STAT3 as a Candidate Transcriptomic Prognosticator of Sepsis Severity Levels

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ABSTRACT

Background. Sepsis is a life-threatening multiple-organ dysfunction caused by a dysregulated host response to infection and is the leading cause of death in non-cardiac intensive care facilities. Early reliable prediction of sepsis outcomes leads to cost-efficient resource allocation and therapeutic strategies. However, there are still no reliable markers to predict the outcome of patients at the initial stage of sepsis. Analyzing transcription profiles enables researchers to predict early outcomes using transcripts and their expression patterns. Transcriptomic profiling of septic patients has been done recently; however, analysis of prognostic outcomes is still scarce.

Objective. This study aimed to determine transcriptional indicators that may be useful in the prognosis of the severity of sepsis.

Methods. This is a prospective cohort study of Filipino patients admitted for sepsis at the national tertiary referral hospital in Manila, Philippines. We conducted differentially expressed gene analysis, network analyses, and area under the curve study of publicly available datasets of surviving vs. non-surviving sepsis patients to identify candidate prognosticator markers. Quantitative PCR was used to characterize the expression of each marker. A model using ordinal logistic regression analysis was done to determine which among the markers can best predict the outcome of sepsis severity.

Results. We identified *ACTB*, *RAC1*, *STAT3*, and *UBQLN1* as candidate mRNA prognosticators. The expression of *STAT3*, a gene involved in immunosuppression, is inversely correlated with the severity of sepsis.

Conclusion. Transcriptomic markers such as *STAT3* can predict the severity of patients with sepsis. Early detection of its inverse expression may prompt early and more aggressive management of patients.

Keywords: sepsis, STAT3, data mining, transcriptomics, prognostication

INTRODUCTION

Sepsis, defined as "life-threatening organ dysfunction caused by a dysregulated host response to infection,"[1] is a common condition encountered in intensive care units[2] and is the leading cause of death in non-cardiac intensive care facilities.[3] The effectiveness of therapy is time-dependent; hence, identifying appropriate treatment through accurate prognostication is important.[4] Current sepsis prognostication is clinically based,[5] and several proteins associated with the disease pathophysiology are also used as biomarkers for prognosis.[6] However, the reliability of these tools to predict the outcome of patients with sepsis at diagnosis is still in question.[6]

Transcriptomics, or the study of transcriptome – the set of RNA transcripts expressed by the genome, has been used in discovering prognostic markers in other diseases such as breast cancer,[7] viral infections,[8] and other inflammatory

Paper was presented in the MedEureka: Trends and Breakthrough in Molecular Medicine on February 10, 2018, at Buenafe Hall, College of Medicine, University of the Philippines Manila.

Paper was presented in the Biological Sciences Graduate Congress 2017, on December 19-21, 2017, at the National University of Singapore, Singapore.

Paper was presented in the 5th International Heidelberg Forum for Young Life Scientists on June 8-9, 2017, at the German Cancer Research Center, Heidelberg University, Germany.

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diseases.[9] This high-throughput technology may be applied in sepsis as the transcriptomic profiling of patients with sepsis has been done recently.[10] However, further analysis of these data regarding prognostic outcomes is scarce.

The lack of reliable prognosticating tools for sepsis with the availability of its transcriptomic profiles, albeit lacking further analyses of these datasets, motivated the work of this study. Therefore, this research aims to screen and evaluate transcripts that can predict the severity of sepsis. Specifically, the study seeks to: (1) identify transcripts associated with survival in sepsis using publicly available datasets; and (2) determine if the gene expression levels of the selected transcripts can be associated with the severity level of Filipino patients with sepsis.

Sepsis is one of the leading causes of mortality worldwide. While appropriate management based on prognosis is needed, available prognosticating tools for sepsis are not reliable, and the use of transcriptomic studies may address such concern.[11] Despite the availability of transcriptomic profiles of sepsis patients, further analysis of these data to generate clinically significant results is lacking. This study identified *ACTB, RAC1, STAT3,* and *UBQLN1* as candidate prognosticators of sepsis mortality through data mining.

MATERIALS AND METHODS

Selection of publicly available datasets

Datasets were obtained from the Gene Expression Omnibus of the National Center for Biotechnology Information (GEO-NCBI) and ArrayExpress of the European Bioinformatics Institute. The string search used was (sepsis and (prognos* or predic* or nonsurviv*)) AND "Homo sapiens" or "sepsis" to include as many datasets as possible. Inclusion criteria for the selection of datasets were as follows: (a) use of human blood samples; (b) inclusion of healthy controls, surviving and non-surviving sepsis patients; and (c) freely available and normalized microarray or RNASeq data. Only the studies with freely available datasets were used in this study.

Screening of candidate prognosticators from selected datasets

Differentially expressed transcripts were identified using Gene Expression Omnibus 2 R-Statistic (GEO2R) – a web-based tool in analyzing the Series Matrix data file directly from each of the datasets – a feature of the NCBI databases site. With a cut-off p-value of less than 0.001, the top 250 differentially expressed genes (DEGs) were determined per dataset using this tool. The gene expression levels used were specifically derived from the time of diagnosis (D0 or D1) since this study aimed to determine the prognosis of patients upon diagnosis, in addition to the top 250 genes per dataset.

Network interaction among the 250 genes per study was analyzed using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) version 9.1. Hub genes and each of the networks were then identified for further analysis. Characterization of selected candidate prognosticators was determined using gene annotation tools, such as Aceview, Database for Annotation, Visualization and Integrated Discovery (DAVID), and Kyoto Encyclopedia of Genes and Genomes (KEGG).

Selection of Study Population and Sample Handling

The identified candidate mRNA prognosticators were verified on the subjects from the UPMREB-approved study of Nevado et al. (n. p., 2014) entitled *Quantitative molecular signatures and predictors of sepsis and the development of its complications using gene expression markers and pathway analyses*. The study, approved by the University of the Philippines-National Institute of Health Ethics Review Board, included clinically diagnosed patients with sepsis from the University of the Philippines-Philippine General Hospital. Inclusion and exclusion criteria were similar to the parameters used to screen the databases. Patients older than 18 years of age and without other co-morbidities were selected for this study. A one-time sample collection was done following the study protocol of Nevado et al. (n. p. 2014). Patients with sepsis who qualified for this study were enrolled from September 2015 to February 2016.

Blood samples were extracted upon enrollment in the study. Total RNA extraction and isolation were done following the protocol of the study of Nevado et al. (n.p. 2014). Extracted RNA was quantified using NanoDrop and was stored in a -80˚C refrigerator. cDNA synthesis was done following the iScript cDNA synthesis kit Biorad.™ The reaction containing 1 µl reverse transcriptase, 4 µl reaction buffer, and 500 ng of RNA sample was added to nuclease-free distilled water to a total of 20 µl per sample. The reaction was performed under the conditions of 25˚C for 5 min, 42˚C for 30 min, 85˚C for 5 min, and 4˚C for the remaining time.[12]

Primer Designing

Intronic/exonic boundaries for each candidate prognosticating transcripts were considered in designing the primers. The transcript sequences were sourced from the NCBI Nucleotide database. The University of California Santa Cruz (UCSC) human genome browser was used to identify the sequence of the selected variant with the lowest single nucleotide polymorphism (SNP) hits. Primer 3™ was used to design the primers from the desired sequence. The designed primers were then tested *in silico* using the USCS genome browser to check if there were probable non-specific binding to other non-target genes. The lines were ordered from Lifeline diagnostics.

Quantification of Expression of Selected Candidate Prognosticators

We examined the clinical significance of the candidate prognosticators by analyzing the gene expression of their transcripts in actual sepsis patients. Gene expressions were

determined using real-time quantitative polymerase chain reaction (qPCR). Instructions from SYBR™ Green were used: 2 ul of cDNA, 8 ul of primers – both forward and reverse – and 10 ul dye was used to have a 20 ul reaction mix. The condition used for the qPCR run 950˚C for 5 min, with 39 cycles consisting of 95˚C for 45s, chosen annealing temperature for 45s, 72˚C for 45s, read at 78˚C, and 95˚C for 45s. 18S RNA was used as the housekeeping gene. All primers used were 500 nM. Expression levels of candidate mRNA prognosticators were measured using Delta-CT.

Clinical Determination of Sepsis Survival Likelihood

Pertinent clinical values were collected from the patients. They were used to evaluate their respective Acute Physiology and Chronic Health Evaluation (APACHE) II score – a measure for sepsis severity - which has a corresponding mortality likelihood. Patients are classified into severity levels 1–8 according to their clinical scores: 0–4, 5–9, 10–14, 15–19, 20–24, 25–29, 30–34, and >34, respectively.[13] The mortality rate for each severity level are as follows: 4, 8, 15, 25, 40, 55, 75, and 85%, respectively.[13]

Statistical analysis

The identified hub genes from the STRING network study were subjected to area under the curve (AUC) analysis using MedCalc statistical software. Genes with an AUC value of 0.9 to 1.0 were selected as the candidate prognosticators of this study.

The mean expression of the level of each candidate gene was analyzed using one-way ANOVA. Gene expression levels were compared with the APACHE II scores of each patient using ordinal logistic regression analysis. Ordered logistic regression analysis was performed using Stata to evaluate if the change in the gene expression per candidate prognosticator significantly varies per severity level. A significant result would indicate that the components of the model – the relative gene expressions of the four candidate genes taken altogether – correlate with the outcome (the sepsis severity level).[14] Individual p-values for each gene were also determined to identify which of the components significantly contributes to the ability of the model to correlate with the outcome.[14] Spearman rank-order correlation was done to determine the relationship between gene expression and sepsis severity level. The significance level for the hypothesis testing used was set at 5%.

RESULTS

Identification and Characterization of Candidate Prognosticators in Publicly Available Datasets

During the literature search, only two studies were able to satisfy the inclusion criteria for datasets. The first dataset was from the study *Patterns of Gene Expression in Peripheral Blood Mononuclear Cells and Outcomes from Patients with Sepsis Secondary to Community-Acquired Pneumonia* by Severino et al., 2014.[15] The study investigated the whole-genome gene expression profiles of mononuclear cells from the survivor (n=5) and non-survivor (n=5) septic patients and three healthy controls. The blood samples were collected and analyzed at the time of sepsis diagnosis and seven days later. The platform that they used in their study was Agilent-014850 Whole Human Genome Microarray 4x44K G4112F. The second dataset was from the survey *Identifying Key Regulatory Genes in the Whole Blood of Septic Patients to Monitor Underlying Immune Dysfunctions* by Parnell et al., 2013.[16] The study investigated the whole blood of patients from survivor (n=26) and non-survivor (n=9) patients with sepsis and healthy controls (n=18). Gene expressions were analyzed from the day of diagnosis for five consecutive days. Illumina HumanHT-12 V3.0 expression bead chip was used in their study to analyze gene expression levels. For both datasets, only the results from D0 or D1, the first day of diagnosis, were used. Only gene expressions from surviving and non-surviving patients were analyzed. Differentially expressed genes from the datasets were then pooled for further analysis.

Genes that highly interact with other genes are more likely to sensitively change in response to any perturbation on a given disease hence making these genes good biomarkers.[17] To determine which differentially expressed genes are highly interacting with other genes, STRING analysis was done. Analyzing gene annotations was also done to identify whether the highly interacting genes have biological implications in the pathophysiology of sepsis. Hence, genes with annotated immunity or related to such associations were identified using functional annotation software such as DAVID and KEGG. Fourteen differentially expressed genes were identified based on these analyses. The selected 14 genes were further narrowed to 4 (Table 1) based on their AUC values which were calculated. AUC analysis shows how a certain expression of a gene predicts the outcome we want to assess, which is in our case, the survivorship of patients with sepsis from the datasets. A value near 1.0 or 0.99 indicates that the expression of the gene highly predicts the outcome being analyzed. From STRING, AUC, and gene annotation analyses, the candidate prognosticators were determined as shown in Table 1.

Clinical Evaluation of Identified Prognosticators

A total of 40 patients from the project *Quantitative molecular signatures and predictors of sepsis and the development of its complications using gene expression markers and pathway analyses* were included in the study.

Most of the patients were men and within the age range of 45–54 years (Table 2). Sixty percent (24 out of 40) were classified under categories 3 and 4, with 12 patients each. None of them were under category 7, and only one patient was classified with a severity score of 8. The mean relative gene expressions of the candidate prognosticators were analyzed at each level as shown in Figure 1.

Table 1. Identified candidate prognosticators

AUC	Gene code	Gene name	Brief gene description
1.000	*UBQLN1*	Ubiquitin 1	Promotes delivery of ubiquitinated proteins to the proteasome for degradation
0.996	*RAC1*	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	Involved in regulating cell cycle progression, specifically in the G2/M transition, and is required for cell proliferation
0.991	*STAT3*	Signal transducer and activator of transcription 3 (acute-phase response factor)	STAT3 codes for transcription factors involved in the TH1 and THαβ immunity, specifically in Th17
1.000	*ACTB*	Actin, beta	Structural gene among the transcripts, codes for actin

Table 2. Patient demographics per category of sepsis severity level

Severity level	1	2	3	4	5	6	7	8
Gender								
Men	4	3	9	8	0	1	0	1
Women	2	1	3	4	3	1	0	0
Total	6	4	12	12	3	2	0	1
Age (yrs.)								
18–24	2	1	0	0	0	0	0	0
25–34	2	1	3	1	1	0	0	0
35–44	2	1	3	2	0	0	0	0
45–54	0	1	4	6	2	1	0	1
55–64	0	0	2	2	0	1	0	0
Total	6	4	12	12	3	2	0	1

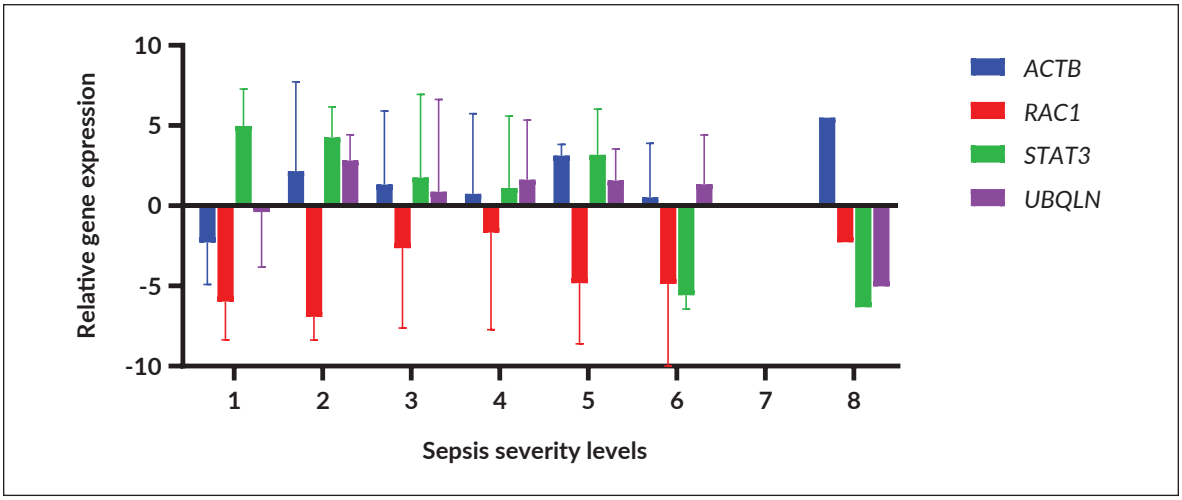

Figure 1. Relative gene expression levels of candidate prognosticators per severity level as per APACHE II scores.

Patients in severity level 1 were all healthy controls (5 out of 40). Comparing the relative expressions per severity level, all candidate prognosticators had significantly different expression profiles compared to control (One-way ANOVA p-value < 0.05). However, as seen in Figure 1, only *STAT3* had a significantly different expression for all levels, especially at sepsis severity levels 3 and 4 (Dunnett's test p-value < 0.05).

The four candidate genes were correlated significantly with the sepsis severity level (p-value of 0.0304) (Table 3) for the model that included all the predictors. Examining the individual p-values of the genes, only *STAT3* expression (p-value = 0.005) had a significant gene expression change across the severity levels.

There was a moderate negative correlation between the *STAT3* gene expression and sepsis severity levels, which was statistically significant ($r_s(40) = -0.429$, $p = 0.006$) (Figure 2). Therefore, 43% of the variation observed is attributed to the relationship between the gene expression level of *STAT3* and the severity level of sepsis.

DISCUSSION

The study identified *ACTB, RAC1, STAT3*, and *UBQLN1* as candidate prognosticators of sepsis mortality through data mining of publicly available datasets. From the ordered logistic regression model of the four transcripts, only the expression of *STAT3* was significantly associated with the severity level of sepsis patients (p-value = 0.05). Furthermore, Spearman's rank-order correlation showed that the downregulation of *STAT3* is correlated with sepsis severity level ($rs(40) = -0.429$, $p = 0.006$).

The involvement of the four candidate genes can be implicated in the pathophysiology of sepsis. The canonical pathology of sepsis is attributed to the unregulated hyper-inflammatory response of the body to an infectious etiology.

Table 3. Ordered logistic regression (including all candidate prognosticators)

Ordered logistic regression — Number of obs = 40; LR chi2(4) = 10.68; Prob > chi2 = 0.0304; Log likelihood = -61.600094; Pseudo R2 = 0.0798

severity	Odds Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
actbdeltact	1.143663	.094035	1.63	0.103	.9734423	1.34365
rac1deltact	.9438809	.0674233	-0.81	0.419	.8205672	1.085726
stat3deltact	.7990868	.0632828	-2.83	0.005	.6842015	.9332626
ubqln1deltact	1.002122	.0658769	0.03	0.974	.8809774	1.139925
/cut1	-2.080804	.5621596			-3.182616	-.9789911
/cut2	-1.357829	.4930203			-2.324131	-.3915269
/cut3	.1075131	.4295545			-.7343982	.9494245
/cut4	1.851763	.5423934			.7886914	2.914834
/cut5	2.762312	.7040095			1.382479	4.142145
/cut6	4.104003	1.107197			1.933936	6.27407

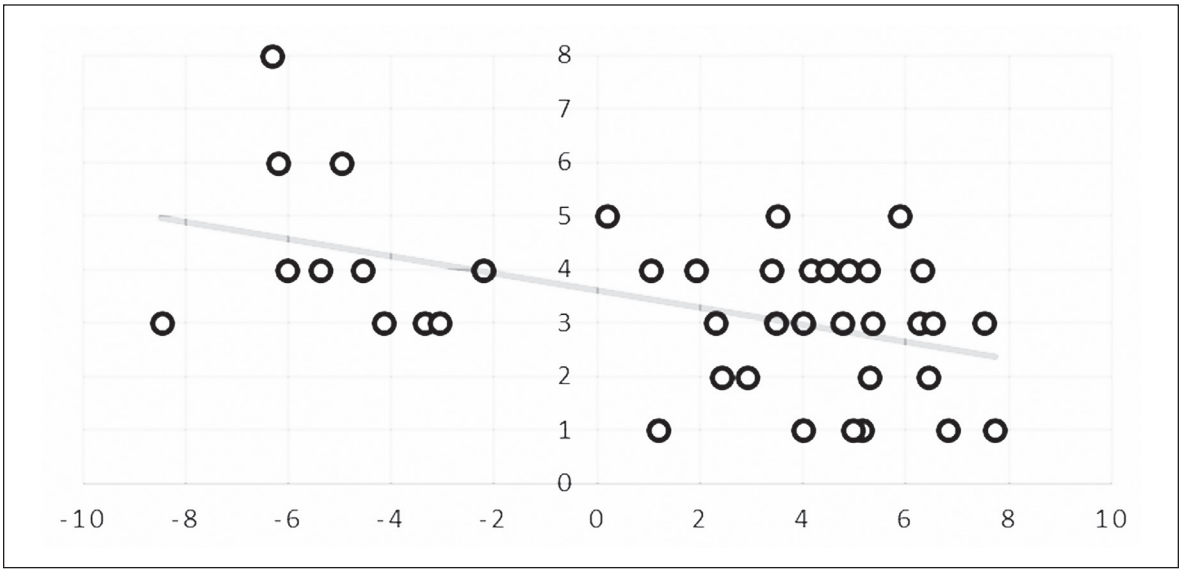

Figure 2. *STAT3* gene expression and sepsis severity levels. Using Spearman rank-order correlation, *STAT3* gene expression contribute 43% of the variability across sepsis severity levels; (rs(40) = -0.429, p = 0.006).

This hyper-inflammation leads to coagulative problems that eventually cause end-organ damage, as seen in severe cases of sepsis.[18,19]

Several cell-signaling pathways are involved in the hyper-inflammatory pathology of sepsis. These pathways include hyper-activation and proliferation of cells as in leukocytosis and cytokine storm observed in septic patients. One of the signaling molecules found in these pathways is *RAS-related C3 botulinum toxin substrate 1 (RAC1*). Being part of the Ras-related superfamily of GTP-binding proteins, it regulates cell cycle progression, specifically in the G2/M transition, and is required for cell proliferation.[20] Aside from cell proliferation, *RAC1* has also been implicated with reactive oxygen species (ROS) production and inflammatory responses.[21] RAC 1, a protein kinase, may also regulate the activation of enzymes involved in the hyperinflammatory response.[22]

UBQLN1 is part of the ubiquitin family of ubiquitin receptors that promote the delivery of ubiquitinated proteins to the proteasome for degradation.[23] It may play a role

in degrading damaged proteins due to the hyperactivity within cells due to increased transcription of cytokines or chemokines in response to sepsis. A specific function of *UBQLN1* involves interacting with *HERC3*, an E3 ligase, that stabilizes the interaction of *HERC3* to proteosome and *NFkB*.[24] This interaction of *HERC* to *NFkB* might also be associated with the inflammatory role of *UBQLN1* in sepsis.

ACTB, the only structural gene among the candidate transcripts, codes for actin.[25] Actin has a variety of cellular processes involved in the immune function, such as intercellular interactions, endocytosis, cytokinesis, signal transduction, and maintenance of cell morphology;[26] therefore, involvement of actin in the pathophysiology of sepsis is not surprising. Molecular transport of substances within and outside cells and maintaining cellular integrity are essential in innate and adaptive immunity; hence, cytoskeletal proteins also play an essential role in regulating inflammation.[27] A recent study compared patients' serum actin/gelsolin ratio with their APACHE II scores and found that they may serve as complementary prognostic markers of sepsis.[28]

Although it is more generally accepted that a hyper-activated innate immune response is the culprit for the pathological effects of sepsis, the emerging concept of adaptive immune suppression is beginning to take part in the pathology of sepsis.[29] Recent studies in non-surviving patients of sepsis show reduced inflammatory responses leading to shock or different susceptibility to other infections that might exacerbate the septic condition.[30] Downregulation of *STAT3*, a gene coding for pro-inflammatory regulator proteins,[31] might be involved in the immunosuppression observed in the pathology of sepsis. *STAT3* codes for transcription factors in TH1 and THαβ immunity, specifically in Th17.[32] Th1 or T-helper 1 cells are part of the CD4+ effector lineage that promote cell-mediated immune response and play a role in defense against intracellular infections;[33] Th17 or T-helper 17 cells are a unique lineage of CD4+T-cells dedicated to producing interleukin 17 (IL-17), a very potent pro-inflammatory cytokine affecting a variety of tissue stromal cells.[34] With these molecules downstream of *STAT3*, downregulation of the gene promotes adaptive immune suppression in sepsis. A study analyzed the immunosuppressive arm of sepsis pathology and showed that most adaptive immunity genes were downregulated, including *STAT3*.[35] This supports the finding that *STAT3* downregulation is associated with the severity of sepsis in patients. Hence, the significant changes in *STAT3* expression across different sepsis severity levels and its significant contribution to the predictive model of sepsis prognosis are all congruent with other studies supporting the immunosuppressive arm of the pathophysiology.

The use of transcriptomics in the field of molecular diagnostics and prognostics proves to be a high-throughput approach in understanding the complexity of sepsis.[36] Data from these studies provide unbiased results in identifying candidate biomarkers, reducing investigator bias. In this approach, almost all known genes are interrogated rather than a specific set of genes chosen by the investigator based on *a priori* and potentially biased assumptions. This neutral approach is particularly suited to sepsis, where the pathophysiology is not well elucidated. Transcriptomics in the discovery of prognosticating biomarkers has been done in other diseases such as cancer and trauma, and sepsis;[37-39] instead of mRNA, most of these studies utilized micro-RNAs.

RNA expression in blood is labile, and its regulation can reflect either the normal or pathological state of an individual.[40] With this, RNA profiles, such as mRNA expression level, have been used in several diseases such as cancer, as a diagnostic or prognostic tool.[41] However, heterogeneity of cells present in blood can generate misleading results; hence interpretation of transcriptional profiles must be in the context of the cellular composition of the blood being sampled.[42] The use of subset cells, peripheral blood mononuclear blood cells (PBMCs), attempt to control the effect of cell heterogeneity present in the transcription profiles.[43]

Due to the limited sample size of non-surviving patients (n=1), ROC curve and AUC analyses were not performed because these tests require at least two samples in a category. Unfortunately, only one patient was classified under severity 7. Ideally, the study should have analyzed different AUC values for single and combinatorial gene panels, with and without a standard clinical scoring system for sepsis progression (e.g., APACHE II, SOFA). Based on the literature, panel biomarkers for sepsis coupled with clinical scoring systems yielded better AUC values.[1] Although we determined that the model with all the four genes is associated with the severity levels of sepsis patients, the study cannot determine whether these gene/s have a better prognosticating value than the APACHE scoring or if combining APACHE scoring with the gene/s can increase sensitivity and specificity of prognosticating sepsis patient outcome.

Nevertheless, the study showed that differentially expressed genes mined from other databases with a highly heterogeneous sample population could be used in a local setting like the Philippine General Hospital. From the four genes - *ACTB, RAC1, STAT3, and UBQLN1* - we selected from data mining two publicly available datasets, we determined that the downregulation of *STAT3* is significantly associated with the degree of sepsis severity.

CONCLUSION

ACTB, *RAC1*, *STAT3,* and *UBQLN1* were identified as candidate prognosticators of sepsis mortality through data mining of publicly available datasets. Various bioinformatics tools such as GEO2R, STRING, were used to identify the transcripts. Among the four transcripts, the gene expression of *STAT3* is significantly associated with the severity level

of Filipino patients with sepsis. Moreover, the expression of *STAT3* is significantly inversely proportional to the severity outcome. Further studies can be done to validate the involvement of *STAT3* in the pathophysiology and severity of sepsis through the use of biological models.

Acknowledgments

This work is funded by the Philippine Council for Health Research, Department of Science and Technology. I would also like to acknowledge the Institute of Human Genetics of the National Institute of Health-University of the Philippines Manila for providing equipment and technical assistance.

Statement of Authorship

The author contributed in the conceptualization of work, acquisition of data and analysis, drafting and revising and approved the final version submitted.

Author Disclosure

The author declared no conflicts of interest.

Funding Source

The study was funded by the Department of Science and Technology - Philippine Council for Health Research and Development (DOST-PCHRD).

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