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Identification of associated molecular events in *Helicobacter pylori* infection and gastric cancer through integrated computational analysis of Microarray and RNA-Seq datasets

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ABSTRACT

According to the World Health Organization, Gastric cancer (GC) is the third leading cause of death worldwide, where, the major precursor of cancer progression is infection with *Helicobacter pylori*. It has been reported that 50% of the total populace is infected with *H.pylori*, while in 80% the ulcer emerges in later stages of the infection. Although extensive separate analysis has been performed on *H.pylori* infection and GC data, however, there is a need to perform comparative analysis to identify the cross-talk between the conditions and to hunt significant molecular events that occurs during *H.pylori* induced GC. The aim of this multi-population study was to identify common molecular events and potential bio-markers against *H.pylori* induced GC. We performed microarray and RNA-seq analysis on publicly available *H.pylori* infection, gastritis, *H.pylori* induced GC and GC datasets to obtain Differentially Expressed Genes (DEGs). After obtaining the DEGs, integrative analysis, functional enrichment analysis and network biology approaches were utilized to identify common markers and hub genes between various disease conditions. Functional enrichment analysis revealed the DEGs of *H.pylori* infection, gastritis, *H.pylori* induced GC and GC were strongly associated with spliceosome, adherens junction, focal adhesion and ribosome. Being one of the common DEG, and highly interactive hub protein in the networks of all the conditions, translationally controlled tumour protein (TPT1) was identified as a significant predictive biomarker for early prognosis and diagnosis of *H.pylori* induced GC. Therefore, the mechanisms behind TPT1 should be further studied using *in vitro* cell-based functional assays, to determine its role in the progression of *H.pylori* induced GC.

Introduction

Helicobacter pylori (*H.pylori*), a gram-negative and spiral-shaped bacteria, is a pathogen that resides in the gastric epithelium¹. Based on poor hygienic conditions in the developing countries, almost 80% of the people having ulcer is due to the *H.pylori* infection². Due to the asymptomatic nature of *H.pylori*, the infection is not timely diagnosed and if it is not treated on time the infection slowly progress towards gastric ulcer which further leads towards gastric cancer (GC)³. GC is the fifth commonly diagnosed cancer, whereas, it is the third leading cause of death worldwide⁴. Although, several socio-economic, environmental, molecular and genetic factors are involved in the progression of GC, however, one of the major culprit behind gastric carcinoma is *H.pylori*^{5,6}.

Virulence factors produced during pathogenesis of *H.pylori* that are responsible for gastric carcinoma are cytotoxin-associated gene A (CagA), vacuolating cytotoxin (VacA) and type 4 secretory system (T4SS) along with outer inflammatory protein A (OipA)⁷. Where, some of the major host signalling pathways regulated or deregulated by *H.pylori* virulence factors include Jak/Stat signalling pathway, NF κ B signalling pathway, focal adhesion, Src/MEK/ERK signalling pathway, autophagy pathway and c-Met-PI3K/Akt-mTOR signalling pathways, where these pathways has also found to be involved in the progression of GC⁸⁻¹¹. Infection with *H.pylori* also instigates methylation of explicit genes; this explicitness is dictated by the epigenetic status in typical cells, including the occurrence of H3K27me3 and RNA polymerase II. These specific sorts of aggravation i.e., driven by the *H.pylori* infection are significant for methylation, stimulation and invasion of monocytes and has all the earmarks of being involved in progression of GC¹².

In previous studies, extensive analysis on *H.pylori* infection and GC datasets has been performed and the link between *H.pylori* infection and GC has been reported with the involvement of similar genes and pathways¹³⁻¹⁵. However, comparative analysis on *H.pylori* infection and GC samples from multi-population studies have not been performed previously by the integration of microarray and RNA-seq gene expression profiling. Therefore, it is necessary to perform a comparative analysis

to describe the common and unique Differentially Expressed Genes (DEGs) between *H.pylori* infection, gastritis, *H.pylori* induced GC and GC. Further, functional enrichment and network analyses of the DEGs is a necessary step to acquire insights into the common molecular features that are involved in all the conditions to identify potential targets.

Based on multi-population data, we aim to analyse the progression of *H.pylori* pathogenesis towards GC by hunting common molecular events with an active role in the conditions. Differential expression analysis was performed on microarray and RNA-seq datasets of *H.pylori* infection, gastritis, *H.pylori* infected GC and GC to obtain significant DEGs. Functional enrichment analysis was performed to identify the role of the DEGs in various biological pathways and molecular functions. Furthermore, to unveil the relationship between proteins encoded by several genes in *H.pylori* infection and GC, and markers involved in the progression of *H.pylori* induced GC, network analysis was performed to identify the hub genes. The analysis revealed translationally controlled tumour protein (TPT1) to be a common marker and one of the highly interactive proteins in the *H.pylori* infection, gastritis, *H.pylori* infected GC and GC networks.

Results

The principal objective of this study was to identify linking molecular events, common patterns and key interactions in the different disease states i.e., *H.pylori* infection, gastritis, *H.pylori* infected GC and GC. For this purpose, we performed differential expression analysis on microarray and RNA-seq datasets followed by integrative, functional enrichment and network analyses.

Differential expression Analysis

Differential expression analysis on microarray and RNA-seq datasets was performed to obtain significant DEGs. The total number of DEGs obtained from microarray and RNA-seq analysis of *H.pylori* infection, gastritis, *H.pylori* infected GC and GC datasets are mentioned in Table 2. Moreover, significant DEGs were visualized through volcano plots, as shown in Figure 2 and 3, which also resulted in visualization of the up-regulated and down-regulated DEGs.

Comparative analysis

For the identification of common biomarkers in *H.pylori* infection, gastritis, *H.pylori* infected GC datasets integrated analysis was performed. The results of the integrative analysis of the DEGs from microarray datasets identified 4 common genes (BIRC3, NAMPT, MMP1, ACSM3) in *H.pylori* infection whereas 119 common genes in GC datasets (Figure 4). Moreover when compared, no common markers were identified in the microarray *H.pylori* infection and GC datasets. Similarly, the DEGs from RNA-seq *H.pylori* infection, gastritis, *H.pylori* infected GC and GC datasets were then integrated, which identified 222 common genes in *H.pylori* infection and *H.pylori* infected GC datasets and 13 common genes in GC datasets (Figure 5). After comparing DEGs of all the four conditions, 2 genes (TPT1 and LYPD6B) were identified to be common in the RNA-seq *H.pylori* infection, gastritis, *H.pylori* infected GC and GC datasets.

Functional Enrichment analysis

Gene ontology (GO) and pathway analysis were performed to identify the biological processes and molecular pathways involved in *H.pylori* infection, gastritis, *H.pylori* infected GC and GC RNA-seq datasets. From the Toppgene function of Toppgene, the top GO results for *H.pylori* infection, gastritis, *H.pylori* infected GC and GC DEGs are mentioned in Table 3. Information mentioned in the table are the GO IDs, functions the DEGs are involved in i.e. molecular function (MF), biological process (BP) and cellular components (CC), significant p.value, false discovery rate (FDR) and the entities represents the DEGs involved in a particular function. The genetic ontologies revealed the involvement of the significant DEGs in molecular binding, RNA binding, cell adhesion molecular binding, cadherin binding and macromolecular junctions and complexes.

Pathway analysis of the DEGs performed through DAVID revealed significant associations and roles of the genes in various similar biological mechanisms. Furthermore, Cytoscape was used to visually represent the common interactive pathways involved in *H.pylori* infection, gastritis, *H.pylori* infected GC and GC as shown in Figure 6. Spliceosome was found to be the common pathway involved in all the conditions, whereas, adherens junction and RNA transport were the most commonly interactive pathways.

Spliceosome, being highly significant pathway identified in all the conditions was further explored to find out common markers across the conditions. HNRNPC, DDX42 and HNRNPU were the differentially expressed common entities found in *H.pylori* infection, gastritis *H.pylori* infected GC and GC. Moreover, other pathways that were enriched in the respective conditions included ribosome, adherens junction, focal adhesion and Alzheimer's disease and RNA transport. The top enriched pathways involved in *H.pylori* infection, gastritis, *H.pylori* infected GC and GC are mentioned in Table 4.

Network analysis and Hub nodes identification

To identify the hub genes and protein-protein interaction (PPI) networks of *H.pylori* infection, gastritis, *H.pylori* infected GC and GC DEGs, or between the conditions, network and module analysis was performed. PPI networks were identified using the

STRING database with the highest confidence score (0.9) which was further modified and visualized by Cytoscape software along with module analysis. The PPI analysis indicated 171 genes in *H.pylori* infection, 112 genes in gastritis, 1105 genes in *H.pylori* infected GC and 183 genes in GC that were found to be differentially expressed in the respective conditions. After analyzing the networks of *H.pylori* infection, gastritis, *H.pylori* infected GC and GC, hub nodes were identified with the highest degree of interactions. These PPI networks were further merged to evaluate the genomic connectivity within the neighboring genes along with common interactions. Network analysis of the *H.pylori* infection network revealed 17 hub genes (RPS20, RPLP0, RPS17, SRSF3, RPL8, POLR2L, SENP3, RPL6, SRRT, RNPS1, RPL39, TRA2B, SLU7, SRRM2, RPL23, RPL35A and RPL32) based on the degree values as shown in plot A of Figure 7 and Table 5.

To identify the core modules, the MCODE app in Cytoscape was used to find the highly connected nodes in the *H.pylori* infection network with Node Score Cutoff: 0.2 Haircut: true Fluff: false and K-Core: 2. In the network, 31 clusters were generated in which 1 cluster was selected based on the highest scores and highly connected nodes shown in plot B of Figure 7 having 32 nodes, 272 edges and a 17.5 density score. Similarly, network analysis revealed 9 hub genes (SRSF7, U2AF2, ITGB1, SF1, TRIM21, HIST1H2AH, RPL6, UBE2G2 and RAP1B) in the gastritis dataset with the highest degree of interaction as shown in plot E of Figure 7. MCODE analysis resulted in 10 clusters out of which 1 highly interactive cluster as shown in plot F of Figure 7 with 9 nodes, 35 edges and an 8.5 density score was selected.

In the network of *H.pylori* infected GC, 82 hub nodes were identified of which UBC, TP53, RPS7, RPS9, UBE2C, RPS4X, RPS3, RPS2, UBR4 and RPS26 were the top highly connected nodes as shown in Figure 7 based on the degree as shown in Table 5. Module analysis formed 43 clusters out of which 1 highly connected cluster was selected from the network having 103 nodes, 2541 edges and 49.8 a density score. Whereas, in the GC network, 9 hub genes (RPL13, RPL11, RPL37A, RPLP0, RPL34, RPL32, RPS15A, RPL3 and RPS12) were selected as shown in plot C of Figure 7, based on the degree as mentioned in Table 5 and high connectivity with the neighboring nodes. After MCODE analysis, 9 clusters were obtained of which 1 cluster was selected as shown in plot D of Figure 7 that depicted high connection within the network of GC having 17 nodes, 128 edges and a density score of 16. Average shortest path length, betweenness centrality, closeness centrality, clustering coefficient, degree, eccentricity and neighbourhood connectivity for all the hub genes of *H.pylori* infection, gastritis, *H.pylori* infected GC and GC are mentioned in Table 5.

Network summary statistics

The summary statistics of *H.pylori* infection, gastritis, *H.pylori* infected GC and GC networks were obtained using the network analyzer tool in Cytoscape. It provided statistics regarding the number of nodes and edges, connected components, average number of neighbours and clustering coefficient in PPI networks as mentioned in Table 6.

Common interactions

To identify common genetic markers between significant DEGs of the four dense networks i.e. *H.pylori* infection, gastritis, *H.pylori* infected GC and GC, nodes from the four PPI networks were merged. In the merged PPI network, the nodes of *H.pylori* infection are given red color, gastritis indicates the green color, *H.pylori* infected GC are represented in purple color and GC is given dark blue color in Figure 8. Highlighted as the yellow node, in the merged PPI network TPT1 was identified to be the common interaction in all of the four conditions. Whereas, LMO7, RAP1B and RPL6 were the 3 common interactions between *H.pylori* infection, gastritis and *H.pylori* infected GC that are shown as pink nodes in Figure 8. Moreover, the light blue nodes (RPLP0, RPL32 and SRRM2) accentuates the highly connected common interactions in *H.pylori* infection, *H.pylori* infected GC and GC. Ultimately, ITGB1 is identified as the common interaction between gastritis, *H.pylori* infected GC and GC and is shown as an orange node in the network in Figure 8.

According to the study, low expression of TPT1 was observed in *H.pylori* infection (GSE55699) ($\log_2FC = -20.07$) and GC (GSE41476, GSE80388) ($\log_2FC = -3.28$, $\log_2FC = -3.42$) datasets, whereas, it was found to be highly expressed in *H.pylori* infected GC (GSE73226) ($\log_2FC = 5.79$) and gastritis (E-MTAB-3689) ($\log_2FC = 1.9$) datasets. The gradual increase in the expression patterns of TPT1 at various stages i.e. *H.pylori* infection, gastritis and *H.pylori* infected GC suggests that TPT1 may have a significant role in the progression of *H. pylori* induced cancer.

Discussion

According to GLOBOCAN's cancer statistics, worldwide, Gastric cancer (GC) is reported to be the third leading cause of death¹⁶, where, one of the major causes of carcinoma is *Helicobacter pylori* infection¹⁷. The pathogen asymptotically resides in the stomach for years and in later stages progress into GC due to numerous *H.pylori* virulence factors i.e. CagA, VacA, T4SS etc¹⁸. To prevent this silent progression, early antibiotic treatment of *H.pylori* infection is required, unfortunately, the pathogen is showing resistance against major antibiotics including Clarithromycin, Metronidazole, Levofloxacin, Amoxicillin, Tetracyclin, Rifabutin and Furazolidon¹⁹. Therefore it is crucial to identify key essential genes and discover common biomarkers that might have a potential role in *H.pylori* infected GC. The advancements in computational approaches and experimental

technologies have opened up new possibilities for the scientific community to explore the role of the gut microbiome in tumor microenvironment on molecular, genetic and epigenetic levels²⁰.

Previously, scientists have performed differential expression analysis on *H.pylori* infection and GC datasets including microarray and RNA-seq analysis^{13–15}. However, these studies have generated, analyzed and explored data of a targeted population to understand the genetic variations in *H.pylori* infection and GC. A multi-population integrative data analysis has not been performed to investigate the cross-talk between *H.pylori* infection and GC. Therefore, it is crucial to perform a multi-population differential expression based study to identify genetic biomarkers and common interactions between *H.pylori* infection and GC.

This study explored the differential expression in *H.pylori* infection, gastritis, *H.pylori* infected GC and GC by analyzing microarray and RNA-seq datasets and resulted in significant differentially expressed genes (DEGs) of the respective conditions. Based on the availability of data, the countries selected for this multi-population differential expression study were Hungary, Portugal, China, Germany, South Korea, Taiwan and Nicaragua (Central America). Once the significant DEGs were acquired from the selected datasets (GSE55699²¹, GSE73226²², GSE41476²³, GSE80388²⁴ and E-MTAB-3689²⁵), functional enrichment and network analyses were carried out to identify potential genetic biomarkers and common interactions. We were not able to achieve commonality between the microarray and RNA-seq DEGs and the reason behind it might be due to the difference in both the approaches as well as selection of data from different populations.

Translationally controlled tumor protein (TPT1) and Ly6/Plaur Domain Containing 6b (LYPD6B) were identified as the common biomarkers for *H.pylori* induced GC. Till now, LYPD6B is only identified as a potential biomarker in the progression of ovarian cancer²⁶ and is not studied in other cancers including GC. Although LYPD6B was one of the common DEGs, however, no interactions were identified with the other DEGs. Being differentially expressed in all the conditions and showing a high degree of connectivity with other interacting genes, TPT1 (p.value<0.05) was identified as a potential biomarker, based on the comparative and network analyses. It has been reported that TPT1 has an essential role in various biological processes including cell growth and survival, cell proliferation, DNA damage and repair, cellular reprogramming, inflammation and biogenesis of ribosomes, RNA and proteins^{27,28}. High expression of TPT1 is proved to have malignancy promoting characteristic in lung, colon and prostate cancers, where, TPT1 is found to be involved in cancer development and progression^{27,28}. Similarly, in gastric carcinoma high expression of TPT1 has revealed its involvement in the later stages of malignancy i.e. invasion and metastasis^{29,30}.

The common pathway where DEGs of *H.pylori* infection, gastritis, *H.pylori* infected GC and GC appeared to be enriched was spliceosome where the three common identified biomarkers in all the conditions were Heterogeneous nuclear ribonucleoproteins C1/C2 (HNRNPC), DEAD-Box Helicase 42 (DDX42) and Heterogeneous nuclear ribonucleoprotein U (HNRNPU). Spliceosome genes are found to be involved in the progression of various cancers including GC^{31–33}, however, its role in *H.pylori* induced GC is not explored. The other significant pathways were ribosome, adherens junction, Alzheimer's disease and focal adhesion that are previously reported to be involved in *H.pylori* induced GC^{34–37}. Alzheimer's disease was one of the common pathways in *H.pylori* infection and GC datasets where Amyloid precursor protein (APP), Cytochrome C Oxidase Subunit 4I1 (COX4I1), ATPase Sarcoplasmic/Endoplasmic Reticulum Ca²⁺ Transporting 3 (ATP2A3), Caspase-9 (CASP9) and Caspase-7 (CASP7) were identified as the common DEGs. Although *H.pylori* infection is reported to be one of the causative agents of Alzheimer's disease^{38,39}, however, its role in *H.pylori* induced GC is yet to be explored. To study the connection between Alzheimer's disease and *H.pylori* induced GC APP, COX4I1, ATP2A3, CASP9 and CASP7 should be studied to get insights into the molecular functions involved in the process.

Moreover, protein-protein interaction (PPI) networks revealed interaction of TPT1 with several 60S subunit ribosomal proteins including ribosomal Protein Lateral Stalk Subunit P0 (RPLP0), ribosomal protein L32 (RPL32), ribosomal protein L11 (RPL11), ribosomal protein L12 (RPL12), ribosomal protein L24 (RPL24) and ribosomal protein L25A (RPL25A). Although ribosomal proteins have a significant role in the progression of several cancers including colorectal cancer⁴⁰, breast cancer⁴¹, prostate cancer⁴², lymphoblastic leukaemia⁴³ and cervical cancer⁴⁴. However, to the best of our knowledge and literature search, the role of ribosomal proteins in *H.pylori* induced GC has not been explored yet. LIM domain only protein 7 (LMO7), Ras-related Protein Rap-1b (RAP1B), Ribosomal Protein L6 (RPL6), RPLP0, RPL32, Serine/arginine repetitive matrix protein 2 (SRRM2) and Integrin Subunit Beta 1 (ITGB1) were identified as other common potential interactions. The hub genes in *H.pylori* infection, gastritis, *H.pylori* infected GC and GC PPI networks were selected based on the degree of connectivity where ribosomal genes were found to be involved in all the above conditions except gastritis. Which shows that in several ways ribosomal proteins contribute to the progression of *H.pylori* induced GC and it is crucial to study their role.

Conclusion

In conclusion, comparative analysis identified translationally controlled tumor protein (TPT1) and Ly6/Plaur Domain Containing 6b (LYPD6B) to be the common DEGs across all the RNA-seq datasets. Overall, the most significant pathways where the genes were found to be enriched were spliceosome, focal adhesion, adherens junction and Alzheimer's disease. Network analysis

helped us identify TPT1 as a potential predictive biomarker for *H.pylori* induced GC due to its presence and highly interactive nature across the networks of all the conditions. In future, to better understand the role of TPT1 in *H.pylori* induced GC, this study needs to be further validated experimentally by using *in vitro* cell-based functional assays, whereas, TPT1 can be targeted by using inhibitors and enhancers.

Methods

The differential expression analysis of microarray and RNA-seq datasets was performed followed by functional enrichment and network analyses for identification of linking molecular events and significant biomarkers. The overall methodology performed on the datasets of *H.pylori* infection and GC is shown in Figure 1.

Data collection

In this study, a total of 11 datasets for Microarray and RNA-seq analysis were retrieved from [ArrayExpress](#)⁴⁵ and [Gene Expression Omnibus \(GEO\)](#)⁴⁶ databases. For the *H.pylori* induced GC datasets, the selection criterias were the organism to be Homo sapiens with normal and disease conditions, the samples being treatment-naive i.e no effect of drugs on the samples and no mutation in the genes in the datasets. Based upon our inclusion criteria, the selected datasets belonged to different regions which ended up making our research a multi-population study, which ended up removing geographic region bias from our analysis. Details of ID, platform, number of samples, the origin of the microarray (i.e. *H.pylori* infection and GC), RNA-seq (i.e. *H.pylori* infection, gastritis, *H.pylori* infected GC and GC) datasets and the literature references are listed in Table 1.

Data processing

Microarray data analysis

Microarray data analysis on *H.pylori* infection and GC datasets was performed using end to end workflow⁴⁷ in R⁴⁸. Different Bioconductor R packages were used i.e [Biobase](#), [oligoClasses](#), [ArrayExpress](#), [oligo](#), [arrayQualityMetrics](#), [RMA](#)⁴⁹, [limma](#)⁵⁰, and [EnhancedVolcano](#)⁵¹. Expression data from all the datasets were imported using ArrayExpress package that fetched ADF, IDF, SDRF and raw data files. Quality control was carried out on *H.pylori* infection and GC datasets. Normalization was then performed to remove the variation and outliers in the raw expression data using the Robust Multiarray average (RMA) package which performed background correction, normalization and summarization. Quality control and normalization were performed for both alignment and control followed by differential expression analysis using limma package to retrieve DEGs. For *H.pylori* infection and GC datasets, significant threshold (cut-off) negative log of p.value<0.05 and a $\log_2$ fold change ($\log_2FC$) ($\log_2FC < -1$ & $\log_2FC > 1$) were set to extract genes that were significantly differentially expressed. Moreover, for visualization of the DEGs, volcano-plot was generated by using the EnhancedVolcano package.

RNA-seq data analysis

A well-annotated pipeline was created and RNA-seq data analysis was carried out using [Galaxy](#)⁵² platform and R. Various tools in Galaxy were used to analyse the RNA-seq datasets including *H.pylori* infection, gastritis, *H.pylori* infected GC and GC datasets. Quality control was performed using the FastQC⁵³ tool on the raw sequence data of RNA-Seq datasets to identify the adapter contents which were further removed using FASTQ Quality Trimmer⁵⁴. The sequence reads were then aligned to the reference genome of Homo sapien i.e. Hg38 (present by default) using HISAT2⁵⁵. Moreover, to check the quality of data and identify sequence and mapping based duplicate reads RSeQC⁵⁶ tool was used. The duplicate reads were then marked using MarkDuplicate [Picard](#) and further removed by the RmDup⁵⁷ tool.

After the alignment, gene expression data were retrieved using StringTie⁵⁸. The retrieved output files were assembled transcripts, exon, intron and transcript-level expression data, along with exon to transcript mapping and intron to transcript mapping files. For differential expression analysis, Ballgown⁵⁸ R script was used, which generated significant DEGs by processing the read count data obtained from StringTie. The significant DEGs of *H.pylori* infection, gastritis, *H.pylori* infected GC and GC datasets were then visualized using EnhancedVolcano plot with a cut-off p.value<0.05 and $\log_2FC < -1$ and $\log_2FC > 1$. The aim of keeping similar $\log_2FC$ values was to obtain a significant amount of DEGs and to make it comparable in further comparative, functional enrichment and network analyses.

Comparative Analysis

For identification of common markers and key genes, [biomaRt](#) was used to perform comparative analysis on the DEGs obtained through the differential expression analysis. To visualize the common DEGs between all the datasets [InteractiVenn](#)⁵⁹ was used.

Functional Enrichment and Network Analysis

Gene Ontology (GO) and pathway analysis was performed to identify the biological and molecular aspects of DEGs involved in the progression of *H.pylori* infection, gastritis, *H.pylori* infected GC and GC. Ontologies were obtained using the Toppfun

function in [Toppgene](#)⁶⁰, and pathway analysis was performed using the [DAVID](#)⁶¹ database, where the [KEGG](#) (Kyoto Encyclopedia of Genes and Genomes)⁶² pathways were considered. Furthermore, common pathways in *H.pylori* infection, gastritis, *H.pylori* infected GC and GC datasets were selected with a p.value<0.05.

To further identify the interactions and hub genes between the DEGs of *H.pylori* infection, gastritis, *H.pylori* infected GC and GC conditions, network analysis was performed. To get an interacting network of each condition, the lists of the DEGs were uploaded to the [STRING](#) database (version: 11.0)⁶³, where, the threshold for interaction score was set to have the highest confidence score of 0.9. For detailed network analysis, [Cytoscape](#) software (version: 3.8.2)⁶⁴ was used for visualization and identification of highly dense clusters in the networks. Ultimately, to identify the interconnected nodes (DEGs) in the *H.pylori* infection, gastritis, *H.pylori* infected GC and GC networks and to understand their functionality, network analyzer⁶⁵ tool and MCODE⁶⁶ app were utilized.

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Author contributions statement

Sabaoon Zeb and Rehan Z. Paracha conceived and designed the study. Sabaoon Zeb conducted the analysis and wrote the manuscript. All authors took part in analytical discussions and critical reviewing of the manuscript.

Additional information

Not applicable

Figure 1. Overall methodology of the research Differential expression analysis was performed on microarray and RNA-seq datasets of *H.pylori* infection, gastritis, *H.pylori* infected GC and GC to obtain differentially expressed genes (DEGs). Further comparative analysis was performed to identify common genetic biomarkers followed by functional enrichment and network analysis for identification of common significant pathways and interactions.

Table 1. Datasets information

ID	Platform	Samples	Country	References
Microarray Datasets				
<i>Helicobacter pylori</i> infection				
E-GEOD-5081	Affymetrix [HG-U133Plus2]	16 (8 non-infected & 8 infected)	Hungary	67
E-GEOD-70394	Agilent whole genome 4x44KV1	6 (3 non-infected & 3 infected)	Portugal	68
E-GEOD-74577	Affymetrix [HTA-20]	6 (3 non-infected & 3 infected)	China	69
Gastric cancer				
E-GEOD-19826	Affymetrix [HG-U133Plus2]	27 (12 tumor & 15 normal)	China	70
E-GEOD-49051	Agilent whole genome 4x44KV2	6 (3 tumor & 3 normal)	China	71
E-GEOD-79973	Affymetrix [HG-U133Plus2]	20 (10 tumor & 10 normal)	China	72,73
RNA-seq Datasets				
<i>Helicobacter pylori</i> infection				
GSE55699	Illumina HiSeq 2500	4 (2 non-infected & 2 infected)	Germany	21
<i>Helicobacter pylori</i> infected GC				
GSE73226	Illumina HiSeq 2000	6 (3 non-infected & 3 infected)	Germany	22
Gastric cancer				
GSE41476	Illumina Genome Analyzer II	5 (3 tumor & 2 normal)	South Korea	23
GSE80388	Illumina MiSeq	8 (6 tumor & 2 normal)	Taiwan	24
Gastritis				
E-MTAB-3689	Illumina HiScan2500	10 (6 tumor & 4 normal)	Nicaragua	25

Table 2. Differentially expressed genes of *H.pylori* infection, gastritis, *H.pylori* infected GC datasets

Microarray Analysis	
ID	Number of obtained DEGs
E-GEOD-5081	254
E-GEOD-74577	2198
E-GEOD-74577	483
E-GEOD-19826	920
E-GEOD-49051	6146
E-GEOD-79973	1898
RNA-seq Analysis	
GSE55699	908
GSE73226	2424
E-MTAB-3689	314
GSE73226	2066
GSE41476	1548

Figure 2. Differential expression analysis of microarray datasets (*H.pylori* infection and GC datasets): Enhanced volcano plots for *H.pylori* infection and GC datasets; **A:** Dataset E-GEOD-5081 **B:** Dataset E-GEOD-70394 **C:** Dataset E-GEOD-74577 **D:** Dataset E-GEOD-19826 **E:** Dataset E-GEOD-49051 **F:** Dataset E-GEOD-79973. In the volcano plots, on the x-axis log₂FC i.e. log₂FC < -1 and log₂FC > 1 was plotted against negative log of p.value < 0.05 on the y-axis. Here, the red dots represents the genes that are significantly differentially expressed. The right side of plots shows the up-regulated DEGs while the DEGs plotted on the left side are down-regulated. The blue dots display gene that are significant but not differentially expressed and the green dots are genes that are differentially expressed but not significant. Whereas, the gray dots represents genes that are neither significant nor differentially expressed.

Figure 3. Differential expression analysis of RNA-seq datasets (*H.pylori* infection, gastritis, *H.pylori* infected GC and GC datasets): Enhanced volcano plots for *H.pylori* infection, gastritis, *H.pylori* infected GC and GC datasets; **A:** Dataset GSE55699 **B:** Dataset GSE73226 **C:** Dataset GSE41476 **D:** Dataset GSE80388 **E:** Dataset E-MTAB-3689, with $\log_2\text{FC} < -1$ and $\log_2\text{FC} > 1$ and its $p\text{-value} < 0.05$. Here, the red dots represents the significant DEGs where on the right side of the plot are up-regulated DEGs and on the left side are the down-regulated DEGs. The blue dots display genes that are significant but not differentially expressed and the green dots are genes that are differentially expressed but not significant. Whereas, the gray dots represents genes that are neither significant nor differentially expressed.

Figure 4. Comparative analysis-Microarray datasets: **A:** From *H. pylori* infection datasets 4 common DEGs identified; **B:** From GC datasets 119 common DEGs identified; **C:** No common DEGs identified from integrative analysis of *H. pylori* infection and GC microarray datasets.

Figure 5. Comparative analysis-RNA-seq datasets: A: From integrating the DEGs from *H.pylori* infection, gastritis, *H.pylori* infected GC and GC, we identified 2 common DEGs (TPT1 and LYPD6B).

Figure 6. Pathway interactions in *H.pylori* infection, gastritis, *H.pylori* infected GC and GC: This network shows the common interactive pathways involved in the four conditions. The yellow node shows spliceosome which is found to be the sole common pathway across all the conditions, whereas, the 2 pink nodes (Adherens junction and RNA transport) were the pathways common in three conditions. Other common pathways are given light blue color based on interactions in two conditions.

Figure 7. Hub genes in *H. pylori* infection, gastritis, *H. pylori* infected GC and GC protein-protein interaction (PPI) networks: **A:** PPI of *H. pylori* infection indicating 17 hub genes along with highly interactive genes cluster **B:** PPI network of gastritis indicating 9 hub genes with a cluster of highly interactive genes in the network. **C:** PPI network of *H. pylori* infected GC indicating 14 hub genes with a cluster of highly interactive genes in the network. **D:** PPI network of GC indicating 9 hub genes along with a highly interactive genes cluster

Figure 8. Common interactions and potential markers in *H.pylori* infection, gastritis, *H.pylori* infected GC and GC: Network indicates common significant interactions in *H.pylori* infection, gastritis, *H.pylori* infected GC and GC. *H.pylori* infection, gastritis, *H.pylori* infected GC and GC. Here TPT1 gene is common in all the conditions.

Table 3. Top enriched ontologies retrieved from Toppgene

ID	Function	Name	P.Value	FDR	Entities
H.pylori infection enriched ontologies					
GO:0003723	MF	RNA binding	1.16E-08	1.69E-05	135
GO:0045296	MF	Cadherin binding	1.39E-07	1.02E-04	37
GO:0050839	MF	Cell adhesion molecule binding	1.23E-06	6.00E-04	47
GO:0044265	BP	Cellular macromolecule catabolic process	8.78E-09	5.55E-05	90
GO:0019752	BP	Carboxylic acid metabolic process	1.77E-08	5.55E-05	85
GO:0006082	BP	Organic acid metabolic process	3.66E-08	5.55E-05	90
GO:0005925	CC	Focal adhesion	5.60E-10	5.33E-07	45
GO:0030055	CC	Cell -substrate junction	1.83E-09	8.73E-07	109
GO:0005912	CC	Adherens junction	1.02E-08	2.53E-06	93
Gastritis enriched ontologies					
GO:0003823	MF	Antigen binding	8.63E-09	6.95E-06	15
GO:0003723	MF	RNA binding	9.99E-06	2.68E-03	49
GO:0050839	MF	Cell adhesion molecule binding	7.46E-05	1.20E-02	19
GO:0006958	BP	Complement activation, classical pathway	3.05E-12	1.38E-08	17
GO:0002455	BP	Humoral immune response	1.48E-11	3.35E-08	17
GO:0030449	BP	Regulation of complement activation	2.94E-11	3.48E-08	15
GO:0019814	CC	Immunoglobulin complex	2.09E-14	1.35E-11	20
GO:1902494	CC	Catalytic complex	3.05E-06	9.84E-04	42
GO:1990234	CC	Transferase complex	6.83E-05	1.47E-02	25
H.pylori infected enriched ontologies					
GO:0045296	MF	Cadherin binding	9.56E-16	1.61E-11	82
GO:0003723	MF	RNA binding	6.68E-15	5.61E-11	289
GO:0050839	MF	Cell adhesion molecule binding	1.73E-14	9.70E-11	107
GO:0016032	BP	Viral process	2.32E-32	2.12E-27	189
GO:0044403	BP	Symbiotic process	2.84E-31	1.30E-26	195
GO:0044419	BP	Interspecies interaction between organisms	5.84E-30	1.78E-25	199
GO:0030055	CC	Cell-substrate junction	2.95E-32	2.09E-28	121
GO:0005925	CC	Focal adhesion	4.54E-32	2.09E-28	119
GO:0005912	CC	Adherens junction	1.07E-30	3.28E-27	141
GC enriched ontologies					
GO:0003735	MF	Structural constituent of ribosome	3.89E-19	9.50E-16	78
GO:0003723	MF	RNA binding	2.43E-16	5.93E-13	285
GO:0050839	MF	Cell adhesion molecule binding	5.98E-13	1.46E-09	130
GO:0044403	BP	Symbiotic process	1.35E-33	1.41E-29	249
GO:0016032	BP	Viral process	3.64E-33	3.82E-29	237
GO:0044419	BP	Interspecies interactions between organisms	3.16E-32	3.16E-32	255
GO:0044391	CC	Ribosomal subunit	1.04E-29	1.41E-26	94
GO:0005739	CC	Mitochondrion	2.36E-28	3.20E-25	399
GO:0019814	CC	Immunoglobulin complex	3.63E-28	4.93E-25	77

Table 4. KEGG pathways retrieved from DAVID

KEGG ID	Term	Count	P.Value
H.pylori infection enriched KEGG pathways			
hsa03040	Spliceosome	16	3.13E-03
hsa01100	Metabolic pathways	80	1.20E-02
hsa03050	Proteasome	7	2.34E-02
hsa05010	Alzheimer's disease	16	2.47E-02
hsa05161	Hepatitis B	14	3.41E-02
Gastritis enriched KEGG pathways			
hsa04520	Adherens junction	6	0.004976
hsa03040	Spliceosome	7	0.017902
hsa05322	Systemic lupus erythematosus	7	0.018509
hsa04141	Protein processing in endoplasmic reticulum	7	0.049395
H.pylori infected Gastric Cancer enriched KEGG pathways			
hsa03010	Ribosome	48	8.56E-13
hsa03013	RNA transport	43	1.32E-06
hsa04510	Focal adhesion	44	6.62E-05
hsa05131	Shigellosis	20	6.81E-05
hsa03040	Spliceosome	24	3.00E-02
Gastric Cancer enriched KEGG pathways			
hsa03010	Ribosome	68	3.15E-19
hsa05016	Huntington's disease	59	1.26E-06
hsa03013	RNA transport	54	1.87E-06
hsa05010	Alzheimer's disease	49	5.19E-05
hsa03040	Spliceosome	35	0.004784

Table 5. Top hub genes in *H.pylori* infection, gastritis, *H.pylori* infected GC and GC protein-protein interaction networks

Gene	Avg. Shortest Path Length	Betweenness Centrality	Closeness Centrality	Clustering Coefficient	Degree	Eccentricity	Neighborhood Connectivity
H.pylori infection Hub genes							
RPS20	1.375	0.282710091	0.727272727	0.234482759	24	3	19.8
RPLP0	1.4	0.060595847	0.714285714	0.316239316	21	2	21.2962963
RPS17	1.444444444	0.181880964	0.692307692	0.276353276	21	3	21
SRSF3	1	0.067611955	1	0.349802372	20	1	19.47826087
RPL8	1.363636364	0.087170271	0.733333333	0.307692308	20	2	21.18518519
POLR2L	1.844444444	2.734273198	0.542168675	0.132132132	19	4	13.05405405
SEN3P	1	0.208449878	1	0.223684211	18	1	18.55
RPL6	1.384615385	0.067164488	0.722222222	0.329230769	18	2	21.76923077
SRRT	1.4	0.030429322	0.714285714	0.371428571	18	2	19.61904762
RNPS1	1.4	0.82547346	0.714285714	0.211111111	17	3	21.16666667
Gastritis Hub genes							
SRSF7	3.036082474	0.02984606	0.329371817	0.470588235	17	6	12.11764706
U2AF2	3.139175258	0.014312771	0.318555008	0.558333333	16	6	13.0625
ITGB1	3.139175258	0.099231964	0.318555008	0.058333333	16	7	4.375
SF1	3.077319588	0.022461931	0.324958124	0.628571429	15	6	13.6
TRIM21	3.170103093	0.058294451	0.315447154	0.272727273	11	7	7.090909091
HIST1H2AH	3.484536082	0.017094311	0.286982249	0.563636364	11	8	9.545454545
RPL6	3.242268041	0.011645183	0.308426073	0.436363636	11	6	10.90909091
UBE2G2	3.164948454	0.03519891	0.315960912	0.377777778	10	7	8.9
RAP1B	3.365979381	0.030676355	0.297090352	0.095238095	7	8	7
H.pylori infected Gastric cancer Hub genes							
UPF2	0	0	0	0.5	42	0	49.5
UPF1	1	0	1	0.5	41	1	49.5
SSR2	0	0	0	0.497560976	41	0	48.41463415
SMG6	1	0	1	0.5	40	1	49.5
RPS9	1	0.038577629	1	0.426470588	39	1	45.63461538
RPS7	1.071428571	0.16607394	0.933333333	0.422699849	39	2	45.71153846
SMG1	1	0	1	0.5	39	1	49.5
RPS4X	1.066666667	0.16607394	0.9375	0.422699849	38	2	45.71153846
SPCS2	1	0	1	0.5	38	1	48.94871795
SRP9	1	0	1	0.5	38	1	48.94871795
GC Hub genes							
RPS12	2.854545455	0.128086055	0.350318471	0.707602339	19	7	14.26315789
RPS15A	2.854545455	0.128086055	0.350318471	0.707602339	19	7	14.26315789
RPL13	2.618181818	0.455179209	0.381944444	0.713450292	19	6	14.42105263
RPL32	2.909090909	0.05652601	0.34375	0.713450292	19	7	14.36842105
RPL11	2.927272727	0.020162374	0.341614907	0.797385621	18	7	15.16666667
RPL3	3	0.004180331	0.333333333	0.867647059	17	7	15.64705882
RPL34	2.945454545	0.017532905	0.339506173	0.852941176	17	7	15.70588235
RPL37A	2.945454545	0.017532905	0.339506173	0.852941176	17	7	15.70588235
RPLP0	3	0.004180331	0.333333333	0.867647059	17	7	15.64705882

Table 6. Networks summary statistics of *H.pylori* infection, gastritis *H.pylori* infected GC and GC protein-protein interaction networks

Network	Nodes	Edges	Connected components	Avg. number of neighbours	Clustering coefficient
H.pylori infection	259	388	20	2.9	0.107
H.pylori infected Gastric cancer	136	1956	1	28.76	0.42
Gastric cancer	96	263	10	5.47	0.284
Gastritis	112	195	8	3.48	0.205

Supplementary Files

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