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Integrative Systems Biology Approaches to Identify Potential Biomarkers and Pathways of Cervical Cancer

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Abstract: Nowadays, cervical cancer (CC) is treated as the leading cancer among women throughout the world. Despite effective vaccination and improved surgery and treatment, CC remains its fatality rate about half of the infected populations globally. The major screening biomarkers and therapeutic target identification have now become a global concern. The present study, we have employed systems biology approaches to retrieve the potential biomarkers and pathways from the transcriptomic profiling. Initially, we have identified 76 of each up-regulated and down-regulated gene from a total of 4,643 differentially expressed genes. The up-regulatory genes are mainly concentrating on immune-inflammatory response and the down-regulatory genes are on receptor binding and gamma-glutamyltransferase. The involved pathways associated with these genes were also assessed through pathway enrichment and they were mainly focused on different cancer pathways, immunoresponse, and cell cycle pathways. After the subsequent enrichment of these genes, we have identified 12 hub genes, which play a crucial role in CC. Furthermore, the survival of the hub genes was also assessed, and among them, finally, CXCR4 has identified as one of the most potential differentially expressed gene that might play a vital role to the survival of CC patients. Thus CXCR4 could be used as a prognostic biomarker and development of a drug target for CC.

Keywords: Systems biology; cervical cancer; prognostic biomarker; differentially expressed genes.

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Introduction:

Cervical cancer (CC), classified as the second most prominent cancer, is one of the most recurrently diagnosed cancers in terms of prevalence and source of cancer-related deaths in women worldwide [1]. According to World Health Organization (WHO), cervical cancer represents almost 6.6% of all cancers of females, with an estimated 570,000 new cases in 2018 where low- and middle-income countries experienced an average of 90% deaths [2-4]. Developed countries have also experienced the fatality of this cancer; for instance, only the USA had accounted for almost 13,170 newly diagnosed cervical cancer cases and 4,250 deaths in 2019 [5].

Human papillomavirus (HPV) infection suggested as the first and foremost cause of cervical cancer pathogenesis [6-8], and studies reported that HPV is responsible for genome aberrations and increases specific target-wise gene expression [9]. Apart from mutations in P53, PIK3CA, PTEN genes, an altered number of oncogenes copies have been reported as the core factor of cervical carcinoma progression [10-12]. Other risk factors such as smoking, pregnancy history, and long term use of oral contraceptives have also been conveyed [13].

The available treatment strategies such as surgery, radiotherapy, chemotherapy are though the prodigious hope for cervical cancer patients, however near about 75% of patients develop further progression and/ recurrent tumour [14, 15]. Disease heterogeneity of patients is another challenge to apply a specific treatment method [5].

On the other hand, diagnosis and prognosis of cervical carcinoma are still poorly recognized, insufficient, insensitive, and not specific to in-time or prior identification of the carcinoma properly in the clinical phase [16]. Therefore, there is a pressing need to identify new suitable molecular markers or models to predict the diagnosis/prognosis of cervical cancer.

A series of studies have already been conducted by relying on gene expression patterns to sort out auspicious molecular gene signatures to use as a recurrence prediction tool. A signature of 7-genes series identified in the early stage, [17] and a predictive prognostic model for recurrent tumour had constructed, which is composed of a 12-genes series [18]. Through the analysis of long non-coding RNA (lnc-RNA), it was revealed that 9-genes signature sets were used to predict patients' chance to develop recurrent tumours [19]. However, searching for an effective series of gene sets is to be under investigation that can be used as a promising prognosis and/ diagnosis purpose to mitigate the disease outcome at the very early stage.

Nowadays integrative systems biology approach is a promising technique, which has been applied to predict novel molecular oncogenes and gene signatures using existing gene expression profiles from Gene Expression Omnibus (GEO) [20-26].

In the current research, we have employed systems biology approaches to explore the differentially expressed genes (DEGs), gene network, pathways, and protein-protein interactions unique to CC to retrieve potential biomarkers and pathways of cervical carcinoma.

2. Materials and Methods

The entire procedure of integrative systems biology analytical approach to identify novel molecular gene signatures and pathways of cervical cancer is shown in **Figure 1** through the schematic diagram.

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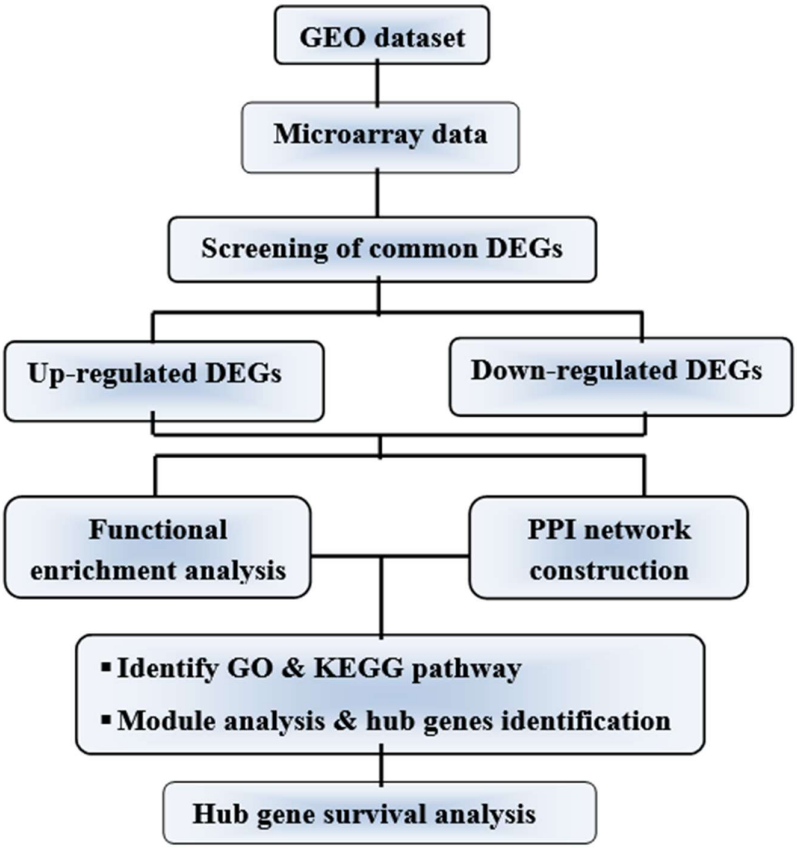

Figure 1: Flow diagram of integrative bioinformatics analytical approach applied in this study.

2.1 Data Retrieval

The gene expression profile (GSE148747) of primary fibroblasts from the normal region vs. tumourous region of the human uterine cervix, based on the platform of GPL4133 (Agilent-014850 Whole Human Genome Microarray 4x44K G4112F), were collected from the Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) database, a public repository that provides free access to a full set of microarray, next-generation sequencing and other forms of high-throughput functional genome data submitted by the different research group [27]. A total of eight samples were used in this dataset (GSE148747). The GSM4478163, GSM4478166, GSM4478167 GSM4478168, and GSM4478170 were used as normal primary fibroblast, and the GSM4478164, GSM4478165, and GSM4478169 were used as tumour-associated fibroblast.

2.2 Screening of Differentially Expressed Genes (DEGs)

We utilized the GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/) statistical tool to calculate and assess the genes that were expressed differently between the primary fibroblasts from the normal region and tumourous region of the human uterine cervix [28]. The Benjamini and Hochberg (false discovery rate) and t-test methods were utilized with the GEO2R tool to

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calculate the FDR and p-values, respectively, to identify the DEGs [29]. We considered P-value $p< 0.05$ and a logFC (large-scale fold changes) > 1 to be statistically significant for the DEGs, and a logFC≥ 1, logFC≤ −1 were considered to indicate up-regulated and down-regulated DEGs, respectively [30-32]. The resultant DEG dataset was collected and used for further analysis. Including all of the DEGs identified in the samples, we constructed a volcano plot by using the pheatmap package in R language.

2.3 Functional Enrichment of Gene Sets

The initial ontology of gene (GO) and KEGG pathway enrichment analyses of the DEGs were annotated ($p<0.05$) using the online bioinformatics tool DAVID v6.8 [33]. The human genome was selected as the background parameter, and Affymetrix based identification was selected as identifier. The KEGG pathway enrichment analyses of the DEGs were cross-checked using the NetworkAnalyst online tool [34]. Gene Ontology (GO) study is a frequently used approach for the functional studies of large-scale transcription or genomic data [35, 36]. Similarly, the Kyoto encyclopedia of genes and genomes (KEGG) is generally used to understand metabolic pathways widely for gene annotation [37, 38].

2.4 PPI Network Construction

The online database STRING (v11.0, http://www.string-db.org/) was used to construct the PPI network of the proteins encoded by DEGs. The String is an online repository with 24,584,628 proteins from 5,090 organisms to predict the relationship between genes [39]. The combined score was set at less than 0.75 (medium confidence score) to be considered significant.

2.5 Selection of Central Hub Proteins from the PPI Network

The obtained PPI networks were visualized by Cytoscape (http://www.cytoscape.org/) [40]. The Cytoscape plug-in Molecular Complex Detection (MCODE) [41] was applied to obtain significant modules with an established score of greater than 3 and nodes of greater than 4. In the PPI network, the number of edges involved determines the degree value of the nodes; nodes with high degree values were considered to be hub genes. We mapped the hub genes to evaluate their PPI information. We use cytoHubba [42] (a Cytoscape plugin) to evaluate hub genes from the constructed PPI network. The cytoHubba is a tool that uses 11 specific methods to calculate hub genes from the PPI network; in this study, we use degree score to identify hub genes.

2.6 Hub gene Survival Analysis

A comprehensive online platform called Gene Expression Profiling Interactive Analysis (GEPIA2) [43] provides fast and customized delivery of functionalities based on TCGA (The Cancer Genome Atlas) and genotype-tissue expression (GTEx) data. GEPIA2 evaluates the survival effect of differentially expressed genes in a given cancer sample. The overall survival effect of hub genes in CC was estimated by calculating the log-rank p-value and the HR (hazard ratio-95% confidence interval) using GEPIA2 single-gene analysis.

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3. Results and Analysis

3.1 DEG Identification

The expression profiling was performed on the CC gene dataset GSE148747, which was retrieved from GEO and the overall patients' information is shown in **Table 1**. To identify the DEGs from these two groups, we conducted GEO2R web-server to calculate the p-values and log2FC values. The resulting genes that met the cutoff criteria (logFC≥ 1, logFC≤ −1, and P-value p< 0.05) were considered DEGs. Overall, 4,643 genes were identified from the GEO dataset using the GEO2R tool. The volcano-plot was constructed by comparing the two groups and is depicted in **Figure 2**. Finally, among them, a total of 152 DEGs were identified based on the cut off criteria (logFC≥ 1, logFC≤ −1 and an adjusted P-value p< 0.05) where 76 and 76 were up-regulated and down-regulated, respectively (**Table S1** and **S2**).

Table 1: Patients information in GSE148747 derived from the GEO database.

Group	Accession	Organism	Disease state	Cell Type
Normal	GSM4478163	Homo sapiens	Normal	Normal fibroblast
	GSM4478166	Homo sapiens	Normal	Normal fibroblast
	GSM4478167	Homo sapiens	Normal	Normal fibroblast
	GSM4478168	Homo sapiens	Normal	Normal fibroblast
	GSM4478170	Homo sapiens	Normal	Normal fibroblast
Tumor	GSM4478164	Homo sapiens	Cervical Cancer	Tumor-associated cervix fibroblasts
	GSM4478165	Homo sapiens	Cervical Cancer	Tumor-associated cervix fibroblasts
	GSM4478169	Homo sapiens	Cervical Cancer	Tumor-associated cervix fibroblasts

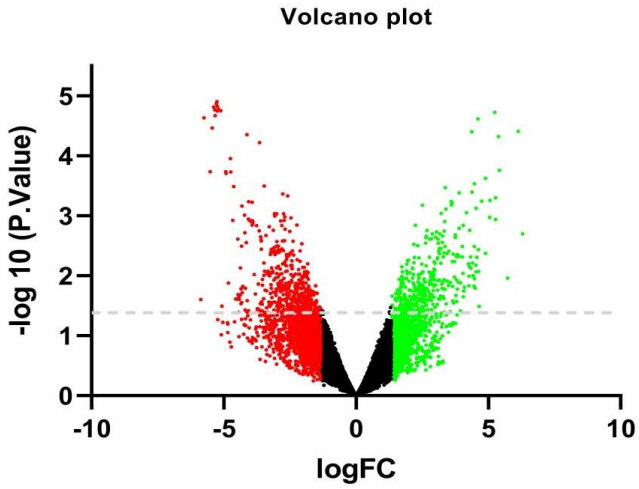

Figure 2: Volcano-plot of the DEGs from the GSE148747 dataset. X-axis: logFC, large-scale fold changes; y-axis: –log10 of the p-value is showing the statistical significance. The green point represents the up-regulated genes where red are the down-regulated genes.

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3.2 Functional analysis of DEGs

By using the DAVID database, the top 10 enrichment analysis outcomes were screened for the up-regulated and down-regulated DEGs of the GO analysis. The biological process (BP), cellular component (CC), and molecular function (MF) enrichment analysis outcomes are displayed in **Figures 3A and 3B**. DAVID and NetworkAnalyst analysis of KEGG pathway outcomes for the significantly enriched DEGs are depicted in **Figure 4**. The overlapped gene lists of up- and down-regulated genes were involved in GO were shown in supplementary **Table S3, S4,** and the gene-lists of the KEGG pathway were shown in supplementary **Table S5**, respectively.

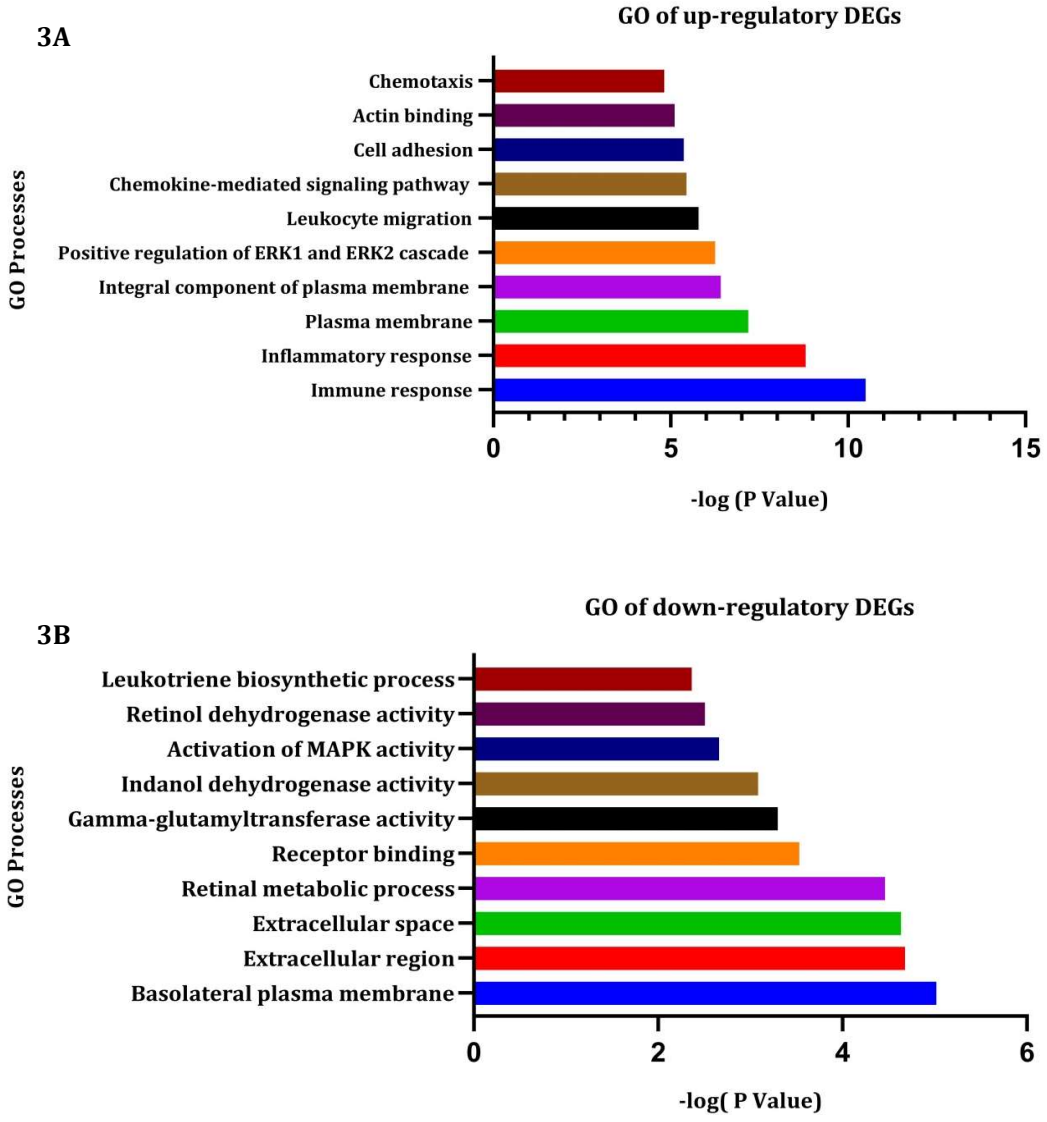

Figure 3: Functional analyses of the differentially expressed genes (DEGs) in cervical cancer. In figure **3A**, GO of up-regulatory DEGs and in **3B**. GO of down-regulatory DEGs. Each of the GO processes was plotted based on the –log (p) value.

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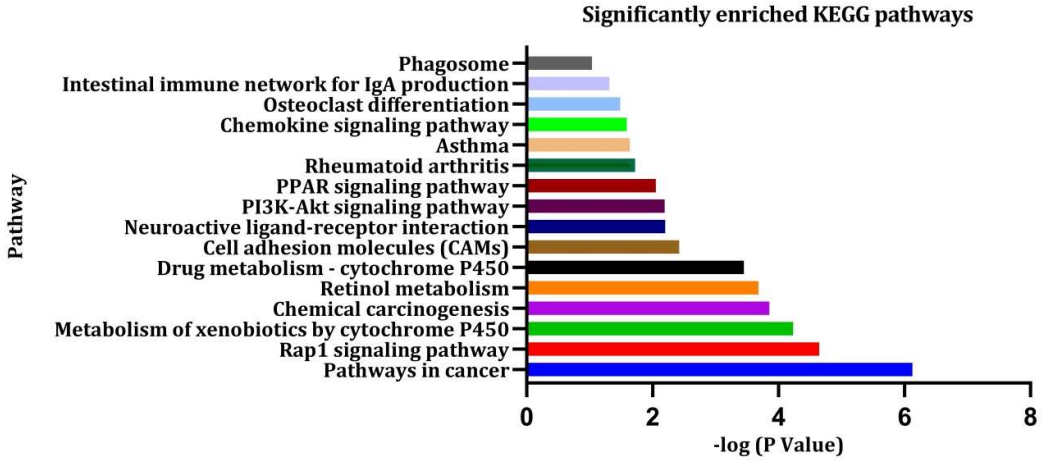
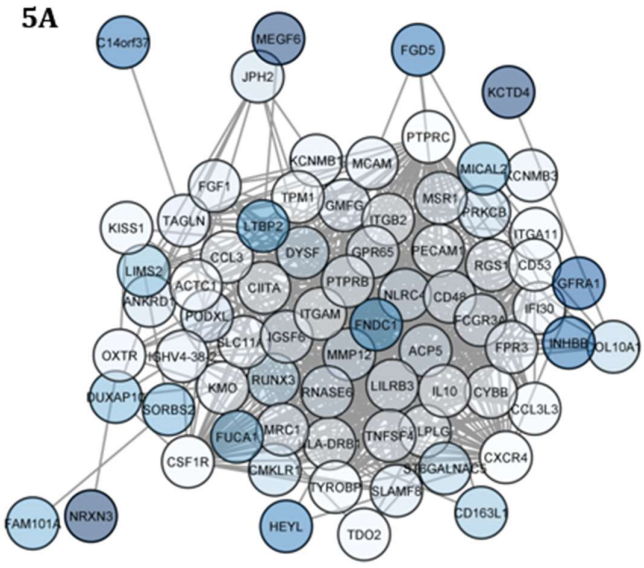


Figure 4: The enriched KEGG pathways generated from the DEGs.

3.3 PPI Network Construction

To evaluate the PPIs between the DEGs, we used the STRING tool to identify the PPI networks for both the up- and down-regulated genes. Thereafter, the resulting PPI network from STRING was exported as a ".txt" file and imported as a.csv file into Cytoscape v3.8.0 software for visualization. After subsequent enrichment of each of the networks, we have generated a merged network of PPI consisting of 152 nodes and 1206 edges (**Figure S1**). The PPI network for up-regulated DEGs had 76 nodes with 656 edges (**Figure 5A**) and the PPI network for down-regulated DEGs had 76 nodes with 151 edges between the nodes (**Figure 5B**).



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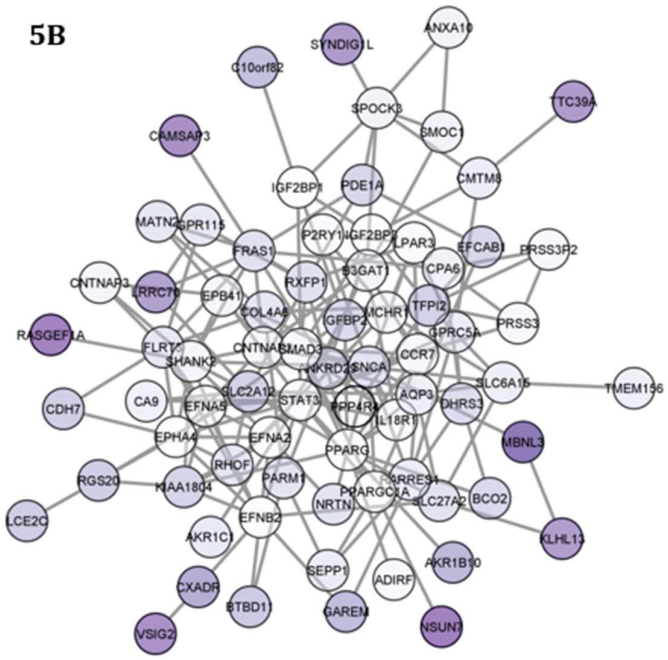
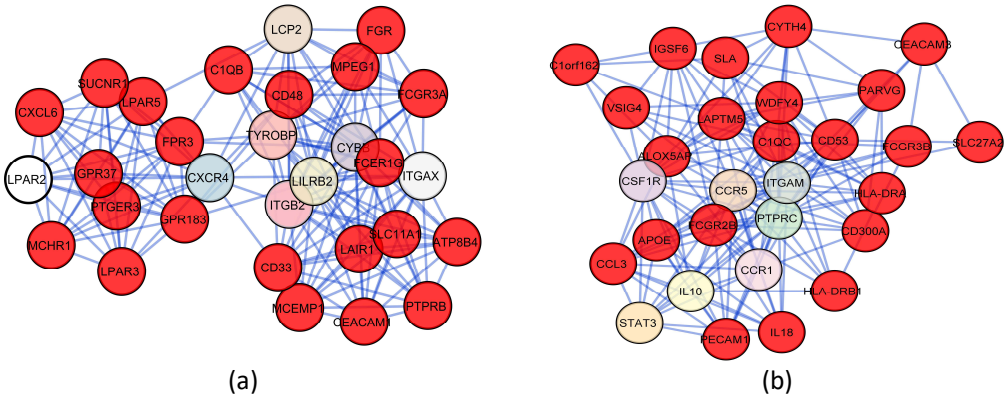


Figure 5: Visualization of the protein-protein interaction network of CC. In figure 5**A**, the PPI network for up-regulated genes with 76 nodes and 656 edges and in figure 5B the PPI network for down-regulated genes with 76 nodes and 151 edges.

3.4 Modules and Hub proteins identification

We identified 3 significant modules from the merged PPI network by using the Cytoscape plugin MCODE. Module 1 had 30 nodes with 187 edges (**Figure 6A);** on the other hand, Module 2 and 3 had 29 and 24 nodes with 166 and 57 edges, respectively (**Figure 6B** and **6C**). The DEGs of the modules had an important role to enrich significant GO terms 'protein binding' and 'cytoplasm'. We also identified the top 12 hub genes (**Table 2**) from the PPI network using the cytoHubba tool and are depicted in **Figure 7**. The topological parameters of the twelve molecular hub genes of the PPI network are also shown in **Table 2**, including the clustering coefficient, degree, the betweenness centrality, and the closeness centrality for the individual gene.



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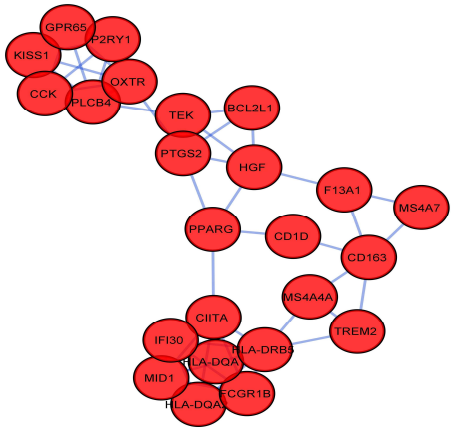

(c)

Figure 6: Module analysis of PPI network. A. Module 1 had 30 nodes with 187 edges. B. Module 2 had 29 nodes and 166 edges. C Module 3 had 24 nodes and having 57 edges.

Table 2: Topological parameters for the twelve potential biomarkers of the PPI network.

Gene Signature Name	Degree	Betweenness Centrality	Clustering Coefficient	Closeness Centrality	Stress
PTPRC	80	18133.72223	0.23196	244.0667	167664
ITGAM	79	14447.28068	0.23337	242.4833	140924
IL10	70	15227.82113	0.18841	234.9833	145074
TYROBP	69	6407.99793	0.29113	227.9833	78988
ITGB2	66	9280.28036	0.28858	226.5595	95454
CCR5	61	8309.33294	0.29836	225.8	98026
ITGAX	60	4722.34853	0.29492	222.35	59082
CSF1R	55	7708.90149	0.32727	221.5333	87030
LILRB2	55	5622.88217	0.34007	217.1333	57172
CXCR4	55	10433.80294	0.24108	225.8333	95538
STAT3	53	15091.4144	0.20682	225.2833	126744
CYBB	50	4529.93215	0.37469	218.95	54562

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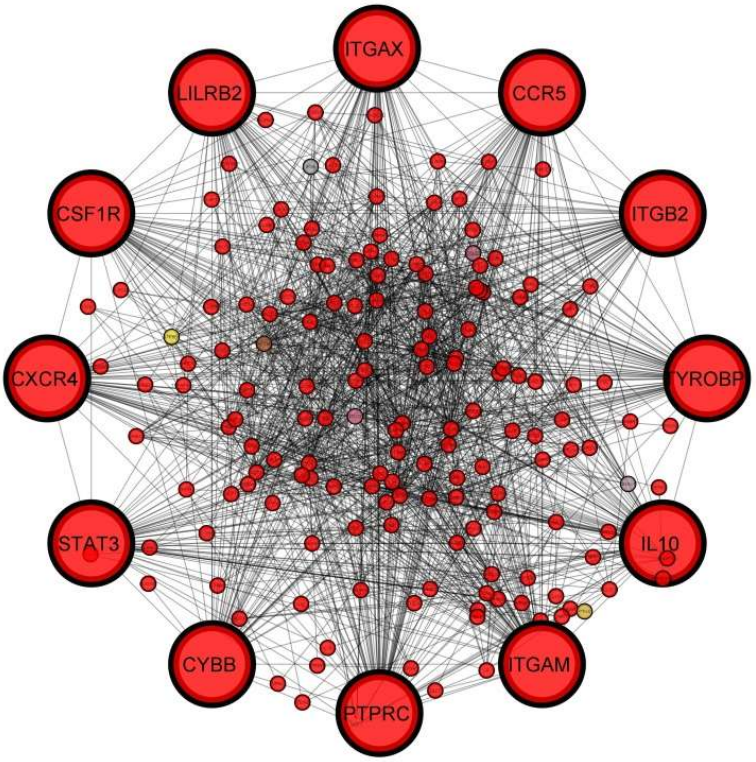

Figure 7: PPI network with identifying hub genes. The highlighted 12 nodes represent the hub genes. The degree score method of cytoHubba considers 94 nodes in this network from the merged PPI network.

3.5 Survival of the Hub Genes

GEPIA survival assessment was used to inspect the overall association with survival of 12 hub genes from both the up and down-regulated DEGs and is depicted in **Figure 8.** Among all the twelve genes, only CXCR4 showed lower overall survival in the higher expression group and having the Hazards Ratio (HR) of 2.6.

Discussion:

The detection of early-stage CC has an admirable prognosis compared to advanced-stage. The early-stage cases cured significantly through surgery, chemo-radiation, or a blend of treatment approaches, where the advanced level CC patients suffered most because of the often recurrences and incurable nature of CC [1].

The patterns of the DEGs from both tumour and control were visualized by the volcano-plot (**Figure 2**). The green points represent the up-regulated genes and the red points are the down-regulated genes.

After subsequent enrichment of all the DEGs, we have found some significant up-regulatory GOs such as immune response, inflammatory response, and chemokine-mediated signalling pathway, positive regulation of ERK1 and ERK2 cascade, and chemotaxis in the biological process. For the cellular component, the plasma membrane and integral component of the

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plasma membrane are enriched significantly. The major molecular function up-regulated in the DEGs were immune response and inflammatory response.

On the other hand, down-regulatory GOs such as retinal metabolic process, activation of MAPK activity, and leukotriene biosynthetic process were found in the biological process. The major molecular function down-regulated in the DEGs were receptor binding, gamma-glutamyltransferase activity, and indanol dehydrogenase activity.

From the pathway analysis, we have found that most of the pathways were mainly concentrated in different cancer pathways, immunoresponse and cell cycle pathways (**Figure 4 and Table S5**).

We identified 12 hub genes based on degree value, clustering coefficient, betweenness centrality, and closeness centrality from the merged PPI analysis; these 12 genes PTPRC, ITGAM, IL10, TYROBP, ITGB2, CCR5, ITGAX, CSF1R, LILRB2, CXCR4, STAT3 and CYBB had the diagnostic value which distinguishes CC from normal individuals (**Table 2**). A higher percentage of cervical tumours occupied by PTPRC+ cells were strongly associated with enhanced tumour-infiltration by Tbet+ cells and Foxp3+ cells. Longer disease-free and disease-specific survival were reported in the type I-oriented PTPRC+ cell to infiltrate occupied tumour areas [44]. ITGAM, ITGB2 genes play a pivotal role in cell adhesion in multiple myeloma and reported as potential diagnostic markers [45]. Type-2 cytokine, IL-10 show immunosuppressive functions and capable of stimulating tumour growth and cervical tumour biopsies showed increased presence for mRNA for IL-10 [46]. The chemotactic receptors, CCR5 expressed in CD8+ T cells which preferentially proliferated in cervical cancer [47], and the proliferation and invasion of cervical cancer can be arrested by down-regulating the expression of CCR5 [48]. Similarly, the reduced level of CSF1R protein significantly contributes to suppressing cervical cancer cell proliferation and motility and induces apoptotic cell death [48]. Therefore, CCR5 and CSF1R might be the new targets for cervical cancer treatment. Cervical tumour invades in adjacent tissues and subsequently into distant organs initiated by the expression of CXCR4 through the link of another factor CXCL12 [49]. Also, CXCR4-deficient cells had lower expression of the proliferation marker Ki-67 and decreased ability to engraft into lungs and spleen [50, 51]. STAT3 accumulation was observed in specific sites especially in basal and suprabasal layers of HPV16-positive early pre-cancer lesions and STAT3 expression and activity were distinctively higher in poorly-differentiated lesions [52]. So, it suggests that the STAT3 gene might be used as a prognostic marker in cervical cancer prognosis at a very primary stage.

Finally, the four genes namely TYROBP, IGTAX, LILRB2, and CYBB have not been previously reported to be associated with cervical cancer. These genes need to be independently validated before they can be useful for evaluating the prognosis and/or diagnosis of CC patients.

According to our predictions, among the identified key 12 hub genes only STAT3 showed the down-regulatory expression patterns whilst all the remained genes are up-regulated but the Kaplan–Meier overall survival analysis denoted that the level of only CXCR4 expression was significantly related to the overall survival of patients with cervical cancer (**Figure 8**). Importantly, previous studies have been revealed the role of CXCR4 in the regulation of tumour growth. Smith MC et., al has been reported the initiation of cell proliferation and/or survival of cervical cancer cells is not possible without the CXCR4 receptor [53]. Furthermore, the CXCR4 is a very important factor in the metastatic process of cervical cancer and inhibition of CXCR4 expression and function significantly impairs the growth of cervical carcinoma [50]. So, CXCR4 could be a novel target for the prevention of cervical carcinoma growth and metastasis.

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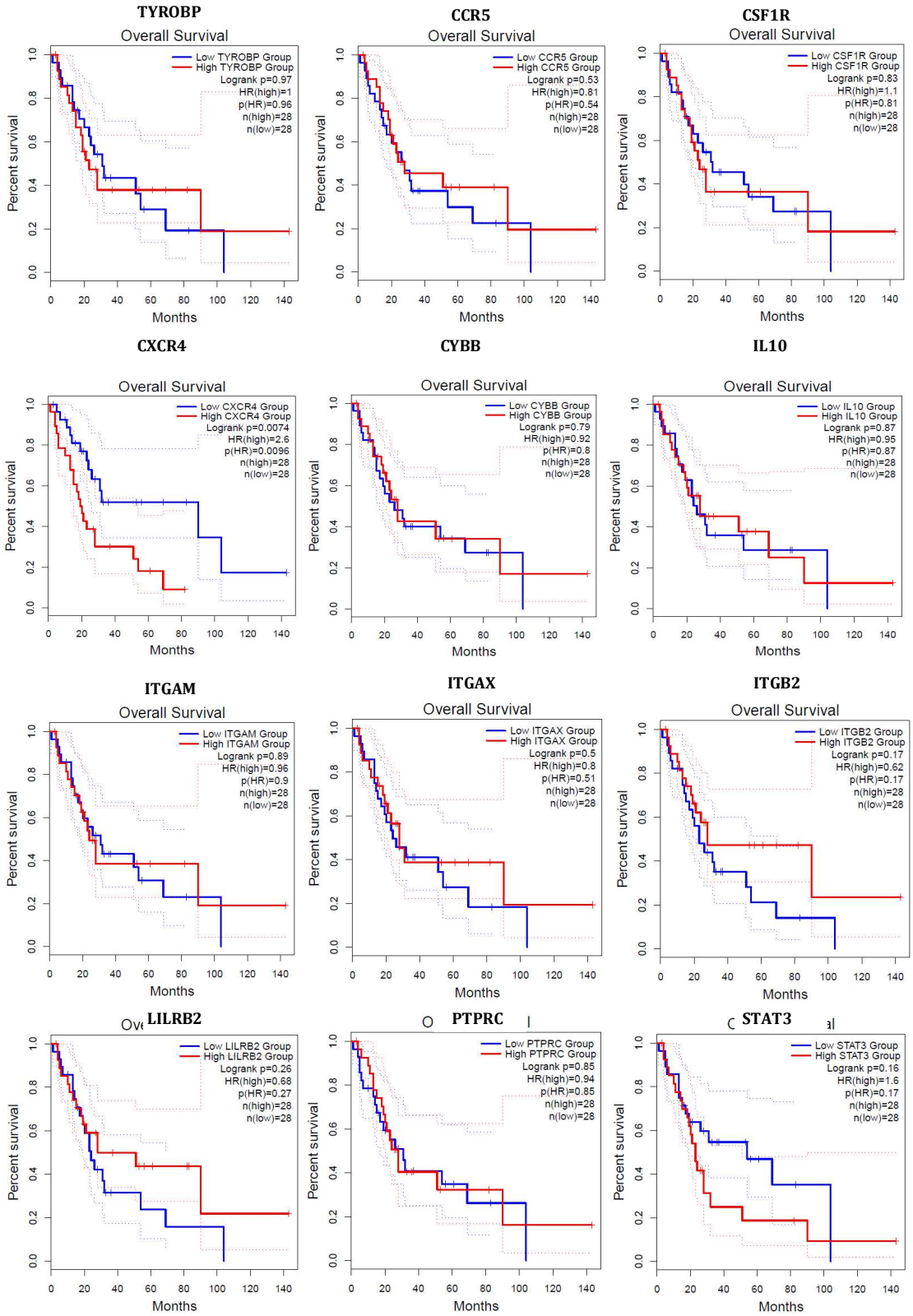

Figure 8 Kaplan–Meier overall survival analysis of the hub genes expressed in CC.

Conclusion

In conclusion, our analysis from the current study provides evidence that candidate genes such as PTPRC, CXCR4, CCR5, and CSF1R and their enriched pathways, respectively, of the pathway in the plasma membrane, inflammatory response, and chemokine-mediated signalling might be involved in the pathogenesis of CC. Moreover, CXCR4 might play an important role in the metastasis of CC. We believe that the results obtained above can provide theoretical guidelines for future works in the laboratory. Further wet lab investigation is required to validate and using as a prognostic, diagnostic and/or unique target to support the treatment management of CC.

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Ethical Approval: Not Applicable.

Author contributions

Arafat Rahman Oany conceived, designed, and guided the study, drafted the manuscript, and analysed the data. Mamun Mia carried out the analysis and drafted the manuscript. Tahmina Pervin drafting the manuscript and helped in analysis. Salem Ali Saleh Alyami and Mohammad Ali Moni participated in coordination, performed critical revision, and helped in drafting the manuscript. All authors read and approved the final manuscript.

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