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ORIGINAL ARTICLE

Diagnostic Role of Circulating Exosomal microRNA-1286 for the progression of Colorectal Cancer

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ABSTRACT

OBJECTIVE: To assess the expression and clinical significance of exosomal miR-1286 for the detection and progression of CRC.

METHODOLOGY: miR-1286 was selected using the Geo dataset GSE39833. Bioinformatics tools were used to analyze its expression in CRC. After the collection of serum samples using the double centrifugation technique from 23 CRC patients, exosomal enrichment was performed, and miRNA extraction was done. After reverse transcription of the RNA template, its expression level was analyzed by qRT-PCR (Quantitative Reverse Transcription Polymerase Chain Reaction). The expression levels of miR-1286 correlated with the clinical characteristics of colorectal cancer, and patient survival was further examined using Kaplan-Meier analysis.

RESULTS: miRNA-1286 was found non-significant in comparing T3 and T4, N1 and N2 stages, and to discriminate colorectal metastatic tumor patients from non-metastatic tumor patients (p-value >0.05). ROC (Receiver Operating Characteristic) curve analysis revealed an AUC (Area under Curve) of 0.69 with 75% sensitivity and 83.3% specificity at the cut-off value of > 1.07.

CONCLUSION: Upregulated expression of miR-1286 in colorectal cancer is statistically not significant to be used as a diagnostic biomarker for the detection and progression of CRC.

KEYWORDS: miRNAs, colorectal carcinoma, CRC, Exosome, real-time PCR, Exosomal miRNA

INTRODUCTION

Colorectal cancer is becoming a global threat. This is the second deadliest after lung cancer, 3rd most common cancer, and 10% of all malignancies. Due to its high annual incidence of 1.93 M cases in 2020, it is one of the most investigated neoplasms. Its predicted rise in prevalence over the next two decades makes this risky. Recent years have shown an alarming rise in CRC prevalence in high HDI (human development index) countries, with more than 40 incidents per 100,000 people and 935,000 fatalities predicted for 2020. The age-standardized mortality rate (ASMR) for colorectal cancer (CRC) is roughly 8.9 per 100,000 people worldwide. The 5-year survival rate for stage IV cancer is 14%, in contrast with stage I CRC, which accounts for roughly 90%[1].

CRC causes altered bowel habits, bleeding per rectum, abdominal pain or mass, weight loss, microcytic hypochromic anemia, anorexia, and deep vein thrombosis. Western lifestyle adoption, obesity, red meat consumption, alcohol intake, sedentary lifestyle, and smoking are risk factors for CRC, which is rising rapidly in emerging nations. A strong family history is associated with a younger CRC diagnosis, especially in proximal colonic cancer compared to rectal cancer[2].

In Pakistan, the WHO reports a steady rise in cancer rates. After 30% of the population was elderly and 20% were adults, 43% of middle-aged people were most at risk, with male predominance (men, 54%; women, 46%). CRC is most common in Sindh and Urdu-speaking males, followed by Baloch, Punjabi, and Pakhtoon males, and is least common in Siraiki males[3]. According to the 2021 annual cancer registry data from Shaukat Khanum Memorial Hospital, colon cancer accounts for 7.7% of all malignancies and is the second most common cancer in Pakistan after breast cancer[4].

DRE (digital rectal examination), FIT (fecal immunological test) for hemoglobin, gFOBT (Guaiac faecal occult blood test), flexible sigmoidoscopy, and optical colonoscopy are used to diagnose CRC. Since gFOBT is not specific for human hemoglobin, various foods and medicines can cause false positives. NICE recommends FIT (which detects human-specific hemoglobin in feces) for suspected CRC with bowel habit changes, iron-deficient anemia, or at age 60. Endoscopic tumour identification, sample collection, and gut examination after patient preparation determine the diagnosis[5]. Validated biomarkers that are minimally invasive, accurate, and enable early detection and prognostic prediction of CRC are needed. microRNAs, evolutionarily conserved short non-coding RNAs of 18-25 nucleotides, regulate cellular and pathogenic processes via diverse pathways[6].

Due to serum and plasma biostability, circulating miRNAs are regarded as blood-based biomarkers. The present research attempted to assess miR-1286 expression levels as early CRC diagnostic and prognostic biomarkers and relate them to the clinicopathological characteristics of CRC patients. For this study, miR-1286 expression was evaluated to correlate with different stages of CRC to deduce the disease at its early stages, prognosis, and treatment response.

METHODOLOGY

This was a cross-comparative experimental investigation with subjects aged 20-70 years. The three groups were C Group 1 (Healthy normal individuals), T Group 1 (Non-metastatic colorectal cancer blood sample), and T Group 2 (Metastatic). The study's process, dangers, and benefits were described to participants who provided consent.

Subject Selection and Recruitment

Over 6 months, 23 patients (10 non-metastatic and 13 metastatic) with histopathologically confirmed CRC diagnosis from Nishtar Hospital, Multan, were enrolled. To meet inclusion and exclusion criteria, all CRC patients were treatment-naïve and had not had any cancer treatment, including chemotherapy or radiotherapy. Healthy controls were 10 people without benign or malignant tumours or chronic systemic illnesses.

Pilot *in silico* analysis

Datamining of exosome datasets

Four public databases, i.e., Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo), Expression Atlas (https://www.ebi.ac.uk/gxa), ArrayExpress (https://www.ebi.ac.uk/arrayexpress), and the Genomic Expression Archive (GEA, https://www.ddbj.nig.ac.jp/gea) were searched for exosome-based miRNA gene expression datasets of colorectal cancer. These public datasets were searched with keywords such as "colorectal carcinoma", "colorectal cancer", "rectal cancer", "colon cancer", "exosomes", "exosome", "exosomal", "miRNA", and "microRNA". A set of criteria was used to filter the datasets.

Pre-processing and differential expression

R software was used to extract RAW Agilent microarray image files of miRNA expression from GEO datasets. Background correction using the "normexpr" method and normalization through the "quantile" method were conducted for each array. Background-adjusted and normalized data were analyzed to remove signals from control, negative, and low-expressed probes. Methods, including RLE plots, boxplots, heatmaps, hclust clustering plots, and PCA plots, were used to ensure data quality. To counter within-sample outlier variance, the "limma" R package calculated array weights for each array, and the "lmfit" and "eBayes" algorithms extracted the overall and within-stage contrasts-based differential expression. Differential significance was determined using FDR-adjusted $p < 0.05$ and $|\log FC| > 1$.

Selection of miRNA for the current study

The study systematically analyzed differential miRNAs, identified those not previously studied for their potential as biomarkers, and selected "has-miR-1286" miRNA with a logFC value of 7.89 based on its significant differences in public literature databases.

Sample Collection

Samples were collected from CRC patients and healthy subjects at Nishtar Hospital, Multan, after approval from the IMBB/University of Lahore ethical review committee with letter no. Ref-IMBB/BBBC/23/186-A, dated February 15, 2023. and further processed at the University of Health Sciences, Kala Shah Kaku Campus, Lahore.

Screening of Subjects

Biochemical and hematological parameters were used to screen suspected CRC patients and healthy subjects, including Hemoglobin, Total leukocyte count, and Carcinoembryonic antigen.

Histopathology-based confirmation of CRC and Staging

The study included all stages of suspected cases confirmed by histopathology after colonoscopy and grouped according to WHO criteria.

Study data collection

Demographic (i.e., age and gender) and clinicopathologic (hematological and biochemical

parameters, tumor stage, nodal involvement, and distant metastases) data of CRC patients were recorded using a dedicated proforma.

Sample Size

The study had 90% power, a 5% significance level, and a logFC of 7.89, with a standard deviation of ΔCt of 2. A sample size of 2 was calculated for each group using the formula from the Statistical Rules of Thumb. 13 samples were selected from the metastatic group, 10 from the non-metastatic group, and 10 from the healthy control group for better results.

Serum separation and storage for miRNA analysis

Blood samples were collected from the CRC patients (before any treatment) and healthy control subjects in serum vacutainer tubes (BD, USA) and centrifuged for serum separation and divided into aliquots of 1 mL and frozen at -20°C for subsequent processing for exosome and RNA purification. For RNA purification, frozen serum was thawed completely by incubation in a 37°C water bath.

Exosomal Enrichment

Invitrogen™ Total Exosome Isolation Reagent (from serum), Catalog number: 4478360, was used for the extraction of exosomes according to the instructions given.

miRNA Isolation

miR-1286 was isolated from CRC and healthy control serum samples using the FavorPrepTM miRNA Isolation kit according to the described protocol.

cDNA Synthesis

cDNA was synthesized using miRcute Plus miRNA First-Strand cDNA Kit following the prescribed protocol. 2×miRNA RT Reaction Buffer, RNA Template, and RNAse-free ddH_2O was thawed fully and transiently centrifuged, and all of them were put on ice. All the steps were performed on ice.

Expression analysis on real-time PCR

miRNA sequence (www.mirbase.org) based primers were synthesized commercially by Macrogen.

Table I: Target microRNA Sequences

Sr#	Item	Specifications
1	RT primer (universal)	CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGG CAATTTTTTTTTTVN
2	Reverse primer for qPCR (universal)	CAGTGCAGGGTCCGAGGT
3	FP_ has miR-1286	UGCAGGACCAAGAUGAGCCCU
4	FP_ hsa-RNU6-1	CGCTTCGGCAGCACATATACTA

miR-1286 was amplified on real-time PCR using miRcute Plus miRNA qPCR Kit (SYBR green). The expression results of real-time PCR were measured and compared using various analyses.

Statistical Analysis

Data was entered and analyzed using IBM SPSS statistical software version 26 (The IBM® SPSS® software) and GraphPad Prism software 8.0 USA. Shapiro-Wilk's and Kolmogorov-Smirnov tests were used to assess the normality of the data. A p-value of < 0.05 was considered significant. Frequencies and percentages were used for qualitative variables (age, gender, TNM stage, tumor invasion (T), tumor nodules (N), metastasis, tumor localization, histological grading of tumor, and patient survival status). The mean (± SD) values were used to express the data of quantitative variables: Carcinoembryonic antigen (CEA), Hemoglobin (Hb), and Total leukocyte count (TLC). The independent t-test was used

to express the association of miRNA with age, gender, TNM stage, tumor invasion (T), tumor nodules (N), metastasis, tumor localization, histological grading of tumor, and patient survival status. For different comparison groups, e.g., CRC patients at different stages and healthy subjects, normalized miRNA expression values were calculated and compared using technique $2^{-\Delta\Delta Ct}$. An unpaired t-test was used to compare stage III with stage IV (non-metastatic versus metastatic), and to compare stages N1 and N2, and stage T3 and stage T4. A receiver operator characteristic (ROC) analysis was used to evaluate the diagnostic accuracy, specificity, sensitivity, and area under the curve (AUC) of dysregulated miRNA-1286.

RESULTS

Cohort characteristics

This study included a cohort of 33 Pakistani CRC patients. Different demographic, clinicopathological, therapeutic, and Survival characteristics, along with their association with CRC serum microRNA-1286 levels, indicated that miR-1286 was non-significant for stage T1-T, N0-N1 histological grading or metastasis in CRC samples, as shown in **Table II**.

Table II: Baseline demographic and clinicopathological features and their association with CRC serum microRNA-1286 levels of Pakistani CRC patients

Characteristics	CRC patients (n =33)	miR-1286 (mean ± SD)
Demographic		
Age in years (Mean ± SD)	51 (21-72)	
Age ≤50 years	09 (39.1 %)	6.64±1.26
Age >50 years	14 (60.9 %)	5.96±0.62
p-value		0.58
Gender, n=23 (%)		
Female	11 (47.8%)	6.09±0.77
Male	12 (52.2%)	6.06±0.77
p-value		0.94
Clinicopathological		
cT stage, n=23(%)		
T1-T2	02 (8.7%)	0
T3-T4	21 (91.3%)	6.07±0.73
p-value		-
cN stage, n=23 (%)		
NX-N0	03 (13%)	0
N1-N2	20 (87%)	6.06±0.73
p-value		-
Overall TNM stage, n=23 (%)		
I-II	03 (13%)	0
III-IV	20 (87%)	6.07±0.73
p-value		-
Histological grading, n=23 (%)		
Well-differentiated	04 (17.4%)	5.92±0.75
Moderately differentiated	09 (39.1%)	

Poorly differentiated	10 (43.5%)	6.18±0.75
p-value		0.56
Tumor localization, n=23(%)		
Colon	10 (43.5%)	5.99±0.59
Rectum	13 (56.5%)	6.13±0.85
p-value		0.740
Tumor metastasis, n=23(%)		
No	09 (39.1%)	6.67±0.45
Yes	14 (60.9%)	5.95±0.72
p-value		0.18
Laboratory		
Median Carcinoembryonic Antigen (CEA)	105.4	
Mean Hemoglobin (Hb)	9.37±1.72	
Mean Total leucocyte count (TLC)	10.04±1.05	
Survival		
Overall Survival in months, n = 23, Median (Range) 12		
Survival status, n (%)		
Alive	21 (91.3%)	6.22±0.70
Dead	02 (8.7%)	5.34±0.18
p-value		0.009
miRNA levels		
Mean miR-1286	6.08	

TNM: Tumor Node and Metastasis; cT: Tumour; cN: Node

Expression analysis of miR-1286 in CRC serum samples

The study evaluated miRNA-1286 expression as a minimally invasive biomarker for colorectal cancer progression in 18 serum samples, using the $2^{-\Delta\Delta Ct}$ quantitative approach and found no statistically non-significant difference, indicating that miR-1286 was not significantly expressed in CRC patient serum.

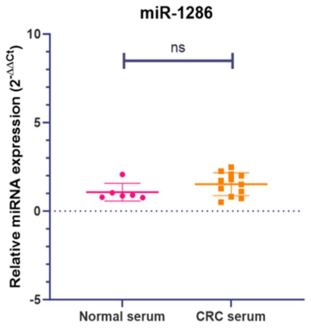
The study evaluated the diagnostic potential of miR-1286, which was not significant in distinguishing colorectal cancer patients from healthy controls. Candidate microRNA-1286 was found to be non-significant in comparing T3 and T4, N1 and N2 stages and to discriminate colorectal metastatic tumor patients from non-metastatic tumor patients (*p*-value >0.05), as mentioned in **Table III** and **Figure 1**.

Table III: Expression analysis of miR-1286 in CRC serum samples

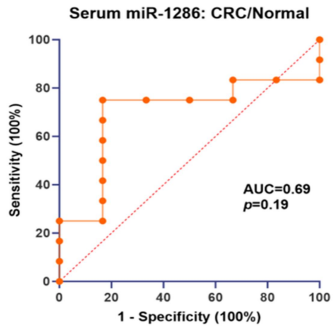
ROC Analysis of serum microRNA-1286 to segregate CRC patients from healthy controls					
AUC	95% CI	Cut-off	Sensitivity	Specificity	*p*-value
0.69	0.43-0.96	>1.07	75	83.33	0.19
Normalized mean expression levels of microRNA-1286 and the fold change in CRC patients versus healthy controls					
No. of samples	Healthy control (mean ± SD)	CRC (mean ± SD)	Fold change	*p*-value	
HC n= 6 CRC n= 12	1.07	1.52	1.42	0.15	
Comparison of miR-1286 expression in stage T3 and stage T4					
No. of samples	T3 (mean ± SD)	T4 (mean ± SD)	*p*-value		
T3 n=04 T4 n=09	6.24±0.74	6.02±0.76	0.67		
Comparison of miR-1286 expression in stages N1and N2					
No. of samples	N1 (mean ± SD)	N2 (mean ± SD)	*p*-value		
N1 n=08 N2 n=03	5.82±0.36	6.01±0.66	0.61		
Comparative analysis of miR-1286 expression in metastatic and non-metastatic CRC patients					
No. of samples	Stage III (mean ± SD)	Stage IV (mean ± SD)	*p*-value		
Stage III n=03 Stage IV n=09	6.30±0.74	5.99±0.75	0.55		

AUC: Area under curve; SD: Standard Deviation; HC: Healthy control; CRC: Colorectal carcinoma

A



B



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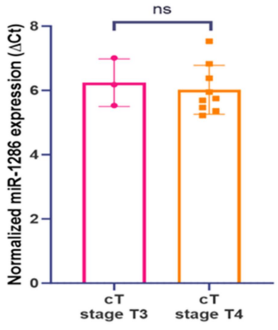

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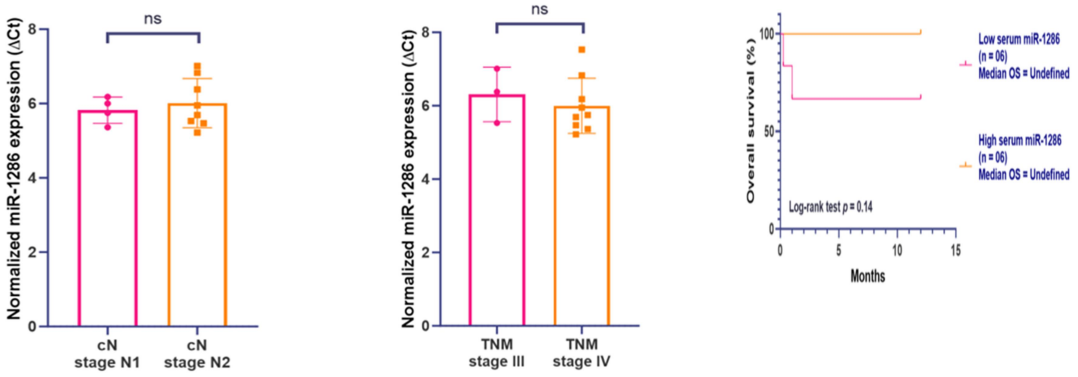

Figure 1: Expression analysis of miR-1286 in Colorectal cancer patients and normal serum samples; A: Relative expression level of microRNA-1286 in CRC and normal serum samples; B: Diagnostic values of serum microRNA-1286 in CRC patients; C: MicroRNA-1286 Expression in stage T3 and stage T4; D: Comparison of miR-1286 expression in stage N1and N2; E: Comparative analysis of miR-1286 expression in metastasis vs. non-metastasis in CRC patients; F: Kaplan Meier survival curve for Overall Survival
CRC; colorectal carcinoma; TNM: Tumor Node and Metastasis

Kaplan-Meier survival curves for Overall Survival

The study analyzed follow-up data for 23 patients, with 8.7% dying due to CRC. The median Survival was 12 months, and the Kaplan-Meier survival curve showed undefined overall Survival for n=06 patients, with both low and high serum miR-1286 levels above 50%.

DISCUSSION

Smaller colorectal cancer is spreading worldwide. CRC is expected to be a monster by 2040, especially in countries with high or very high HDI, as it accounts for 10% of all malignancies, which are the second most deadly, third most diagnosed, and most studied neoplasms. Men get colon cancer more than women[7]. Young patients most often have rectum type and stage III[8]. In age groups >50 and 40, a strong family history of CRC alone or with diabetes mellitus increases risk. CRC risk increases with obesity, alcohol, tobacco, and inactivity[9]. This study found a non-significant laboratory biochemical profile in CRC patients. DRE, FIT, gFOBT, flexible sigmoidoscopy, optical colonoscopy, MRI, CTC, FAPI-PET, and DW-MRI are utilized to diagnose colorectal cancer[10]. Considering CEA and CA19-9 as predictive and prognostic blood biomarkers, however, these methods are not sufficient for accurate, early diagnosis[11]. A tumor forms when persistent DNA mutations prevent apoptosis[12]. Furthermore, the majority of patients would be treated if this massive cancer were found and removed in a precancerous stage, since most colorectal cancers proceed along the serrated pathway or a slow adenoma-carcinoma cascade[13]. Thus, detecting early colorectal neoplasms, including precancerous lesions, is crucial to minimizing colorectal cancer mortality. Data suggest that lymph node metastases may predict disease-free and overall Survival in colorectal cancer patients without distant metastasis[14]. Early tumour detection improves CRC patients' survival rates, emphasizing the need for precise, sensitive, and non-aggressive molecular biomarkers[15]. Early detection and treatment of metastatic colorectal cancer may help patients who have relapsed recover[16]. MiRNA-encoding genes are widely distributed across the genome; therefore, their transcription could be coordinated with that of protein-coding genes. Certain miRNAs have oncogenic properties that influence cell proliferation and other biological processes associated with cancer. Some hypotheses suggest miRNAs can diagnose and prognose cancer[17]. Growing research shows that miRNAs are essential to the formation and progression of human tumors, making them promising biomarkers for cancer diagnosis, prognosis, and treatment. Even though miRNAs may be screening biomarkers, functional prediction analysis is needed to understand their role in CRC carcinogenesis[18]. Santos et al. (2021) found that exosomal miRNAs modulate the immune system, chemoresistance, and metastasis in diverse tumours[19]. Exosomes enable CRC cells to release miRNAs into the extracellular environment regardless of their intracellular expression levels. miRNA profiles have been generated using flow cytometry, microarrays, and cationic lipoplex nanoparticles. However, qRT-PCR remains the favored method due to its excellent sensitivity and specificity[20]. Due to their high sensitivity and specificity, miRNAs can differentiate CRC patients from normal tissue in a minimally invasive manner[21]. Despite a 75% sensitivity and 83.33% specificity, miR-1286 was not a diagnostic marker in this investigation. Although CircCOL1A2 sponges miR-1286 to regulate gastric cancer cell invasion and migration[22]. Overexpression of miR1286 through MAFB downregulation inhibits osteosarcoma cell growth. Mechanism-enhancing head and neck squamous cell cancers and osteosarcoma involve miR1286[23.]. In GC, miR-1286 behaved as a tumour suppressor and biomarker and was strongly linked to HP infection and the emergence of peritoneal metastases[24]. A study found that miR-1286 inhibits NSCLC proliferation through aerobic glycolysis, making it a potential biomarker[25]. MiR-1286 was not previously studied in CRC. A magnitude difference of 1.42 fold change of elevated miR-1286 in CRC samples did not correlate with normal serum samples in this investigation. Demographic, clinical, and pathological associations of miR-1286 were not significant. Two-fifths of miRNAs were not significantly expressed in tumors versus non-tumors. Given that many colon cancer cases are identified at stage T3, the prognosis is unpredictable due to high variability. Many limitations plagued this study. First, clinical samples were few. There was

no availability of Stage I samples, and a smaller number of Stage II samples were available. MicroRNAs for CRC detection and progression need further study. While there is still more to be done, we remain confident that microRNA-related diagnostics and treatments have considerable potential for the prognostic prediction and treatment of CRC.

CONCLUSION

In our study, we found that CRC affects all ages. A considerable percentage of patients acquired a diagnosis beyond the age of 50, and it is more prevalent in older adults. This is consistent with the documented pattern of growing CRC risk with aging. Due to the high rate of advanced-stage patients, early identification methods, such as screening, may be crucial to improving patient outcomes. This significant fold change (FC) of 1.42 in miRNA-1286 expression in CRC patients compared with healthy controls prompted us to assume a correlation with a disease process. The mean normalized expressions of the two groups were not statistically different. At the cut-off value, miRNA-1286 had good AUC, sensitivity, and specificity. Except for histological grading, in which a greater percentage of patients reported having poorly differentiated, advanced cancer and higher levels of miRNA, the miRNAs did not show any substantial relationships. Using miRNA expression to predict severity, stage III and stage IV colorectal cancer patients have aberrant miRNA-1286 expression. There is no evident distinction in miRNA expression between metastatic and non-metastatic patients. MiRNA expression did not differ between colorectal cancer patients with stages T3 and T4, despite the expected misexpression of miRNA-1286. Overall survival analysis was not significant, as individuals with high and low serum levels had similar survival curves above 50%. Few prospective miRNA panels as CRC biomarkers are needed to improve disease prediction. Reflective circulating miRNAs can be increased by minimally invasive screening.

Ethical Permission: University of Lahore, Lahore, Pakistan, ERC approval letter No. IMBB/BBBC/23/186-A.
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Data Sharing Statement: The corresponding author can provide the data proving the findings of this study on request. Privacy or ethical restrictions bound us from sharing the data publicly.

AUTHOR CONTRIBUTION

Nasreen G: Performed the experiments and wrote the original draft
Altaf A: Supervised and administered the entire project
Javaid Z: Assisted with experimentation and manuscript preparation
Maqbool T: Validated the results and proofread the manuscript
Amar A: Facilitated experimentation and data analysis
Bokhari SMR: Provided support for bioinformatic analysis.

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