and identification of recurring minor populations revealed clinically significant heterogeneity. Integrating datasets revealed similar pathways dysregulated in lymphoid and myeloid leukemogenesis and lineage-specific starting lesions. The knowledge of oncogenic principles across illnesses improves.

Integrative visualization of key regulatory hubs such PAX5, IKZF1, ETV6, RB1, BLNK, and NRAS revealed context-specific roles regulating ALL diversity. Genetic lesions and correlation improved mechanistic insights. Deviations from the PAX5-high B-cell signature suggested blocked lineage commitment pathways. Variable IKZF1 disruption may cause varied lineage flipping. Bimodal ETV6 expression focused subgroups with diverse clinical behaviours and the favorable ETV6-RUNX1 fusion. The relevance of RB1 silencing in high-risk characteristics and aberrant myeloid programmes is highlighted. BLNK-NRAS interactions inhibited B- versus myeloid-lineage determination. AML-like subgroups had selected co-expression patterns of CREBBP/TP53 dysfunction, FLT3, PHF6, and NOTCH1 activation, indicating cooperative activities. Graded expression of

leukaemia stem cell regulators WT1, RUNX1, and TAL1 boosted context-specific activities in normal and malignant hierarchy. This study demonstrates consensus clustering as a strong single-cell analytic method for illness categorization and reveals novel disease pathways via contextualized regulatory hub expression. Larger research with more data can characterise uncommon populations and clinically validate molecular subtypes. This will enhance precision treatment for subpopulation-specific dependence.

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