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ORIGINAL RESEARCH

Ensemble Learning for Higher Diagnostic Precision in Schizophrenia Using Peripheral Blood Gene Expression Profile

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Introduction: Stigma contributes to a significant part of the burden of schizophrenia (SCZ), therefore reducing false positives from the diagnosis would be liberating for the individuals with SCZ and desirable for the clinicians. The stigmatization associated with schizophrenia advocates the need for high-precision diagnosis. In this study, we present an ensemble learning-based approach for high-precision diagnosis of SCZ using peripheral blood gene expression profiles.

Methodology: The machine learning (ML) models, support vector machines (SVM), and prediction analysis for microarrays (PAM) were developed using differentially expressed genes (DEGs) as features. The SCZ samples were classified based on a voting ensemble classifier of SVM and PAM. Further, microarray-based learning was used to classify RNA sequencing (RNA-Seq) samples from our case-control study (Pune-SCZ) to assess cross-platform compatibility.

Results: Ensemble learning using ML models resulted in a significantly higher precision of 80.41% (SD: 0.04) when compared to the individual models (SVM-radial: 71.69%, SD: 0.04 and PAM 77.20%, SD: 0.02). The RNA sequencing samples from our case-control study (Pune-SCZ) resulted in a moderate precision (59.92%, SD: 0.05). The feature genes used for model building were enriched for biological processes such as response to stress, regulation of the immune system, and metabolism of organic nitrogen compounds. The network analysis identified *RBX1, CUL4B, DDB1, PRPF19*, and *COPS4* as hub genes.

Conclusion: In summary, this study developed robust models for higher diagnostic precision in psychiatric disorders. Future efforts will be directed towards multi-omic integration and developing “explainable” diagnostic models.

Keywords: Schizophrenia, peripheral blood, gene expression, machine learning, ensemble learning

Introduction

Schizophrenia (SCZ) is a complex neuropsychiatric disorder characterized by a disruption in thinking and sense of self. The death rate is two times higher in schizophrenia-affected individuals, with cardiovascular diseases and suicide as the leading causes of death.[1–3] The global burden of disease 2019 estimates that almost 24 million people are affected by SCZ globally, which indicates its universal presence irrespective of cultural differences worldwide.[4] Significant issues in treating psychiatric disorders are delayed diagnosis and limited certainty of the diagnosis itself. The current diagnostic procedure for SCZ is based on psychiatric evaluation, making it clinician-dependent. The Diagnostic and Statistical Manual of Mental Disorders (DSM-5) based diagnosis for SCZ requires symptoms to be persistent for six months or more.[5] The delay in the treatment accounts for a higher number of years lived with disability associated with SCZ.[6] Recent studies suggest that treatment outcomes can be improved if the time elapsed before the treatment is reduced.[7] Thus, having a sensitive and specific blood test can strengthen and hasten the current diagnostic process for SCZ.

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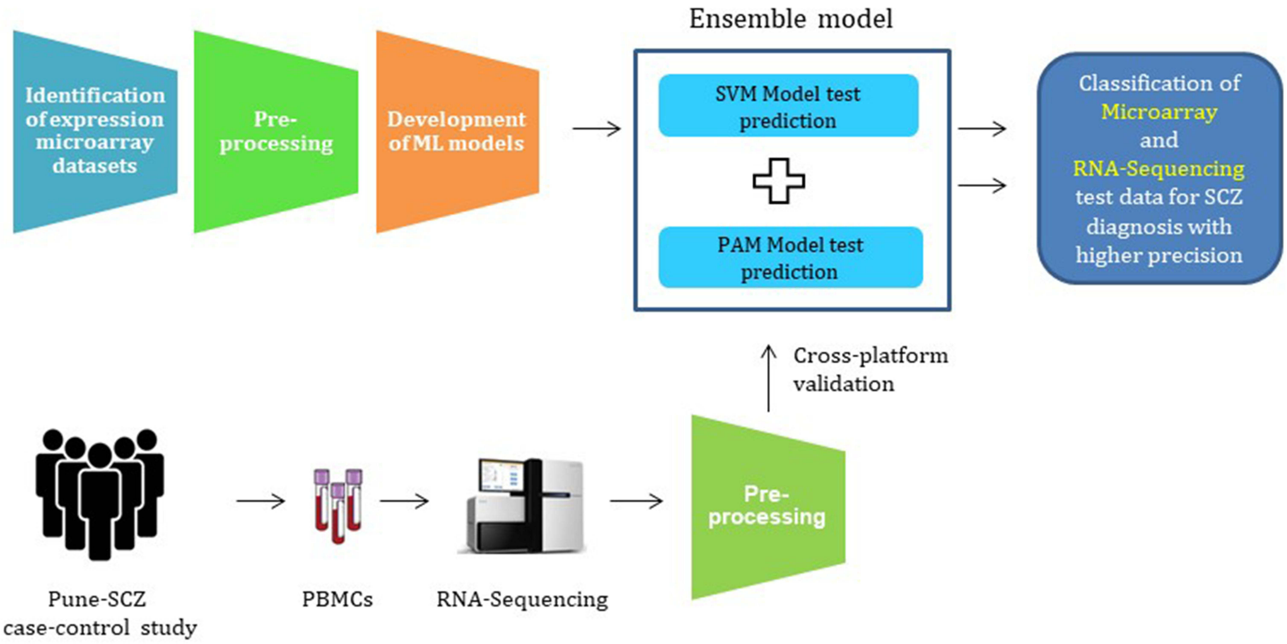
Graphical Abstract


Ensemble model
Identification of expression microarray datasets
Pre-processing
Development of ML models
SVM Model test prediction
PAM Model test prediction
Classification of Microarray and RNA-Sequencing test data for SCZ diagnosis with higher precision
Cross-platform validation
Pune-SCZ case-control study
PBMCs
RNA-Sequencing
Pre-processing

Cellular alterations, such as gene expression changes associated with the disorder, have been proposed to be useful as potential biomarkers. A previous study provides substantial evidence for using peripheral blood gene expression profiles for biomarker discovery.[8] The recent use of machine learning (ML) tools has accelerated the biomarker discovery process for psychiatric disorders.[9–11] ML models employ statistical methods to learn from the data to achieve specific objectives. Support vector machines (SVM) and nearest shrunken centroids (NSC) are popular examples of supervised learning ML algorithms used in genomics, particularly in transcriptomics.[12]

The ML tools have already provided gene expression markers with higher diagnostic potential.[9,10,13] Currently, the ML-based in silico approaches are limited to publicly available microarray datasets. ML-based in silico studies using biomarkers have reported higher diagnostic performance for psychiatric disorders.[10,11,14,15] However, few studies have validated their ML models using independent datasets.[9,13,16] Moreover, we found no studies addressing potential data leakage from preprocessing steps like quantile normalization and feature selection in transcriptomic data. Application of the diagnostic models into clinics would need extensive validation and appropriate data scaling methods to develop models with real-world applications. In addition, most of the studies focused on accuracy and area under the receiver operating characteristic curve (AUROC) as evaluation parameters for the performance of ML models. However, SCZ is associated with a higher degree of stigmatization.[17,18] The stigmatization, especially in developing countries, can lead to unwarranted distress in the lives of those falsely diagnosed with SCZ.[19,20] Thus, a disorder like SCZ demands a higher precision diagnostic test [True positives / (True positives + False positives)] with minimal false positives.

This study employs SVM and PAM for the binary classification of SCZ samples. SVM excels in managing high-dimensional and non-linear datasets commonly encountered in transcriptomics. It achieves this by effectively identifying optimal decision boundaries between the closest data points (support vectors) to separate different classes.[21] PAM is a modified version of NSC tailored explicitly for transcriptomic data. It accurately captures gene expression patterns (centroids) associated with each class and classifies new samples by assessing their similarity to those of the class centroids.[22] We have ensembled SVM and PAM to combine their strengths and enhance the discriminative power for high-precision classification in SCZ.

We selected publicly available gene expression microarray datasets for this analysis. The raw data from each platform was processed independently to avoid data leakage. ML models were developed using SVM and PAM algorithms. We made use of differential gene expression analysis (DGEA) for selecting features with potential diagnostic values. The analysis pipeline used in this study does not result in data leakage, producing ML models more likely to succeed in clinical settings. Further, the ensemble of two ML models classifies samples from test and independent microarray datasets with higher precision. This study also highlights the importance of preprocessing RNA sequencing (RNA-Seq) data for its compatibility with prediction analysis. Our approach is not restricted to microarray data: it classifies RNA-Seq samples from our case-control study (Pune-SCZ) with moderate precision. Finally, gene ontology analysis of differentially expressed genes (DEGs) highlights the biological processes associated with SCZ, further validating our approach. Subsequent network analysis of these feature genes opens avenues for discovering novel diagnostic biomarkers for SCZ.

Materials and Methods

Identification of Datasets

Peripheral blood gene expression microarray datasets for SCZ were identified from the Gene Expression Omnibus (GEO)[23] and ArrayExpress[24] using keywords, "Gene expression", "Peripheral blood", "Biomarkers" and "Schizophrenia" or "Schizophrenia spectrum". A similar search was performed on databases such as PubMed and Google Scholar. Studies with immortalized cell lines, specific cell types, and custom microarray platforms were excluded. The analysis included studies with available raw data, while the authors of studies without publicly available data were contacted to obtain the data necessary for the analysis.

Importing and Preprocessing of the Raw Data

Raw data for each dataset was imported and processed independently in R.[25] Probe filtration was performed for Illumina datasets (eg Illumina probes with detection P.val <0.05 in ≥ 3 samples were retained). Probe IDs of all the arrays were mapped to HUGO Gene Nomenclature Committee (HGNC) gene symbols.[26] Gene expression values for multiple probes were averaged out for individual genes, and common genes across all the datasets were retained. Of the datasets identified, one was set aside for independent dataset validation. The remaining datasets were combined to obtain a meta-file. This meta-file with raw gene expression values (raw meta-file) was processed for ML-based prediction analysis.

Data Scaling for Machine Learning

The raw meta-file (raw gene expression matrix) was divided into train and test data before normalization and batch correction to avoid any data leakage. To achieve this, samples were shuffled and then subjected to a random selection of train (90%) and test (10%) data. This random selection was repeated to obtain ten iterations of train and test datasets. Samples within the training data were quantile normalized based on the microarray platform. Training data was further batch-corrected independently. In contrast, test data was normalized using quantile targets[27] from train data and batch corrected using train data as a reference.[28] Each iteration of normalized and batch-corrected train data was used for feature selection and development of ML models. In contrast, test data was used to evaluate ML models. Similarly, the independent dataset was normalized and batch-corrected using each iteration of the train data as a reference and further used for evaluation of the models (Figure 1).

Feature Selection and Development of ML Models

We used differential gene expression analysis (DGEA) as a feature selection method. Differentially expressed genes (SCZ vs healthy controls, CNT) were identified using limma.[29] The top differentially expressed genes were used as features for model building. We used SVM[30] with different kernels (linear, polynomial, radial, and sigmoid) and PAM[31], both with a probability threshold of 0.5, for binary classification. Each iteration of train data was used for the development of ML models. These models were tuned and cross-validated (k=10) to asses over-fitting. The performance of the machine learning models was assessed using respective test data generated from the random sampling. In addition,

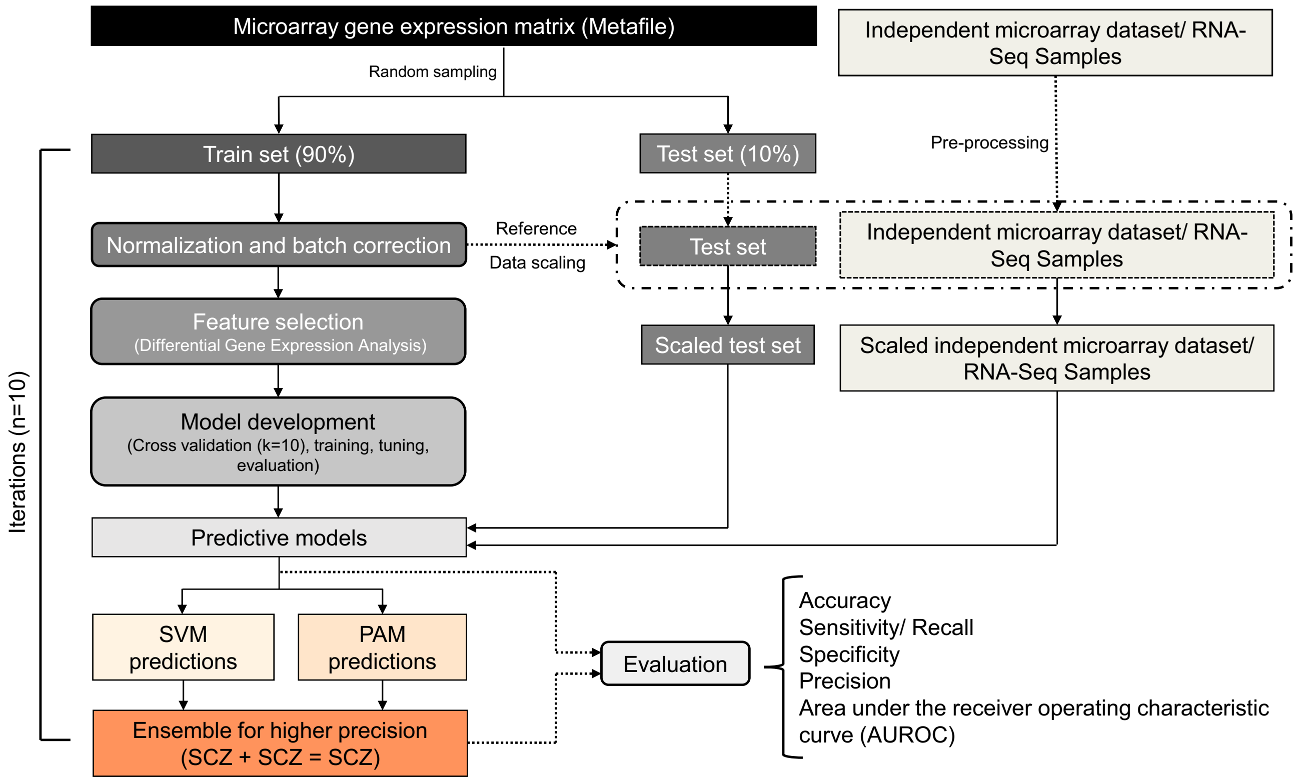

Microarray gene expression matrix (Metafile)
Random sampling
Train set (90%)
Test set (10%)
Independent microarray dataset/ RNA-Seq Samples
Pre-processing
Normalization and batch correction
Reference
Data scaling
Test set
Independent microarray dataset/ RNA-Seq Samples
Feature selection
(Differential Gene Expression Analysis)
Scaled test set
Scaled independent microarray dataset/ RNA-Seq Samples
Model development
(Cross validation (k=10), training, tuning, evaluation)
Iterations (n=10)
Predictive models
SVM predictions
PAM predictions
Evaluation
Accuracy
Sensitivity/ Recall
Specificity
Precision
Area under the receiver operating characteristic curve (AUROC)
Ensemble for higher precision
(SCZ + SCZ = SCZ)

Figure 1 The workflow depicts the steps involved in sample processing and class prediction analysis. A raw meta-file with common genes (rows) across microarray datasets and samples (columns) was used for the analysis. Samples from the raw meta-file were shuffled and subjected to a random selection of train and test data (9:1) generating ten iterations each. Each iteration of train data was independently quantile normalized. The preprocessed train data was used for feature selection and model training. Normalization and batch correction for test data, independent dataset, and RNA-Seq data were performed using train data as a reference. The performance of the individual models was compared with the ensemble learning. The models were also evaluated for their cross-platform compatibility using RNA-Seq samples.

the model's prediction ability was further validated using an independent dataset. The ML models were evaluated based on their prediction accuracy, sensitivity, specificity, precision, and AUROC of test data and independent dataset. Further, ML models were ensembled for higher precision (Figure 1).

Ensemble Learning and Evaluation

To improve the precision of class prediction, we ensembled SVM and PAM models (Figure 1). In brief, only samples predicted as cases by both ML algorithms were labelled as cases in ensemble learning. The performance of the ensemble learning was evaluated in comparison with individual models using parameters such as precision, accuracy, sensitivity, specificity, and AUROC. Of the ten iterations, the one with the highest precision in ensemble learning was further used for functional enrichment analysis.

Functional Enrichment and Network Analysis

Functional enrichment analysis of the genes of interest was carried out using g:Profiler.[32] Search tool for the retrieval of interacting genes/proteins (STRING)[33] based protein-protein interaction (PPI) network for these genes was established in Cytoscape.[34] The essential nodes (hub genes) of the PPI network were identified by the maximal clique centrality (MCC) method of the cytoHubba plugin.[35] A network of hub genes and their first-stage nodes were later visualized in Cytoscape.

Establishment of Case-Control Study

This study complies with the Declaration of Helsinki. Protocol for the Pune-SCZ study was approved by the KEM Hospital Research Centre Ethics Committee (KEMHRC ID No. 2001) and Symbiosis International (Deemed University) Independent Ethics Committee (SIU/IEC/99). We recruited 20 participants of the age group 18–65 years from the Psychiatry Unit KEM Hospital, Pune. A written informed consent was obtained from all the participants before recruitment. For participants affected by

SCZ, written informed consent from a spouse or first-degree relative aged 18 or above was obtained, regardless of the affected participant's capacity to consent. The exclusion criteria for healthy control (CNT) and schizophrenia (SCZ) groups were the presence of a) acute or chronic infections, b) coronary heart disease, c) metabolic disorders, d) arrhythmia, e) heart disorders, f) hyper and hypothyroidism, g) inflammatory bowel disease and h) multiple sclerosis. Female participants with polycystic ovary syndrome, pregnant and lactating mothers, and women on in-vitro fertilization (IVF) treatment at the time of recruitment were also excluded from the study. The participants with a suspected case of schizophrenia and schizophrenia spectrum disorder were considered for recruitment under the SCZ group. Age and sex-matched participants with no history of psychiatric disorders were considered for recruitment under the healthy control group. All the participants were screened to confirm or refute the diagnosis of schizophrenia using structured clinical interview for the DSM-5 research version (SCID-5-RV).

Recruitment of Participants and Clinical Assessment

SCZ diagnosis was made using SCID-5-RV (version 1.0.0).[36] The SCID-5-RV was administered by a trained psychiatrist and a psychologist. The SCZ diagnosis was later confirmed by a senior psychiatrist from the team. SCZ-diagnosed participants were also administered with positive and negative syndrome scale (PANSS).[37] The presence of SCZ and other psychiatric disorders, including past history, was ruled out in the control group participants. Age, sex, family history for psychiatric disorders, medical history, and medication status were recorded for all the participants.

Blood Collection and RNA Extraction

Random (non-fasting) venous blood samples were collected in dipotassium ethylenediaminetetraacetic acid (K2EDTA) vacutainers and processed on the same recruitment day. A blood cell count (hemogram) was performed on the samples collected. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque (Sigma, Catalogue: GE17-5442-02) density gradient centrifugation and re-suspended in TRIZOL (ThermoFischer Scientific, Catalogue: 15596026). Samples were subjected to RNA sequencing using commercial services. In brief, polyA enriched RNA samples were sequenced using the NovaSeq 6000 system - Illumina to obtain a minimum of 60 million paired-end reads of 150 nucleotide length.

Preprocessing of RNA Sequencing Data

The quality of each sample was confirmed using FastQC.[38] The sequences were aligned to the human genome (GENECODE hg38)[39] using HISAT2.[40] The aligned files were subjected to gene assignment using featureCounts[41] to create a count matrix. The gene expression values were locally normalized by converting the raw count to counts per million (CPM) and transcript per million (TPM). The raw counts (RC), CPM, and TPM matrices were further quantile normalized and batch corrected in reference to microarray training datasets independently. The quantile normalized and batch-corrected RC, CPM, and TPM matrices were further used to evaluate the cross-platform performance of ML models developed using microarray data.

Statistical Analysis

Data from processed microarray datasets and samples from the Pune-SCZ study were analyzed using Microsoft Excel-Real Statistics[42] and PAST.[43] Microarray datasets processed using PAM, SVM, and ensemble approach were tested for normality using the Shapiro–Wilk test. For data with normal distribution, a paired version of one-way ANOVA, repeated measures ANOVA (RMA), with an alpha of 0.05, was performed. Following this, Tukey's post hoc test was applied with Greenhouse-Geisser and Huynh-Feldt corrections. The AUROC was plotted using ROCR.[44] For clinical samples, age and blood cell count data were tested for normality, as mentioned previously. The difference between case and control groups was studied using an unpaired *t*-test for normal data and a Mann–Whitney *U*-test for non-normal data. A chi-square test was conducted to assess the variations in categorical variables, such as sex, between the groups.

Table 1 Peripheral Blood Gene Expression Microarray Datasets for Schizophrenia

Dataset	Platform	Control /SCZ	Female (%)	Medication Status	Genes Analyzed	Ethnicity or Origin
GSE18312[45]	Affymetrix Human Exon 1.0 ST Array	8+13	33	Medicated	17,131	San Diego and Taiwan
GSE27383[46]	Affymetrix Human Genome U133 Plus 2.0 Array	29+43	NA	Mix	21,826	Multi-ethnic groups
GSE38481[47]	Illumina HumanRef-8_V3 beadchip	22+15	27	Mix	12,647	Denmark and Netherland
GSE38484[47]	Illumina HumanHT-12_V3 beadchip	96+106	42	Mix	17,233	Denmark and Netherlands
GSE48072[48]	Illumina HumanHT-12_V4 beadchip	31+35	53	NA	15155	Finland, Sweden, Caucasian
GSE54913[49]	Arraystar Human LncRNA microarray V2.0	12+18	NA	Treatment naive	13,003	Han Chinese
Kumarasinghe et al 2013[50]	Illumina HumanHT-12_V3 beadchip	11+10	38	Treatment naive	10,544	Sinhalese

Notes: The "Mix" medication status indicates that the participants with and without drug treatment were part of the study. While "NA" (not available) indicates the unavailability of information on medication status.

Results

Comparative Performance of the ML Models in Test and Independent Dataset

We identified seven peripheral blood expression array datasets for SCZ (Table 1). Participants from these datasets belonged to different ethnic groups. Most of the datasets identified had medicated or a mixed population of SCZ participants. For Kumarasinghe et al paired study, only the "before treatment" samples were considered for the analysis to avoid the over-representation of the same samples. Of the seven datasets identified, GSE27383 was placed aside as an independent dataset. Only the genes common to the other six datasets were retained for the analysis. The resulting metafile with 377 samples and 6858 genes was used for class prediction analysis. The preprocessing of the test data and independent dataset (GSE27383) resulted in normalized (Supplementary Figures 1 and 2) and batch-corrected (Supplementary Figure 3) data. The DGEA of each train data iteration resulted in the identification of differentially expressed genes (DEGs) as features (Supplementary Figure 4). ML models were built using these feature genes from training datasets.

We used two different ML models, SVM and PAM, for prediction analysis. SVM models with kernels such as "linear", "polynomial", "radial", and "sigmoid" did not show any significant difference in the test data prediction accuracy. We, therefore chose the more popular SVM-radial for further analysis. The trained SVM-radial and PAM models were used to classify the test data samples (generated from random sampling) and an independent dataset (GSE27383). The performance of SVM-radial and PAM was evaluated based on the mean test data prediction accuracy, sensitivity, specificity, precision, and AUROC. We did not observe any significant difference in the model performance for the test and independent dataset sample classification (Supplementary Figure 5). The results discussed below are from the independent dataset. In contrast, the results from test data have been included as Supplementary Material.

The Number of Features Affects the Performance of SVM but Not PAM

A comparison of SVM-radial models with different numbers of feature genes (top5, top25, top100, top400, top1600, and all genes) revealed that its performance increases with the number of DEGs (Table 2). However, the different number of feature genes did not affect the performance of the PAM models (Table 2). We did not observe any significant difference in the performance of either of the ML models with 400 DEGs and above (Table 2). The performance of the SVM-radial

Table 2 Performance of SVM-Radial and PAM Models in Independent Dataset Class Prediction Analysis

SVM-radial					
Features	**Accuracy**	**Sensitivity**	**Specificity**	**Precision**	**AUROC**
Top5	54.44 (0.06)	66.05 (0.12)	37.24 (0.13)	60.87 (0.04)	53.98 (0.05)
Top25	60.56 (0.04)	71.4 (0.06)	44.48 (0.15)	66.12 (0.05)	60.60 (0.06)
Top100	67.08 (0.04)	75.35 (0.05)	54.83 (0.1)	71.50 (0.04)	73.34 (0.05)
Top400	67.64 (0.04)	76.28 (0.05)	54.83 (0.09)	71.69 (0.04)	75.26 (0.04)
Top1600	71.39 (0.03)	75.81 (0.05)	64.83 (0.06)	76.27 (0.03)	75.27 (0.02)
All genes	70.83 (0.03)	66.98 (0.08)	76.55 (0.06)	81.22 (0.03)	82.57 (0.02)
PAM					
Top5	61.53 (0.05)	69.07 (0.09)	50.35 (0.10)	67.44 (0.04)	63.56 (0.04)
Top25	65.56 (0.04)	64.42 (0.07)	67.24 (0.04)	74.36 (0.03)	70.76 (0.03)
Top100	64.45 (0.03)	63.26 (0.03)	66.21 (0.07)	73.67 (0.04)	74.91 (0.02)
Top400	67.09 (0.01)	63.72 (0.02)	72.07 (0.03)	77.20 (0.02)	75.40 (0.02)
Top1600	67.22 (0.02)	63.26 (0.03)	73.10 (0.04)	77.78 (0.02)	74.89 (0.01)
All genes	67.09 (0.02)	62.56 (0.03)	73.79 (0.02)	77.98 (0.02)	73.92 (0.01)

Notes: Support vector machine (SVM)-radial and prediction analysis of microarrays (PAM) models were developed using different number of top differentially expressed genes (DEGs). These models were evaluated for their performance based on their ability to predict samples of GSE27383 as independent dataset. The models were compared using RMA followed by Tukey's post hoc test with Greenhouse-Geisser and Huynh-Feldt corrections for each parameter separately. SVM-radial models performed better with higher number of feature DEGs. The values mentioned in the tables are in percentage and the standard deviation for the ten iterations in bracket.

and PAM with 400 DEGs was visualized using receiver operating characteristic curve analysis (Figure 2A and B). A similar performance of the models was observed in the prediction of test data samples (Supplementary Table 1). To further improve the precision, SVM-radial and PAM models were used for combinatorial analysis.

Ensemble Learning Results in Higher Precision for Schizophrenia Diagnosis

Selected ML models of SVM-radial and PAM were used for the combinatorial analysis. In brief, only the samples identified as cases by both algorithms were classified as cases to reduce false positives in prediction analysis. Ensemble learning of models with a hundred and more feature genes resulted in significantly higher precision and accuracy than those with fewer DEGs. The performance of ensemble learning did not significantly differ in test data and the independent dataset (data not shown).

In the independent dataset, the highest precision of 84.55% (SD: 0.04) was observed for ensemble-all genes with an accuracy of 66.39% (SD: 0.02). Also, we did not observe a significant difference in sensitivity and specificity of ensemble-400, ensemble-1600, and ensemble-all genes (data not shown). We chose ensemble-400 for further analysis. The precision of ensemble-400 (80.41%, SD: 0.04) was significantly higher than the individual models of SVM-radial (71.69%, SD:0.04) and PAM (77.20%, SD: 0.02) without any significant drop in accuracy (Figure 3A and B).

Microarray-Based ML Models Classified RNA-Seq Samples with Moderate Precision

A case-control study (Pune-SCZ) was established with 20 participants of Indian origin (SCZ: 10, CNT: 10). There was no significant difference in age, sex, and blood cell counts between case and control group participants (Supplementary Table 2). All the SCZ-affected participants recruited in this study were on antipsychotic medication. RNA was isolated

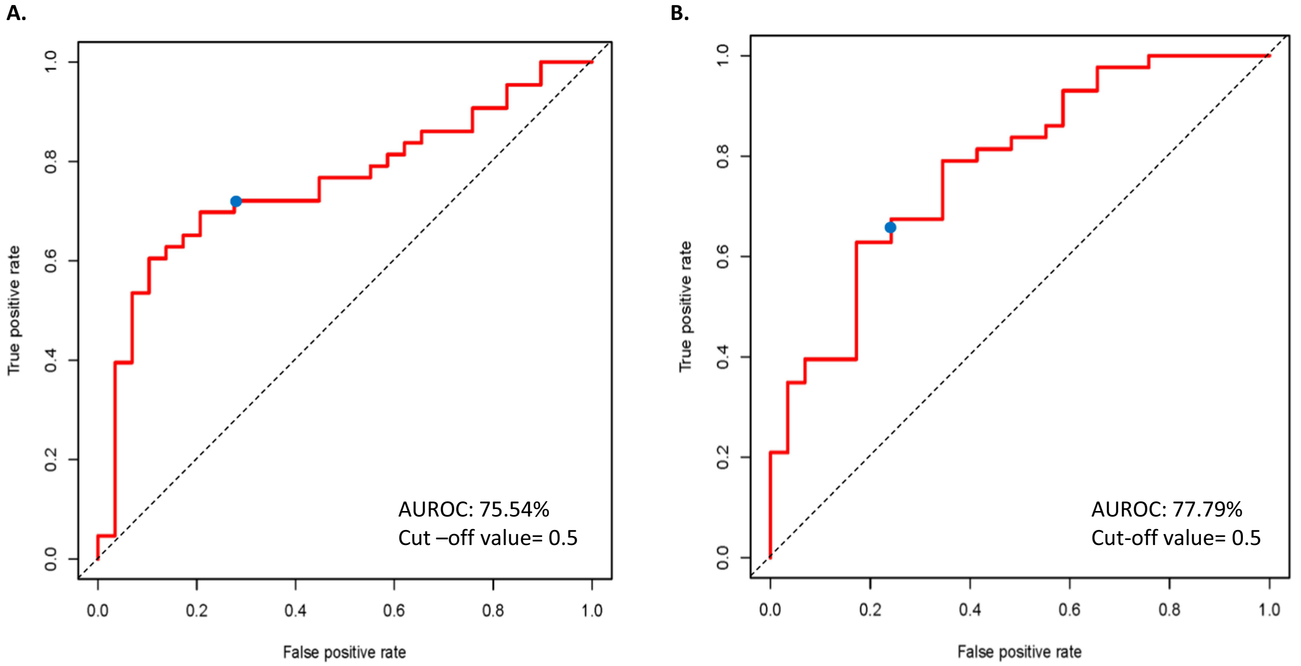


Figure 2 Receiver operating characteristic (ROC) curves for ML models in the independent dataset prediction analysis. (**A**) The AUROC for SVM-radial with 400 DEGs was found to be 75.54%. (**B**) Similarly, an AUC of 77.79% was observed for PAM models. The ROC curves of iteration one was used as representative images.
Note: The blue marker denotes the probability cut-off of 0.5.

from the peripheral blood samples of the participants and subjected to RNA-Seq analysis. The sequencing resulted in ~80 million reads per sample. The quality of sequencing was satisfactory (Supplementary Figure 6), which resulted in an acceptable percentage of alignment (Supplementary Table 3). The RNA-Seq data was used for external cross-platform validation of the models. The class prediction analysis was performed using raw counts (RC), counts per million (CPM), and transcript per million (TPM) matrices, as mentioned in the methodology section. The prediction accuracy of ML models with TPM counts was relatively better compared to CPM and RC. The performance of ML models with TPM counts is reported in this analysis. The ensemble-400 was able to classify SCZ samples with moderate precision (59.92%, SD: 0.05), which was significantly higher than the SVM-radial (52.20%, SD: 0.07) (Figure 3C). However, SVM-radial (52.00%, SD: 0.07), PAM (59.50%, SD: 0.02), and ensemble with top 400 DEGs (58.00%, SD: 0.03) predicted RNA-Seq test samples with low accuracy (Figure 3D). The higher precision of ensemble models was accompanied by higher specificity and lower sensitivity compared to the individual models (Supplementary Figure 7).

Functional Enrichment and Network Analysis Identified Biological Processes and Hub Genes Associated with Schizophrenia

The ensemble learning with the top 400 differentially expressed genes (DEGs) predicted SCZ samples in the independent dataset with higher precision (Figure 3A). Since the independent dataset mimics the clinical settings, the top 400 DEGs from its best iteration of the ensemble- 400 (iteration one) were chosen for functional enrichment analysis. The enrichment analysis of the 400 DEGs identified organonitrogen compound metabolic process, response to stress, and cellular response to the stimulus as the top three biological processes (adjusted P value < 0.05). Among the enriched processes, the biological processes related to immune function appeared more frequently, followed by processes related to protein metabolism (Supplementary Figure 8A). We identified the top five hub genes (*RBX1, CUL4B, DDB1, PRPF19*, and *COPS4*) from the PPI network of the top 400 DEGs from iteration one of the training data (Supplementary Figure 8B).

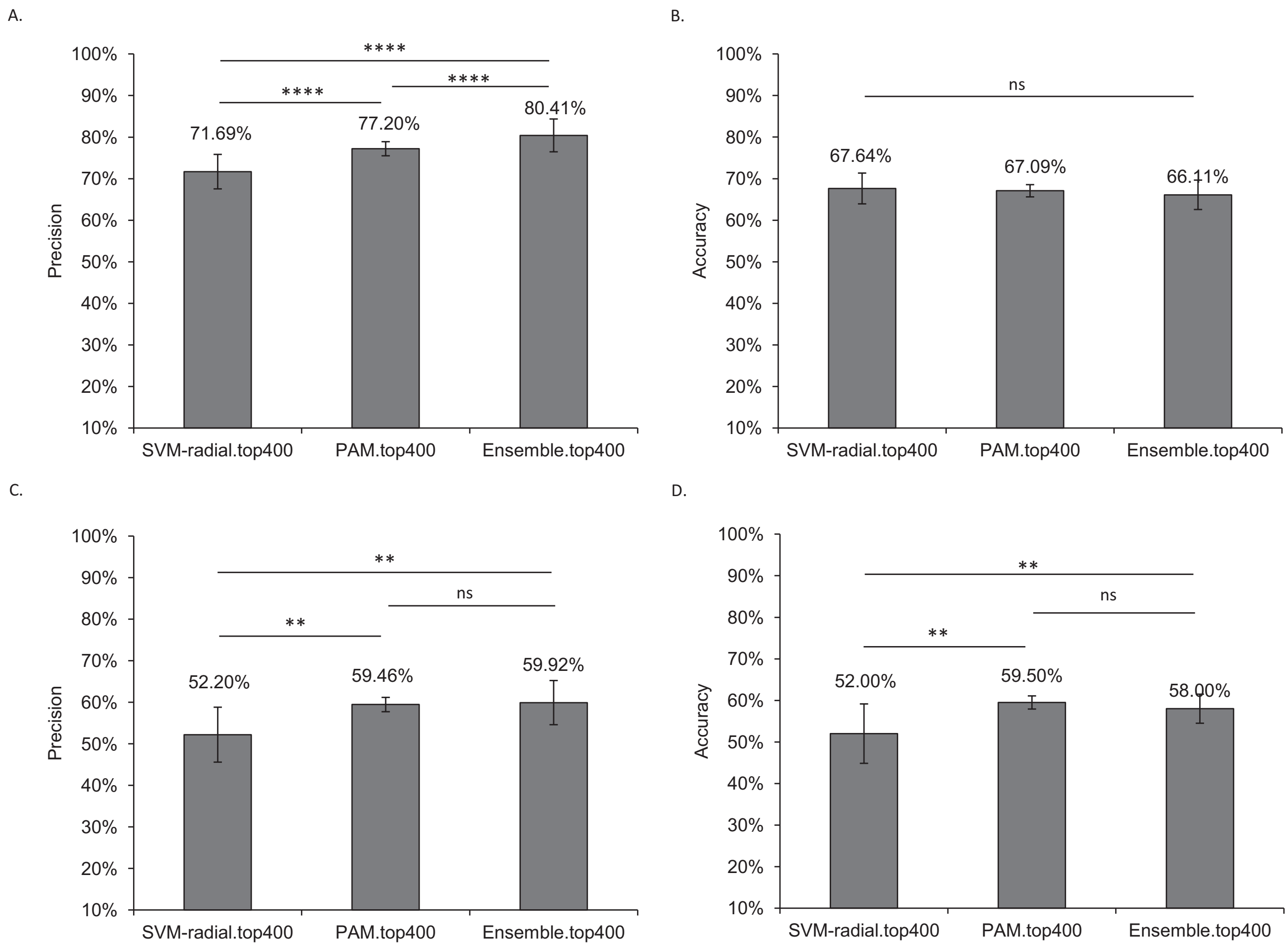


Figure 3 Performance of ensemble learning in the independent microarray dataset and RNA-Seq samples class prediction analysis. SVM-radial, PAM, and their ensemble with 400 DEGs were compared using RMA followed by Tukey's post hoc test with Greenhouse-Geisser and Huynh-Feldt corrections (adjusted P value <0.05). (**A**) Ensemble learning classified samples from the independent microarray dataset with significantly higher precision compared to individual models. (**B**) The high precision was obtained without any significant drop in accuracy. Similarly, the cross-platform performance of ensemble learning was tested using RNA-Seq samples from the Pune-SCZ study. (**C** and **D**) Ensemble learning classified RNA-Seq samples with high precision and without a significant drop in accuracy when compared to the individual models.
Note: Error bars represent standard deviation for ten iterations, "**" (adjusted P value ≤ 0.01), "****" (adjusted P value ≤ 0.0001), "ns" (adjusted P value > 0.05).

Discussion

Neuropsychiatric disorders such as schizophrenia are associated with stigmatization. Hence, diagnostic tests with high precision are needed. In this study, we developed an ensemble learning model with higher precision for classifying SCZ samples. We developed a unique strategy of preprocessing microarray data to build a robust model that can be used to analyze a single sample. We used the feature selection approach (DGEA) for better "explainability" in clinical settings[12] and SVM and PAM for binary classification analysis. The two algorithms use different logic for classification, and a consensus rule resulted in higher precision without any significant drop in accuracy compared to the individual models (Figure 3A and B). The increase in precision was associated with increased specificity and an expected but moderate decline in sensitivity (Supplementary Figure 9A and 9B).

We could identify a limited number of studies that have used ML models for classification analysis in SCZ using peripheral blood gene expression data. Most of these studies have focused on validation of the previously reported DEGs.[10,51–53] Zhu et al reported AUROC of 0.993 for SVM using previously reported DEGs in brain samples.[10] Tsuang et al (n=30) and Middleton et al (n=33) used DEGs from their cohort to classify SCZ and bipolar disorder with an accuracy of 95–97%.[54,55] Several merged data-analyses of publicly available microarray datasets have also been performed to address the confounding effects of different ethnicities. Zhang et al, in their study, achieved a remarkable classification accuracy of 100% with locally weighted learning (LWL).[56] The study highlighted the use of a correlation-based feature selection algorithm for SCZ class prediction analysis. Similarly, Jonathan Hess et al reported an AUROC of 0.72 to 0.77 for SCZ vs CNT and 0.607 for bipolar disorder vs SCZ in two separate studies with independent datasets.[9,16]

We did not come across a study with the aim of developing ML models with higher precision. In this regard, we ensembled predictions of the best performing models of SVM-radial (Precision: 71.69% SD: 0.04) and PAM (Precision: 77.20%, SD: 0.02) with 400 DEGs for high precision. The ensemble learning resulted in higher precision (80.41%; SD: 0.04) without a significant drop in accuracy (Figure 3A and B). The models' performance remained consistent despite biological variations such as drug status and technical differences in the datasets, indicating its robustness, especially for clinical applications (Supplementary Figure 10). Previous studies have suggested a need to integrate biological and clinical information for better performance of ML models.[11,43] However, the unavailability of clinical information for each sample in the publicly available GEO datasets restricted our analysis to only gene expression markers.

We tested the cross-platform performance of ensemble learning with 400 DEGs using RNA-Seq data from the Pune-SCZ study. Similar to the independent microarray dataset (GSE27383), these samples were not part of the ML model development and hence served as independent data for validation of the models. We established a case-control study with age and sex-matched participants (Pune-SCZ). The validation datasets included TPM, CPM, and RC values generated from the RNA-Sequencing results. The prediction accuracy of test data with TPM values was expected to be higher compared to CPM and RC since the TPM values are not influenced by gene length. Similar to microarray data, ensemble learning using TPM resulted in significantly better precision as compared to individual models (Figure 3C). PAM models performed significantly better when compared to the support vector machines (SVM-radial); however, the overall low accuracies of individual models suggest a need for the development of better cross-platform normalization techniques (Figure 3D).

The 400 DEGs from the best-performing iteration of ensemble learning were chosen for functional enrichment analysis. The enrichment analysis identified organonitrogen compound metabolic process (GO: 1,901,564) as one of the enriched biological processes. These results are in accordance with the previous report.[57] The other top biological processes, such as response to stress (GO:0006950) and its child-term cellular response to stimulus (GO:0051716), have not been directly reported in association with schizophrenia before. However, the response to oxidative stress (GO:0006979), a child term of response to stress, has been implicated in the pathogenesis of psychiatric disorders.[58,59] In addition to this, other biological processes related to immune function and cell cycle regulation have also been associated with SCZ earlier (Supplementary Figure 8A).[60–62]

We also identified the key regulators (hub genes) of the protein-protein interaction network (Supplementary Figure 8B). The hub genes identified in this study have been previously reported in association with neuropsychiatric disorders, including SCZ. The genome-wide association studies have also associated *RBX1, DDB1, PRPF19*, and *COPS4* with SCZ.[63,64] Further, *RBX1, CUL4B, DDB1, PRPF19*, and *COPS4* are known to be differentially expressed in the brain of SCZ-affected individuals.[65] Of the hub genes identified, only *RBX1* and *CUL4B* have been reported to be differentially expressed in the peripheral blood.[66,67] Interestingly, Sun L et al reported upregulation of CUL4B in drug naïve and first-onset schizophrenia participants.[67] Of the 400 DEGs *SIGIRR, SRPK1, TIPARP, RPRD1A*, TGFA, *IL18BP, POLR3H, NKG7, GOT2, MAP4K1, C11orf1, MCM3, CUL4B, CTBP1, NELFCD, MAGED1, NAT1, ATIC, SCAP* were also highlighted in our previous study for their association with SCZ.[8]

Strengths and Limitations of the Study

To the best of our knowledge, this is the first study that uses ensemble learning for the analysis of genomics data for schizophrenia (SCZ) diagnosis with higher precision. The similar performance of ML models in test data and independent dataset prediction indicates no data leakage in the sample processing workflow. The preprocessing of raw microarray data in this analysis also allows the prediction of a single test sample, suggesting its reliable application in real-world clinical settings. Further, the higher precision offered by ensemble learning seems to perform equally well, irrespective of the biological and technical variations in the datasets. Notably, cross-platform validation confirms the compatibility of transcript-per-million (TPM) normalization of RNA-Seq data with microarray-based ML models for prediction analysis. The ensemble learning approach developed in this study can be used for other disorders that carry a high burden of stigma.

However, there are several limitations to this study. The proposed method for model development is in its preliminary stage and lacks validation with a larger sample size. The unavailability of clinical information for each sample in publicly available datasets restricted its use in developing multi-modal ML models. Additionally, the absence of clinical attributes prevented us from testing the effect of medication status, ethnicity, age, and sex on the performance of the models. The ML model performance in cross-platform validation was relatively poor, suggesting scope for developing better normalization methods. The analysis was restricted to only SCZ and did not attempt multi-class classification as Yang et al did in their study.[68] The modest sample size of the case-control study remains a limitation.

Conclusions and Future Directions

In conclusion, our study offers a proof of concept for the development of class prediction models for SCZ with higher precision, indicating its potential application in clinical settings in the future. The current strategy effectively deals with problems like data leakage and preprocessing of single microarray samples. The feature genes and biological pathways identified in this study can be pursued to explore their potential role in the disorder. Most importantly, this study attempted cross-platform class prediction using RNA-Seq data as test samples. However, a relatively poor cross-platform performance indicates the need for better cross-platform normalization techniques. In addition, the availability of data from other high throughput genome-wide studies may create novel avenues for developing multi-modal learning. Specifically, the multi-omic approach integrating genomic, transcriptomic, and proteomic data will surely result in the precise diagnosis of psychiatric disorders.

Abbreviations

ANOVA, Analysis of Variance; AUROC, Area under the Receiver Operating Characteristic curve; CNT, Healthy controls; CPM, Counts per Million; DEG, Differentially Expressed Genes; DGEA, Differential Gene Expression Analysis; DSM-5, Diagnostic and Statistical Manual of Mental Disorders, 5th Edition; GEO, Gene Expression Omnibus; HISAT2, Hierarchical Indexing for Spliced Alignment of Transcripts limma, linear models for microarray data; MCC, Maximal Clique Centrality; ML, Machine learning; PAM, Prediction Analysis of Microarrays; PANSS, Positive And Negative Syndrome Scale; PAST, PAleontological STatistics; PBMCs, Peripheral blood mononuclear cells; PPI, Protein-Protein Interaction; RC, Raw Counts; SCID-5-RV: Structured Clinical Interview for the DSM-5 Research Version; SCZ, Schizophrenia; SD, Standard Deviation; STRING: Search Tool for the Retrieval of Interacting Genes/proteins; SVM, Support Vector Machine; TPM, Transcripts per Million.

Data Sharing Statement

The datasets generated for this study can be found in INDA-CA, INCARP000275.

The R scripts used for the analysis are available on GitHub. (https://github.com/macdlab/2023_VW_SCZ_Ensemble).

Compliance with Ethical Standards

Two independent ethical committees approved the study protocol: the KEM Hospital Research Centre Ethics Committee (KEMHRC ID No. 2001) and Symbiosis International (Deemed University) Independent Ethics Committee (SIU/IEC/99). Written informed consent was obtained from all participants. For participants with schizophrenia, written informed consent was supported by written informed consent of a spouse or a first-degree relative aged 18 and above. Parents, siblings, and children were considered first-degree relatives. Clinical interviews were administered by a trained psychiatrist and a psychologist in private. The diagnosis was confirmed by a senior psychiatrist. The identity of the participants was protected by using a unique identification number. The data collected in the study are securely stored with restricted access. Any data sharing with other researchers will prioritize participant confidentiality, ensuring identities remain undisclosed. All the participants were compensated for their travel and time.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors state that there is no conflict of interest.

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