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The role of mir96 in predicting CTC status and prognostic evaluation in gastric cancer patients

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Abstract

In this study, 20 patients clinically diagnosed with advanced gastric cancer were selected as subjects. Circulating tumor cells (CTCs) in the peripheral blood of gastric cancer patients were detected and counted by collecting peripheral blood samples at Ningbo No. 2 Hospital and using the Cell Rich TM system combined with a negative enrichment strategy. In addition, routine pathological examination and immunohistochemical staining were performed on surgical specimens, including HER2, EBER, E-cadherin, and vimentin indicators. These indicators were correlated and analyzed with CTC counts and routine clinical tests. At the same time, miRNA groups were performed to explore the miRNAs with or without correlation with CTC and to try to construct a predictive model for CTC status. Tumor tissue from patients whose CTC counts did not match the results of the miRNA prediction model was subjected to second-generation gene sequencing to analyze indicators such as tumor heterogeneity and immune microenvironment. The results of the study showed that CTCs were detected in the peripheral blood of 50% (10/20) of gastric cancer patients using the Cell Rich TM system, serum fibrinogen was negatively correlated with CTC counts, whereas TNM staging elements, HER2 receptor and p53 were not correlated with CTC counts. miRNA assays showed that the expression levels of miR218, miR1207, miR96, miR409, miR149, miR148a, miR155, miR370 and miR223 were significantly different between the two groups of CTC≥2 and CTC<2. Among them, miR-96 showed good efficacy as an indicator for predicting CTC status and assisting in determining the prognosis of gastric cancer. In conclusion, the present study demonstrated that the Cell Rich TM system combined with the negative enrichment strategy can effectively detect CTCs in the peripheral blood of gastric cancer patients and that the expression of miR-96 can be used as an effective indicator to predict CTC status and assist in determining the prognosis of gastric cancer. These findings have important implications for the diagnosis and treatment of gastric cancer and provide new clues for the further study of the tumor immune microenvironment and tumor heterogeneity.

Keywords: Circulating tumor cells, Gastric cancer, Tumor metastasis, Gene detection, miRNA; EMT

1. Introduction

Gastric cancer is a prevalent disease, ranking fifth in terms of incidence and fourth in terms of cancer-related deaths worldwide in 2020 [1, 2]. More than 70% of the global incidence of gastric cancer is found in developing countries, and once tumors have metastasized, they become highly lethal [3, 4]. Although the incidence and mortality rates of stomach cancer have declined in recent decades, it remains a major public health concern [5]. However, despite improvements in many other digestive tract cancers, there has only been a small increase in relative survival [6]. Gastric cancer with distant metastases is considered advanced and has a poor prognosis [7]. Approximately 3-14% of gastric cancer patients have liver metastases at the time of initial diagnosis [8]. In terms of anatomy, the liver is where hematogenous metastases from advanced stomach cancer most frequently occur [8]. Metastasis and post-treatment recurrence of gastric cancer are the primary causes of deterioration and death in tumor patients [9]. The clinical staging of gastric cancer currently relies on the tumor primary-lymph node-metastasis (TNM) model for diagnosis. Tumor markers, such as carcinoembryonic antigen (CEA), carcinoembryonic antigen 19-9 (CA19-9), carcinoembryonic antigen 72-4 (CA72-4), and carcinoembryonic antigen 125 (CA125), have been extensively used in the diagnosis and treatment of gastric cancer patients [10]. However, the use of serum tumor markers for early diagnosis of gastric cancer is challenging due to their low sensitivity and specificity [11]. Furthermore, most markers have not yet demonstrated a clear association with the development of gastric cancer or clinical prognosis [12]. Furthermore, even if early-stage gastric cancer undergoes timely curative surgery, there is no guarantee that it will not recur or metastasize in the future. Therefore, more accurate biomarkers are required to predict the prognosis of patients with gastric cancer.

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Circulating tumor cells (CTCs) are cells that have been shed from the primary tumor into the bloodstream [13]. Recent studies in gastric cancer have shown a potential correlation between circulating tumor cells (CTCs) in the peripheral blood and tumour recurrence/metastasis [14]. Overall survival (OS) has been linked to a decline in the presence of CTCs [15, 16]. CTCs survive in the bloodstream and interact with the distant microenvironment, promoting the formation of metastases [13]. Therefore, as a non-invasive method, the detection of circulating tumour cells (CTCs) is an alternative to surgical and/or biopsy specimens, which are invasive. This is due to changes in various molecular biological markers of tumor cells during treatment, as well as the spatial and temporal heterogeneity of tumor tissues [17]. The detection of CTC status is expected to serve as a link between molecular phenotype and clinical manifestations. Currently, the clinical applications of CTCs are mostly limited to simple counting. Therefore, the relationship between their phenotype and tumour metastasis needs to be studied in detail. To achieve this, the process of CTCs detection needs further optimization, and the link between CTCs status and tumor metastasis and recurrence must be determined. Additionally, it is important to explore whether there are any blood or tumor primary markers that can accurately reflect the status of CTCs.

This study investigates the relationship between the primary tumor foci's features and the presence of peripheral blood CTCs in gastric cancer patients. It explores the development of CTCs and their role in gastric cancer metastasis. Firstly, the enrichment, detection methods, and confirmation criteria of CTCs were optimized to improve the sensitivity and accuracy of detection. To identify potential predictors or biomarkers associated with CTC status, various clinical features of the primary tumor foci, molecular phenotypes, markers related to the EMT process, and micro RNAs closely linked to tumor metastasis were examined. This study may have a positive impact on the application of CTCs in the precise staging of gastric cancer, clinical prognosis judgement, and medication guidance. It may also further optimize the Cell Rich TM detection process and improve the sensitivity and specificity of CTC detection.

2. Materials and Methods

2.1. Subjects

This study recruited 20 patients with clinical stage II, III or IV gastric cancer between January 2021 and July 2021 in the Department of Gastrointestinal Surgery of Ningbo No. 2 Hospital. There were no oncological treatments, such as chemotherapy or radiation, being administered to the patients. The requirements for inclusion were being older than eighteen, having a histological diagnosis of stomach cancer, and giving consent for blood work and follow-up care. Patients with malignant tumors or benign gastric lesions were not included in the study, nor were those who had recently undergone anti-tumor medication. The Ethics Committee of Ningbo No.2 Hospital reviewed and agreed with this study protocol. Patients were informed of the collection criteria beforehand, and those who met the inclusion criteria were enrolled in the study after providing written informed consent.

2.2. Enrichment of peripheral blood cells by Fish method

The study enrolled subjects based on established criteria and collected peripheral blood specimens on an empty stomach in the early morning of the following day. Radial artery cannulation was used to obtain a total of 7 milliliters of peripheral blood samples from patients with gastric cancer before surgery. A 50 ml centrifuge tube was filled with 4 ml of whole blood (including anticoagulant), and the tube was centrifuged at 700 g for 5 minutes at room temperature. The haemolysate was added to the precipitated cells for lysis at a rate of 20 r/min and at room temperature for 10 minutes. Magnetic particles were then added dropwise to the lysed sample at a rate of 100 μL/person. The centrifuge tube was fixed at a 45° angle on a shaker and shaken at 120 rpm and room temperature for 20 minutes. The incubated liquid was transferred to the top layer of the sample density separator and centrifuged at 300 g and room temperature for 5 minutes. After centrifugation, three layers of solution were visible. Transfer the top 2 layers of the solution to a new centrifuge tube. Add 14 mL of 1× Sample Diluent and mix thoroughly. Centrifuge the mixture at room temperature for 5 minutes at 1000 g. Discard the supernatant until 300 μL remains. Add 1mL of 1× Sample Diluent and gently blow and resuspend the precipitated cells. Transfer the resuspended liquid to a centrifuge tube and mix well for automated capture of magnetic particles.

2.3. Observation and judgement of CTCs results

Place the sample under the 20× objective of a fluorescence microscope and scan the entire area of the sample in an "S" pattern. If a suspected positive signal is detected, the specimen is moved to the 40× objective for identification and a positive signal is confirmed and counted as a positive cell. Positive cells are counted if there are more than 2 signal dots on the probe and no blood-borne leukocyte surface antigen staining (red circle or red flake). Cells are counted as negative if blood-borne leukocyte surface antigen (red circle or red flake) is present or if there are less than or equal to 2 signals.

2.4. HE staining

After isolation of the surgical specimens, the tumor and paracancerous tissues were sectioned and fixed in paraformaldehyde. After graded dehydration with ethanol, 4 μm tissue sections were prepared after wax impregnation and embedding. The sections were then stained with haematoxylin for 7 minutes and eosin for 1 minute and observed under a microscope (Olympus, Tokyo, Japan).

2.5. IHC Immunohistochemistry

Sections of paracancerous and tumor tissue fixed in paraffin were cut at 4 μm. Sections underwent a 40-minute baking process at 60°C, xylene deparaffinization, and a graded ethanol series dehydration. The sections were then heated by boiling them in a microwave oven for 15 minutes with 0.01 mol/L citrate buffer (pH 6.0). After that, the sections were stained with antibodies against E-cadherin and vimentin for antigen retrieval and repair using the SP method. The endogenous peroxidase activity was then blocked with a 3% H2O2 solution, and the tissue sections were left overnight at 4°C to be incubated. The tissue slices were treated with a secondary antibody conjugated

with horseradish peroxidase (Product No. PV-9001, dilution ratio 1:1,000, Beijing Zhongsui Jinqiao Co., Beijing, China) at 37°C for one hour on the following day. The sections were dehydrated, stained with hematoxylin, and exposed to diaminobenzidine chromogen for 15 minutes.

2.6. Total RNA extraction

Using the mirVana TM miRNA isolation kit, total RNA was obtained from tissue cell samples, and 1 mL of Trizol was added to extract total RNA from tumor tissue. CDNA was generated by reverse transcription with oligo primers using reverse transcriptase, and then amplified by PCR using the cDNA as a template for gene expression analysis. Conditions for the qPCR reaction were as follows: 50°C for two minutes and 95°C for ten minutes; the internal reference used was GAPDH. GAPDH was used as an internal reference when analyzing the relative expression of target genes using the 2-ΔΔCt technique. Conditions for the qPCR reaction were as follows: 40 cycles of 50°C for 2 min, 95°C for 10 min, 95°C for 30 s, and 60°C for 30 s; Table 1 displays the primer sequences for the qPCR reaction.

2.7. Whole exome sequencing and somatic mutation detection of key genes

Genomic DNA was extracted from the tumor and paracancerous tissues with Rapid DNA Extraction Kit (KG203), the extracted DNA was subjected to quality control, and the samples that passed the quality control criteria were then used for subsequent library construction experiments. The whole exon region and COSMIC sites were highly enriched using the Agilent Liquid Chip Capture System. To create libraries and conduct capture studies, the Agilent SureSelect XT Human All Exon V6 + COSMIC Kit was utilized. Following the development of the library, Qubit 3.0 was used for preliminary quantification, and the Agilent 2100 was used to determine the size of the library insert. To guarantee the library's quality, the effective concentration (4 nm) was precisely measured using the qPCR method once the detection value had fulfilled expectations. The QC-qualified libraries' effective concentration and the necessary data output were taken into consideration when sequencing the libraries using the Illumina HiSeq X Ten Platform PE150. To evaluate the raw sequencing data, the sequencing data were divided by index. Cancerous and paracancerous tissues were paired and analyzed for specific mutations in tumor tissues, including single SNV, insertion and deletion mutations, CNV, etc.

2.8. Transcript sequencing

RNA was extracted from the patient's cancerous tissue using the Tissue Total RNA Extraction Kit (DP431). Using the NEBNext poly(A) mRNA magn (NEB) kit, mRNA was extracted from total RNA using Oligo d(T)25 beads. The NEBNext Ultra RNA Library (NEB) was then used to create the library. Qubit 3.0 was used for initial quantification following library development. After using Agilent 2100 to verify that the library's insert size was as anticipated, quantitative PCR was used to precisely measure the library's effective concentration. The QC-qualified libraries' effective concentration and the necessary data output were taken into consideration when sequencing the libraries using the Illumina HiSeq X Ten Platform PE150.

Table 1. The primers of sequence.

miRNA	5'-3'
miR-107	F: AACCAAACCTACCCACAACG- R: ACCACTAAGTCAATCCCAGGTG
miR-218	F: ATCACCATCTTCCAGGAGCGA R: CCTTCTCCATGGTGGTGAAGAC
miR-146a	F: GGGACCTGAAACCAGAGAACG- R: ACAGAGGAGGGCATAGAGGATG
miR155	F: GGGGTTAATGCTAATCGTGA R: CAGTGCGTGTCGTGGAGT
miR-370	F: CTCGCTTCGGCAGCACA R: AACGCTTCACGAATTTGCGT
miR-409	F: AGGUUACCCGAGCAACUUUGCA R: GGUUCGUACGUACACUGUUCA
miR-148a	F: AGCCACATCGCTCAGACAC R: GCCCAATACGACCAAATCC
miR-223	F: CAGAGCAGCTACTAAGCGACT R: AAAAGGGGAGATTCGGACAGA
miR-1207	F: GCCCGCCAGUUUGUCAAAUA R: GTGCAGGGTCCGAGGT
miR-96	F: TTGGGTGAAATATATTGTGCGTC R: AGCCGAAGTGAGCCACTGAA
miR-21	F: GCACCGTCAAGGCTGAGAAC R: AGCCGAAGTGAGCCACTGAA
U6	F: GCTTCGGCAGCACATATACTAAAA T R: CGCTTCACGAATTTGCGTGTCAT

2.9 Statistical analysis

Clinical information, including patient age, laboratory test results, corresponding CTC counts, and pathological data, were collected and imported into SPSS 20.0 software for statistical analysis. Continuous measurements were expressed as mean ± standard deviation. Spearman's method was used to analyze multiple indicators that did not satisfy normal distribution, including the correlation analysis between CTC counts and clinical and pathological indicators, with $p<0.05$ considered statistically significant. The TCGA database was analyzed using R studio, and Cox survival curves were plotted.

3. Results

3.1. Basic data of cases

This study involved 20 patients with gastric cancer in total. Table 2 summarizes the patients' names, sexes, ages, diagnoses, pathological stages, pathological T, N, and M data, and CTC counts. Blood tests such as routine blood count, coagulation function and tumor markers were also performed simultaneously in all patients when blood samples were taken preoperatively for CTC detection. The results of neutrophils, lymphocytes, monocytes, platelet count, fibrinogen and D-dimer are shown in Table 3.

3.2. CTCs were discovered in the patient's peripheral blood

In this experiment, CTCs were detected in the peripheral blood of tumor patients (Figure 1). According to the number of CTCs, out of 20 tumor patients, 10 gastric cancer patients had 0 CTCs in peripheral blood. Another 10 gastric cancer patients had CTCs detected, totaling 18 CTCs, and the average count of CTCs in positive patients was 1.8. Among them, CEP>3/CD45-/DAPI+, which is polyploid CEP>3. CD45 is a marker of immune cells, while CEP is a marker of polyploidy. Further Spearman's correlation analysis of CTC counts with age and some pathological indicators (T-stage \N-stage \vascular cancer embolism \p53\ki-67) in Table 2 showed no significant correlation. The results are shown in Table 4, with P values in parentheses. In addition, the CTC counts were analyzed by Spearman's correlation with the general clinical tests in Table 3, and the results are shown in Table 5.

Apart from the serum fibrinogen level, which exhibited a negative correlation with the CTC count (r= -0.455, P=0.044), none of the other parameters demonstrated a significant correlation. The ROC chart of serum fibrinogen level for distinguishing whether CTC is zero or not showed that when the cut-off value is 455 mg/dl, its AUC reaches 0.790, P=0.02, which has certain predictive significance (Figure 1F). However, if the differentiation of circulating tumor cells (CTC) is greater than or equal to 2, as the detection target, it fails to reflect sufficient detection efficiency.

3.3. The expression of the tumor marker for gastric cancer and the quantity of CTCs are uncorrelated

The study selected gastric cancer tumor markers, inclu-

Table 3. The clinical laboratory findings in patients with gastric cancer.

Peripheral blood specimen number	WBC (10^9/L)	NE#(10^9/L)	LY# (10^9/L)	NE#/ LY#	MO# (10^9/L)	PLT (10^9/L)	DD (ng/ml)	FIB (mg/dL)
GR2021-11522	4.90	2.7	1.8	1.50	0.40	227	76	326
GR2021-11433	6.30	4	1.8	2.22	0.50	211	263	542
GR2021-11242	8.60	7.3	1.0	7.30	0.30	302	234	451
GR2021-10905	5.90	4	1.5	2.67	0.40	172	92	553
GR2021-10177	5.40	3.6	1.4	2.57	0.40	201	109	498
GR2021-09010	4.50	3.1	1.0	3.10	0.40	182	121	453
GR2021-07879	6.00	4.9	0.7	7.00	0.40	224	258	573
GR2021-06428	10.50	9.1	1.0	9.10	0.40	452	277	567
GR2021-03599	5.50	3.41	1.6	2.13	0.49	277	73	369
GR2021-04523	7.70	6.3	1.0	6.30	0.40	279	110	561
GR2021-03571	5.70	3.68	1.5	2.45	0.52	148	74	628
GR2021-00096	3.90	1.9	1.8	1.06	0.20	270	101	524
GR2021-04230	5.80	3.98	1.1	3.62	0.72	160	197	383
GR2021-05689	6.10	4.5	1.2	3.75	0.40	192	131	383
GR2021-05887	5.60	3.9	1.3	3.00	0.40	290	179	415
GR2021-12686	6.00	3.7	1.9	1.95	0.40	114	327	458
GR2021-04313	5.80	4.1	1.3	3.15	0.40	174	70	322
GR2021-03624	4.80	2.7	1.6	1.69	0.50	272	4	338
GR2021-11105	5.80	3.9	1.4	2.79	0.50	262	169	536
GR2021-11432	6.70	4.6	1.6	2.88	0.50	229	86	597

Table 4. The Spearman's correlation analysis of CTC counts with age and some pathological indicators.

	Age	T-stage	N-stage	Vascular embolism	Ki-67	p53	her2
R (p)	-0.22 (0.351)	-0.264 (0.261)	0.020 (0.933)	0.064 (0.790)	-0.281 (0.231)	0.014 (0.954)	0.211 (0.371)

Table 2. The basic information about the case.

Number	Sex	Age	Diagnosis	T-stage	N-stage	M-stage (distant metastases)	Pathology staging (AJCC8th)	Vascular embolism	CTC number	KI-67	Her-2 GC	p53	EBER
GR2021-11522	man	53	pancreatic cancer	4a	2	0	IIIB	+	0	60%	+	+	0
GR2021-11433	man	74	GC	4a	3a	0	IIIB	+	0	70%	0	+++	0
GR2021-11242	man	69	pancreatic cancer	4a	2	0	IIIA	+	0	70%	++	±	0
GR2021-10905	man	76	GC	4a	0	0	IIB	-	0	60%	+	+	0
GR2021-10177	man	72	GC	4a	3a	0	IIIB	+	0	70%	0	++	0
GR2021-09010	man	76	GC	4a	2	0	IIIB	+	1	70%	+++	0	0
GR2021-07879	man	60	GC	4a	3b	0	IIIC	+	0	70%	0	+	0
GR2021-06428	man	51	GC	4a	3a	0	IIIC	+	0	60%	++	+	0
GR2021-03599	man	71	GC	1b	1	0	IB	-	1	80%	+	±	0
GR2021-04523	man	67	GC	3	2	0	IIIA	+	3	80%	+++	+++	0
GR2021-03571	women	52	GC	3	3b	0	IIIC	+	0	40%	+	+	0
GR2021-00096	man	64	GC	4a	0	0	IIIA	0	1	60%	++	+	0
GR2021-04230	man	53	GC	3	2	0	IIIA	+	3	70%	0	±	0
GR2021-05689	women	48	GC	1	0	0	IA	-	1	70%	0	++	0
GR2021-05887	man	58	GC	4a	3b	0	IIIC	+	4	30%	++	+	0
GR2021-12686	women	77	GC	4a	3b	0	IIIC	+	0	90%	0	+	0
GR2021-04313	man	54	GC	2	3a	0	IIIA	+	1	70%	0	±	0
GR2021-03624	man	63	GC	2	3a	0	IIIA	+	2	60%	0	±	0
GR2021-11105	women	67	GC	4a	2	0	IIIb	+	1	70%	+	+	0
GR2021-11432	man	68	GC	4a	2	0	IIIb	+	0	80%	++	+	0

Table 5. The spearman correlation analysis of CTC counts and clinical laboratory items.

	WBC	NE#	LY#	NE#/LY#	DHXB	PCT	DD	FIB
R (p)	-0.324 (0.164)	-0.194 (0.413)	-0.215 (0.362)	0.076 (0.750)	0.114 (0.631)	0.215 (0.364)	-0.193 (0.414)	-0.455 (0.044)

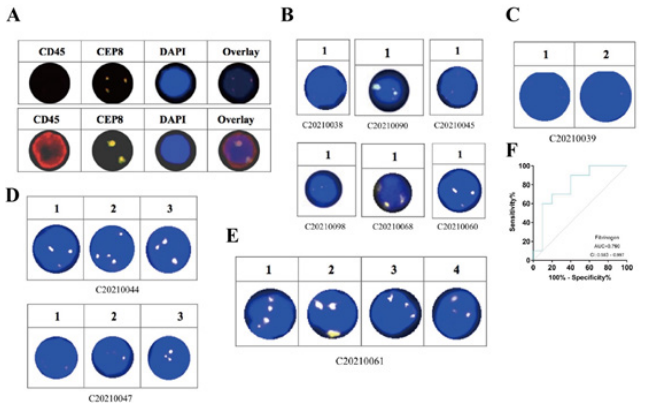

Fig. 1. The number of CTCs in peripheral blood of patients with gastric cancer. (A) CTCs positive vs. negative control. (B) The sample of CTCs numbered one. (C) The sample of CTCs numbered two. (D) The sample of CTCs numbered three. (E) The sample of CTCs numbered four. (F) Serum fibrinogen levels are important in distinguishing CTC from ROC.

ding alpha-fetoprotein, carcinoembryonic antigen, Ca199, and Ca125. The objective was to look for any relationships between the quantity of CTCs and each tumor marker's expression (Table 6). However, Spearman's correlation analysis of CTC counts with each tumor marker did not show any statistically significant association (Table 7).

3.4. During the patients' brief follow-up, no deaths were recorded

The patients' tumor and survival status were monitored simultaneously after the start of data collection. In the short term, one case of recurrent tumor metastasis was observed. Multiple metastases to bilateral ribs were found in July postoperatively due to chest pain, and the patient is currently undergoing follow-up 2-line systemic therapy. As of 30 March 2022, no additional cases were found to have clinical metastases, and there were no fatalities.

3.5. Expression of E-cadherin and Vimentin in tumor tissues of patients with gastric cancer

The study analyzed the expression of E-cadherin and Vimentin in tumor tissues of 20 gastric cancer patients who underwent surgery at Ningbo No.2 Hospital. The results of the study are presented in Figure 2. The correlation between E-cadherin immunohistochemical expression and clinical and pathological factors was examined in both gastric cancer and surrounding mucosa. Figure 2A shows that the rows of cells retained some polarity and exhibited

Table 6. The patient's serum alpha-fetoprotein and oncoprotein test results.

Peripheral blood specimen number	CEA (IU/ml)	AFP (ng/ml)	CA199 (IU/ml)	CA125 (IU/ml)
GR2021-11522	1.930	66.300	35.740	10.10
GR2021-11433	2.400	2.700	26.480	6.80
GR2021-11242	10.670	7.900	0.900	4.90
GR2021-10905	281.410	6.700	548.200	6.20
GR2021-10177	14.600	5.600	1.100	10.60
GR2021-09010	12.110	1.800	133	6.40
GR2021-07879	1.660	2.700	20.960	46.10
GR2021-06428	0.400	1.020	10.270	8.10
GR2021-03599	0.200	4	8.030	9.30
GR2021-04523	2.360	1	60.800	9.90
GR2021-03571	2.070	4	28.260	6.70
GR2021-00096	2.950	3.300	0.900	9.30
GR2021-04230	5.270	6.200	11.180	14.60
GR2021-05689	1.250	2	8.640	10.30
GR2021-05887	1.020	9.800	16.230	5.10
GR2021-12686	11.730	97.100	5.430	7.40
GR2021-04313	0.990	3.600	4.300	10.20
GR2021-03624	5.300	2.600	61.490	9.20
GR2021-11105	11.250	2.300	2036.050	24.60
GR2021-11432	1.670	2.400	1.570	2.80

Table 7. The spearman correlation analysis of CTC counts with each tumor marker.

	Ca199	Ca125	CEA	AFP
CTC number	r=0.194 P=0.412	r=0.221 P=0.349	r=-0.150 P=0.527	r=-0.223 P=0.345

glandular duct morphology. E-cadherin was well-colored and continuous in most basement membranes. However, as the number of CTCs increased, the cell arrangement became more disordered, resulting in the loss of glandular duct morphology. The coloration of E-cadherin became discontinuous or even appeared in a large unstained area. However, in some patients (as shown in Figure 2C), although the E-cadherin staining was good, the corresponding CTC count was 3. Spearman's correlation analysis of E-cadherin staining (negative/+/++) and CTC count showed no significant correlation with a result of r=0.200, P=0.399.

The study found that Vimentin expression was elevated in the tumor tissues of patients with high CTC counts (Figure 2f-2J). However, there is a lack of specific grading criteria in the literature for determining the degree of Vimentin staining in gastric cancer tissues, which makes it difficult to interpret the results as hierarchical information. When interpreting the results, it is important to exclude the influence of various types of interstitial tissues, such as lymphatic tissues in the tumor tissues or granulation tissues in the ulcerated tumor tissues, as they may affect the interpretation of the images to a certain extent. Figure 2F shows many lymphatic infiltrations in the right figure, which corresponds to the patient's late clinical stage. Despite careful interpretation, the results of the tumor cells with Vimentin staining are still negative.

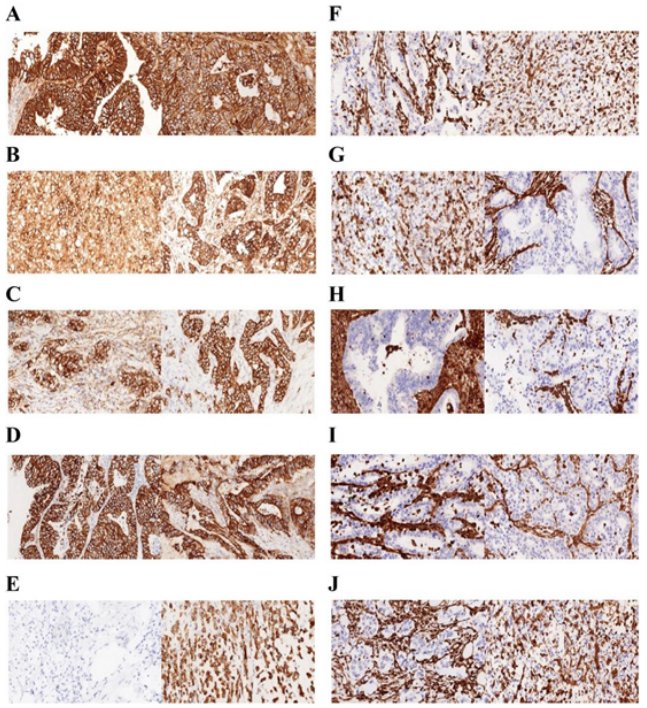

Fig. 2. The expression of E-cadherin and Vimentin in GC patients. (A-E) Expression of E-cadherin in tumor tissue of patients with zero to four CTCs. (F-J) Expression of Vimentin in tumor tissue of patients with zero to four CTCs.

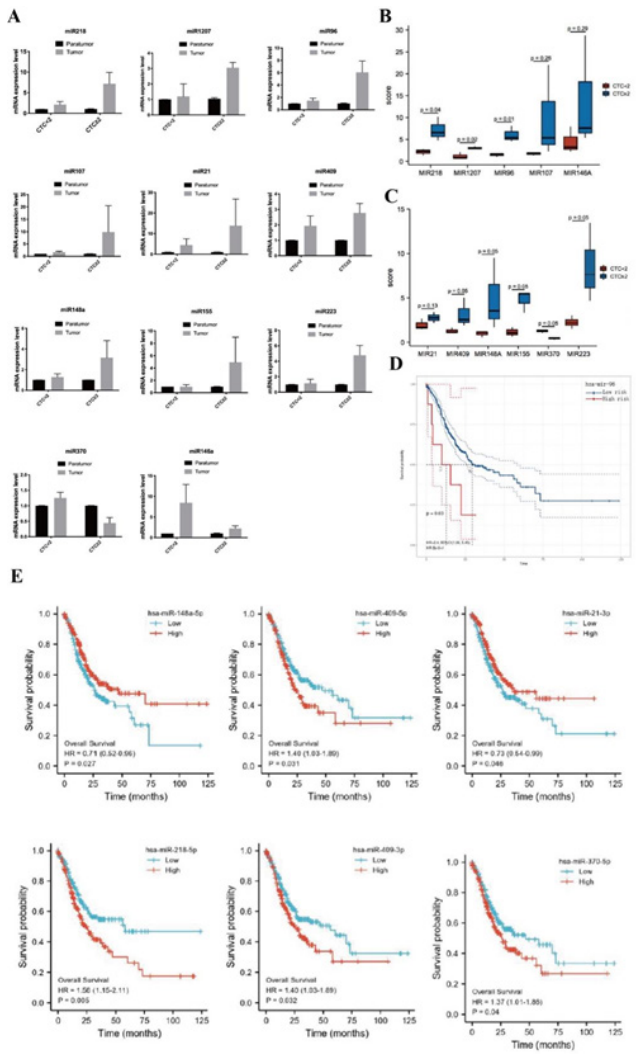

Fig. 3. The miRNA expression in gastric cancer tumor tissues and Kaplan-Meier survival analysis results of different miRNAs. (A) The miRNA expression in gastric cancer tumor tissues. (B-C) The miRNA expression in different CTC groups. (D) Kaplan-Meier survival analysis results for miR-96. (E) Kaplan-Meier survival analysis results for different miRNAs.

3.6. Metastasis-related miRNAs were discovered to be expressed at greater levels in gastric cancer tumor tissues

The qRT-PCR assay results (Figure 3A) indicate an increase in the expression of metastasis-related miRNAs in tumor tissues compared to paraneoplastic controls. Furthermore, miRNA expression increased with the number of CTCs. Upon observing the differences between groups with varying CTC counts, it was found that the expression of metastasis-associated miRNAs in the CTCs ≥2 group significantly differed from that in the CTCs <2 group. Specifically, the expression of miR218, miR96, miR107, miR21, miR155, and miR223 was significantly higher, while miR146a was significantly lower. miR-148a was more specific in the CTC=2 group and significantly lower

than in the CTC=1 group, but significantly higher than in the CTC=2 group. In the CTC=0 group, the expression in tumor tissues and para-carcinoma tissues was similar. However, in the CTC=1 or 2 group, the expression level was significantly different, with both higher and lower expression than that in para-carcinoma tissues. In the CTC=3 group, the expression was high, but the trend of the change was uncertain.

3.7. Correlation between miRNA expression and circulating tumor cells (CTCs) in gastric cancer tissue

The study analyzed the expression of miRNAs in tumor specimens collected from gastric cancer patients. The specimens were grouped based on the CTC count of peripheral blood, with a classification criterion of a CTC count greater than or equal to 2. The expression values were analyzed using t-test or non-parametric test, depending on whether they were normally distributed or not. The study results indicate that miR218, miR1207, miR96, miR409, miR148a, miR155, miR370, and miR223 exhibited significant differences between the CTC≥2 and CTC<2 groups. Additionally, it was observed that the data for miR107, miR148a, and miR223 were more dispersed among the miRNAs with significant differences between the groups (Figure 3B-3C).

3.8. The expression profile of miRNA has an impact on the survival of patients

The study selected miRNAs that showed significant differences in expression levels in different CTCs. These miRNAs include miR218, miR1207, miR96, miR409, miR148a, miR155, miR370, and miR223, which were used to differentiate between high and low expression groups. Prognostic data for gastric cancer patients (STAD set) were obtained from the TCGA database, and survival curves were plotted based on the follow-up results. Among them, miR-96, miR-148a-5p, miR-409-5p, miR-218, miR-370, and miR-21-3p were analyzed for their prognostic value. The survival curves were plotted using the KM method to show the differences in prognosis between the two groups. The 5-year overall survival rates of miR-96, miR148a-5p, miR409-5p, miR218-5p, miR409-3p, and miR370-5p in the low-expression group among gastric cancer patients differed significantly from those in the high-expression group. Similar results were found for miR21-3p (see Figure 3E). The hazard ratio (HR) for the miR-96 high-expression group was 2.4 (95% CI 1.06-5.45), indicating that miR-96 could serve as a complementary prognostic indicator for gastric cancer patients. This finding provides a basis for guiding clinical treatment (Figure 3D).

3.9. MiR96 can serve as a marker for predicting CTC status in the peripheral blood of patients with gastric cancer

Based on the ROC curve analysis, miR-21 (0.837), miR-96 (0.832), and miR107 (0.86) exhibited an AUC of 0.8 or higher. By combining the results of the miRNA group differences and KM curve analysis, miR-96 was identified as a marker for predicting CTC status in the peripheral blood of gastric cancer patients. High expression of miR-96 (>2.5-fold in tumour foci/paraneoplastic areas) was found to be associated with an increased probability of CTC appearance and poor prognosis. Based on the results, a single test for miR-96 was performed on 9 additional surgical specimens from progressive gastric cancer patients who had undergone preoperative CTC testing (refer to Table 8). This was done to externally validate the model. After combining the data from the present study with previous data, miR-96 showed a high sensitivity of 85.7% and specificity of 86.4% in predicting CTC counts of ≥2 in this series. The Youden index of 0.721 further supports the high expression of miR-96 for CTC status in tumor foci/paraneoplastic. Therefore, miR-96 can be considered a reliable predictor of CTC status in tumor foci with a sensitivity of 85.7% and a specificity of 86.4%.

4. Discussion

In contrast to traditional cell screening techniques, Cell rich TM is a new technology for detecting circulating tumors using multi-field capture in nano-chips. The method is simple, painless, and free of radiation contamination. The cell-rich TM system offers higher capture efficiencies than comparable imported equipment and brings significant savings.

The presence of circulating tumor cells (CTCs) has been used to assess the risk of tumor recurrence and as an indicator of metastasis. CTCs have also been employed for real-time tumor response detection and tailored therapy [18]. However, CTC concentrations in human peripheral blood are relatively low and vary widely between individuals, necessitating the use of modern technology to enrich and detect them [19]. This study utilized the Cellrich TM system, which is based on cell surface immunorecognition, to detect circulating tumor cells (CTCs) in the peripheral blood of patients with gastric cancer. Previous studies have shown that neutrophils are associated with tumor growth, metastasis, and tumor angiogenesis. Additionally, an increased number of neutrophils in GC tumor

Table 8. The results of the miR-96 mono-assay in 9 surgical specimens of advanced gastric cancer that underwent preoperative CTC testing.

Patient's number	miR-96 (T/P)	pTNM stage	CTC number
A1	2.810	IIIa	2
A2	5.177	IIIa	2
A3	1.612	IIIc	1
A4	1.005	II	0
A5	1.812	IIIa	0
A6	2.339	IIIa	1
A7	1.054	IIIb	0
A8	11.402	IIIc	3
A9	1.018	IIIa	1

tissue has been linked to a low overall survival (OS) rate in GC patients [20]. A study of 1220 non-surgical advanced gastric cancer patients indicated that individuals with a higher neutrophil/lymphocyte ratio had a lower overall survival. The neutrophil/lymphocyte ratio has been established as a distinct prognostic factor [21]. Preoperative neutrophil/lymphocyte ratio values were revealed as independent risk variables for overall survival and progression in gastric cancer patients having radical resection and postoperative adjuvant chemotherapy [22]. However, this study found no significant correlation between the number of CTCs measured by the Cell rich TM system and the preoperative white blood cell count, absolute neutrophil value, neutrophil/lymphocyte ratio, or platelets in patients with stage III/IV GC. This suggests that the prognostic effect of these factors may not be achieved by influencing the CTC status.

The relationship between CTCs and clinicopathological serum GC markers remains unclear. In this study, the proportion of tumor markers with abnormalities increased significantly with the progression of staging. Two cases with hepatoid adenocarcinoma with directional differentiation showed typical elevation of alpha-fetoprotein (AFP). This is consistent with the general literature, which reports that tumor markers can be used for prognosis and efficacy determination. However, this study found that several commonly used tumor markers, including alpha-fetoprotein, carcinoembryonic antigen, Ca19-9, Ca72-4, and Ca125, did not significantly correlate with CTC. Therefore, it is difficult to predict the status of CTC using these tumor markers. One possible explanation for this is that the concentrations of the detected tumor markers are more related to the overall molecular characteristics of the primary tumor foci, rather than the presence of CTC. Subclinical circulating tumor cells (CTCs) are present in such low numbers that they are unlikely to cause significant fluctuations in tumor marker concentration. Clinically detectable changes in CTCs may not occur until they form metastatic foci. It is also possible that the mechanisms leading to the elevation of these tumor markers do not have a high and stable crossover with key nodes in the CTC formation pathway, and thus do not show sufficient correlation. Further exploration of assays related to circulating tumor cell (CTC) status is necessary, including marker sites located in the epithelial-mesenchymal transition (EMT) or mesenchymal-epithelial transition (MET) pathways.

CTCs are a heterogeneous population of cells. A commonly used method to isolate CTCs is an epithelial cell adhesion molecule (EpCAM)-based enrichment technique. However, this technique failed to detect a subpopulation of CTCs that had undergone EMT, as reported by Gorges et al. [23] and Yu et al. [24]. Furthermore, Yu et al. [25] discovered that circulating tumor cells (CTCs) undergo dynamic changes in both epithelial and mesenchymal components. Specifically, mesenchymal CTCs were found to be associated with metastasis and resistance to chemotherapy [25]. These findings suggest that epithelial-mesenchymal transition (EMT) could serve as a potential biomarker for characterizing CTCs. However, the heterogeneity of CTCs and the characteristics of blood samples from some cancer patients limit their broad clinical application. The tissues of progressive gastric cancer mostly expressed E-cadherin (+~++) in varying degrees. The expression of Vimentin, although difficult to quantify, was consistent with the CTC status in describing tumor invasiveness through clinical staging or tumor subtypes, as demonstrated in most cases. However, no direct correlation was observed between the above metrics and CTCs counts, either jointly or independently. This may be partly due to the assessment of the whole tumor lesion after IHC and the difficulty in effectively differentiating between the various expressions of tumor cells and tumor mesenchyme. Predicting the status of CTCs solely from epithelial or mesenchymal markers in the primary tumor foci or marker sites in the EMT pathway remains challenging. Further exploration of the mechanisms and factors influencing the emergence of CTCs at a more upstream molecular level is necessary.

Through the analysis of selected miRNA microarrays in the primary foci of tumors, certain miRNAs were found to be closely associated with circulating tumor cell (CTC) status in patients with gastric cancer. In this study, we found significant differences in the expression levels of miR218, miR1207, miR96, miR409, miR149, miR148a, miR155, miR370, and miR223 between the groups with CTC ≥ 2 and CTC < 2. We considered previous literature reports and aimed to determine the existence of CTC and differences in prognosis between the two groups. The TCGA database reveals that varying expression levels of miR-148a, miR-409 (5p/3p), miR-21-3p, miR-218, and miR-370 can serve as reliable prognostic indicators. The study revealed that miR-96 expression was normal in healthy tissues but abnormal in cancerous tissues, and the abnormal expression levels could differentiate between different CTC states. Kong et al. [26] and others [27-30] reported a significant up-regulation of the miR-183 family in gastric cancer tissues. However, Li et al. [31] reported that miR-182 acts as an oncogenic factor in gastric adenocarcinomas and is down-regulated through the downregulation of CREB1 (cyclic adenosine monophosphate-responsive element binding protein). The study indicates a correlation between miR-96 expression level and cell adhesion and apoptosis. Additionally, several miRNAs showed significant differences in expression levels among different CTC groups. These findings suggest that up-regulation of miR-96 expression is closely associated with tumor cell infiltration into the stroma, CTC appearance, and metastasis formation. The expression of miR-96 is up-regulated and is closely associated with the ability of tumor cells to infiltrate the stroma and form metastasis. This suggests its potential for prognostic segmentation in patients with progressive gastric cancer.

5. Conclusions

The Cell Rich TM system was used to detect CTCs in the peripheral blood of gastric cancer patients. The negative enrichment and complex IHC of the Cell Rich TM system can effectively detect and confirm CTCs in peripheral blood. The statistics of various clinical tests and routine pathological indexes of gastric cancer patients in the present study showed that high fibrinogen levels indicate a lower probability of the appearance of CTCs. However, the other indexes are not useful in distinguishing different CTC statuses in patients with progressive gastric cancer. Meanwhile, the statistics from various clinical tests and routine pathological indexes of gastric cancer patients in this study showed that high fibrinogen levels were associated with a low probability of circulating tumor cells (CTCs). However, the other indexes did not provide suf-

ficient information to distinguish between different types of CTCs in patients with progressive gastric cancer. After detecting miRNA in patients' tumor tissues, the results showed that miRNA could serve as a marker for the status of CTCs. Additionally, mir-96 detection of tumor foci could supplement the detection of CTC status in clinical practice. The second-generation gene sequencing of the failed cases of predicting CTC status based on mir-96 has revealed the tumor heterogeneity and the immune microenvironment of the tumor foci. This suggests that the immune microenvironment affects CTC status, and the presence of active immune cell infiltration and up-regulation of related immune indexes in the tumor area can lead to a decrease in the level of CTCs, which is a sign of a better prognosis.

Conflict of Interests
The author has no conflicts with any step of the article preparation.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
This study was approved by the ethics committee of Ningbo No.2 hospital.

Informed Consent
Signed written informed consents were obtained from the patients and/or guardians.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions
QZ and HG designed the study and performed the experiments, QZ collected the data, HG analyzed the data, QZ and HG prepared the manuscript. All authors read and approved the final manuscript.

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References

1. Guan WL, He Y, Xu RH (2023) Gastric cancer treatment: recent progress and future perspectives. J Hematol Oncol 16:57. doi: 10.1186/s13045-023-01451-3
2. Siegel RL, Miller KD, Fuchs HE, Jemal A (2021) Cancer Statistics, 2021. Ca-Cancer J Clin 71:7-33. doi: 10.3322/caac.21654
3. Warsinggih, Syarifuddin E, Marhamah, Lusikooy RE, Labeda I, Sampetoding S et al (2022) Association of clinicopathological features and gastric cancer incidence in a single institution. Asian J Surg 45:246-249. doi: 10.1016/j.asjsur.2021.05.004
4. Patel TH, Cecchini M (2020) Targeted Therapies in Advanced Gastric Cancer. Curr Treat Option On 21:70. doi: 10.1007/s11864-020-00774-4
5. Yang WJ, Zhao HP, Yu Y, Wang JH, Guo L, Liu JY et al (2023) Updates on global epidemiology, risk and prognostic factors of gastric cancer. World J Gastroentero 29:2452-2468. doi: 10.3748/wjg.v29.i16.2452
6. Klint A, Engholm G, Storm HH, Tryggvadottir L, Gislum M, Hakulinen T et al (2010) Trends in survival of patients diagnosed with cancer of the digestive organs in the Nordic countries 1964-2003 followed up to the end of 2006. Acta Oncol 49:578-607. doi: 10.3109/02841861003739330
7. Riihimaki M, Hemminki A, Sundquist K, Sundquist J, Hemminki K (2016) Metastatic spread in patients with gastric cancer. Oncotarget 7:52307-52316. doi: 10.18632/oncotarget.10740
8. Luo Z, Rong Z, Huang C (2019) Surgery Strategies for Gastric Cancer With Liver Metastasis. Front Oncol 9:1353. doi: 10.3389/fonc.2019.01353
9. Caspers IA, Sikorska K, Slagter AE, van Amelsfoort RM, Meershoek-Klein KE, van de Velde C et al (2021) Risk Factors for Metachronous Isolated Peritoneal Metastasis after Preoperative Chemotherapy and Potentially Curative Gastric Cancer Resection: Results from the CRITICS Trial. Cancers 13:4626. doi: 10.3390/cancers13184626
10. Gao Y, Wang J, Zhou Y, Sheng S, Qian SY, Huo X (2018) Evaluation of Serum CEA, CA19-9, CA72-4, CA125 and Ferritin as Diagnostic Markers and Factors of Clinical Parameters for Colorectal Cancer. Sci Rep-Uk 8:2732. doi: 10.1038/s41598-018-21048-y
11. Jelski W, Mroczko B (2022) Molecular and Circulating Biomarkers of Gastric Cancer. Int J Mol Sci 23:7588. doi: 10.3390/ijms23147588
12. Zhou B, Zhou Z, Chen Y, Deng H, Cai Y, Rao X et al (2020) Plasma proteomics-based identification of novel biomarkers in early gastric cancer. Clin Biochem 76:5-10. doi: 10.1016/j.clinbiochem.2019.11.001
13. Liu Y, Gai J, Fu L, Zhang X, Wang E, Li Q (2018) Effects of RSF-1 on proliferation and apoptosis of breast cancer cells. Oncol Lett 16:4279-4284. doi: 10.3892/ol.2018.9172
14. Hou J, Yu Z, Xiang R, Li C, Wang L, Chen S et al (2014) Correlation between infiltration of FOXP3+ regulatory T cells and expression of B7-H1 in the tumor tissues of gastric cancer. Exp Mol Pathol 96:284-291. doi: 10.1016/j.yexmp.2014.03.005
15. Huang X, Gao P, Song Y, Sun J, Chen X, Zhao J et al (2015) Meta-analysis of the prognostic value of circulating tumor cells detected with the CellSearch System in colorectal cancer. Bmc Cancer 15:202. doi: 10.1186/s12885-015-1218-9
16. Zhan Q, Liu B, Situ X, Luo Y, Fu T, Wang Y et al (2023) New insights into the correlations between circulating tumor cells and target organ metastasis. Signal Transduct Tar 8:465. doi: 10.1038/s41392-023-01725-9
17. Mohamed SN, Foong YM, Lee DY, Phyo WM, Cima I, Lee EX et al (2015) Non-invasive sensitive detection of KRAS and BRAF mutation in circulating tumor cells of colorectal cancer patients. Mol Oncol 9:850-860. doi: 10.1016/j.molonc.2014.12.011
18. Hong B, Zu Y (2013) Detecting circulating tumor cells: current challenges and new trends. Theranostics 3:377-394. doi: 10.7150/thno.5195
19. Hazra RS, Kale N, Aland G, Qayyumi B, Mitra D, Jiang L et al (2020) Author Correction: Cellulose Mediated Transferrin Nanocages for Enumeration of Circulating Tumor Cells for Head and Neck Cancer. Sci Rep-Uk 10:16607. doi: 10.1038/s41598-020-72920-9
20. Ni Z, Xing D, Zhang T, Ding N, Xiang D, Zhao Z et al (2021) Tumor-infiltrating B cell is associated with the control of progression of gastric cancer. Immunol Res 69:43-52. doi: 10.1007/s12026-020-09167-z
21. Yu L, Lv CY, Yuan AH, Chen W, Wu AW (2015) Significance of the preoperative neutrophil-to-lymphocyte ratio in the prognosis of patients with gastric cancer. World J Gastroentero 21:6280-6286. doi: 10.3748/wjg.v21.i20.6280
22. Li K, Duan WC, Zhao HB, Wang L, Wang WW, Zhan YB et al (2019) Preoperative Neutrophil to Lymphocyte Ratio and Pla-

telet to Lymphocyte Ratio are Associated with the Prognosis of Group 3 and Group 4 Medulloblastoma. Sci Rep-Uk 9:13239. doi: 10.1038/s41598-019-49733-6

23. Gorges TM, Tinhofer I, Drosch M, Rose L, Zollner TM, Krahn T et al (2012) Circulating tumour cells escape from EpCAM-based detection due to epithelial-to-mesenchymal transition. Bmc Cancer 12:178. doi: 10.1186/1471-2407-12-178
24. Yu M, Bardia A, Wittner BS, Stott SL, Smas ME, Ting DT et al (2013) Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. Science 339:580-584. doi: 10.1126/science.1228522
25. Zeisberg M, Neilson EG (2009) Biomarkers for epithelial-mesenchymal transitions. J Clin Invest 119:1429-1437. doi: 10.1172/JCI36183
26. Kong WQ, Bai R, Liu T, Cai CL, Liu M, Li X et al (2012) MicroRNA-182 targets cAMP-responsive element-binding protein 1 and suppresses cell growth in human gastric adenocarcinoma. Febs J 279:1252-1260. doi: 10.1111/j.1742-4658.2012.08519.x
27. Kazemi E, Kahrizi D. (2017). The repeatability of PCR-RFLP method for study of association between gastric cancer and manganese superoxide dismutase mutant (Val-9Ala). Biharean Biologist, 11(2), 112-114.
28. Kazemi E, Kahrizi D. (2016). Lack of association between gastric cancer and hopq alleles in Helicobacter pylori. Genetika. 48(3): 893-902. https://doi.org/10.2298/gensr1603893k.
29. Kazemi E, Kahrizi D, Moradi MT, Sohrabi M, Yari, K. (2016). Gastric Cancer and Helicobacter pylori: Impact of hopQII Gene. Cell Mol Biol 62(2): 107-110. https://doi.10.14715/cmb/2016.62.2.18.
30. Kazemi E, Kahrizi D, Moradi MT, Sohrabi M, Amini A, Mousavi SAR, Yari K. (2016). Association between Helicobacter pylori hopQI genotyping and human gastric cancer. Cell Mol Biol 62(1): 6-9. https://doi.10.14715/cmb/2016.62.2.18.
31. Li X, Luo F, Li Q, Xu M, Feng D, Zhang G et al (2011) Identification of new aberrantly expressed miRNAs in intestinal-type gastric cancer and its clinical significance. Oncol Rep 26:1431-1439. doi: 10.3892/or.2011.1437